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Dietary supplementation and intragastric infusions of L-tryptophan reduce food intake

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Abstract

Select free amino acids have been suggested to promote early satiation. Initial studies indicate that L-tryptophan may play an important role in reducing appetite. The current set of experiments examined whether dietary and intragastrically administered L-tryptophan decreases food consumption and whether this effect is specific to eating stimulated by energy (calorie) needs or by pleasant taste (reward). A link between a satiating action of L-tryptophan and activity of the anorexigenic oxytocin circuit has also been investigated. Supplementation of milk formula-based diets with tryptophan reduced energy deprivation-induced consumption of these formulas in mice. Tryptophan enrichment had no effect on water intake, which precludes the involvement of taste- or thirst-related mechanisms in tryptophan-driven hypophagia. Intragastrically administered L-tryptophan decreased deprivation-induced chow intake. It also reduced hedonics-driven consumption of palatable saccharin and milk, but not of sucrose solutions in non-deprived mice, suggesting a link with feeding reward mechanisms unrelated to sucrose. Finally, oxytocin receptor blockade with very low doses of an antagonist just prior to intragastric L-tryptophan administration, completely abolished early termination of deprivation-induced food intake by this amino acid. Overall, the data indicate that dietary supplementation and intragastric delivery of free L-tryptophan reduce eating behaviour stimulated by energy needs and palatability, and that the effect of L-tryptophan is mediated by the anorexigenic oxytocin system.

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AP, area postrema; CCK, cholecystokinin; DIT, diet-induced thermogenesis; DVC, dorsal vagal complex; GLP-1, glucagen-like peptide-1; IDO, indoleamine 2,3-dioxygenase; LAT1, large neutral amino acid transporter; LNAA, large neutral amino acid; NAD^+, nicotinamide adenine dinucleotide; NMDA, N-methyl-d-aspartate; NTS, nucleus of the solitary tract; OT, oxytocin; OTA, oxytocin receptor antagonist L-368,899; TDO, tryptophan 2,3-dioxygenase; TPH, tryptophan hydroxylase.

Chapter 1: Introduction

1.1 Obesity and appetite

1.1.1 The obesity epidemic

Over the past several decades obesity has become one of the most significant health crises facing society on a global scale [1]. New Zealand suffers from the third highest rate of obesity within the OECD. In 2015, the New Zealand Health Survey found that almost one third of adults within New Zealand were clinically obese and a further third considered overweight [2]. Obesity rates within New Zealand are the highest in Māori and Pacific Island communities [3]. An obese individual carries a significantly increased risk of developing a number of diseases, including certain forms of cancer, type 2 diabetes and coronary heart disease [4]. Obesity is now one of the leading causes of preventable deaths worldwide, and has reached epidemic proportions within New Zealand.

Weight gain occurs when overall energy expenditure is outstripped by energy intake [5]. A number of factors have been attributed as the cause of obesity, including high-stress lifestyles, changes in social attitudes towards weight and physical inactivity [6]. While these factors play a role in the increasing incidence rate of obesity, the most significant contributing environmental factor has been an increase in calorie consumption [7]. New Zealanders on average consume 2810 kilocalories per day [8]. This quantity of energy intake is significantly more than is needed to maintain basic functions of the organism and most individuals do not expend excess energy through physical activity [7]. Therefore, an important

strategy to reduce the incidence of obesity is to decrease the average rate of energy intake via dietary manipulations.

1.1.2 Dietary macronutrient composition

There are numerous factors that influence food consumption. Key aspects that contribute to eating behaviour are associated with food characteristics. Those characteristics include, among others, flavour, texture, temperature, and energy density of a food item [9-11]. Importantly, macronutrient composition of a diet can have a profound impact on the perceived attractiveness as well as on satiating properties of a given food item [12]. Macronutrient composition of a diet thus represents a significant factor associated with energy intake.

The variation in macronutrient composition of otherwise similar, isocaloric food items can impact food consumption. For example, both high-sugar and high-fat foods are associated with the overconsumption of calories and, ultimately, weight gain [13]. This is attributed two effects. Firstly, sugars and fats are perceived as highly palatable, improving not only the scent and flavour of food items, but also food texture [14]. Meats with high fat content are considered to be juicier and more tender [15]. Secondly, high-sugar and high-fat food items tend to enhance activity of neuroendocrine pathways that promote consumption (simultaneously suppressing activity of satiety mechanisms), thereby increasing meal volume and, consequently, energy intake, and shortening the time before additional food items are consumed [16, 17]. It is important to note that in the industrialised world, the most affordable and most accessible food items also tend to contribute the most to weight gain, via unfavourable macronutrient composition and high energy

density. These foods are high in sugars, fats and overall energy content, but low in nutritive quality [6]

Early attempts to manipulate dietary macronutrient composition to benefit weight loss focused on reducing energy density of a diet, especially through minimisation of dietary fat content [18]. These low-energy diets promoted promising reductions in the body mass of participants during short-term eating behaviour studies, however, later long-term monitoring of individuals given these diets revealed issues in adherence associated with greatly reduced satiation following meals [19]. Since these initial investigations, a substantial body of research has been conducted on the effects of various macronutrient compositions on energy intake, satiety, appetite regulation and weight loss [20-24]. This research has repeatedly demonstrated the importance of macronutrient composition in relation to feeding behaviours. A strong consensus has emerged within the scientific literature which holds that of the three significant macronutrients, fats, carbohydrates and proteins, it is the protein content of a diet that has the most potent suppressive effect on energy intake and appetite.

1.1.3 The effects of high-protein diets on appetite

Macronutrients are a source of calories. Consumption of proteins and carbohydrates provides 4 kcal/g of energy, whereas energy-dense fats, 9 kcal/g [25]. In addition to ensuring that energy requirements are met, protein consumption serves as the sole source of exogenous amino acids [26]. Proteins are comprised of amino acids linked by peptide bonds, forming large polymer chains. During digestion, proteins are broken into amino acids before absorption within

the small intestine. These amino acids are vital for numerous biological processes, notably, for general biosynthesis. Of 21 proteinogenic amino acids, 9 cannot be synthesised within the mammalian body and are considered “essential”. In order to prevent malnutrition, these amino acids must be obtained from a protein-rich diet [27].

The proportion of proteins to other macronutrients in the diet has a significant effect on food intake [20]. High-protein diets are considered to be, based on average protein intake, diets in which 20-30% of total energy-intake is sourced from protein [22]. High-protein diets have been thoroughly demonstrated to enhance the development of satiety in humans and other animals, delaying the onset of hunger [28]. This satiating effect of protein is of a much greater magnitude than what has been observed after consumption of high-carbohydrate or high-fat diets [29].

Food intake studies where humans are provided isocaloric meals rich in either proteins, fats or carbohydrates best illustrate the unique impact high-protein diets have on the development of satiety and resultant energy intake [30]. For example, Poppitt et al. provided subjects with identical isocaloric meals to which 1 MJ of either carbohydrate, fat or protein was discretely added. Subjects unknowingly given protein-enriched meals had the lowest energy intakes, as well as the lowest self-reported levels of hunger following meal consumption [31].

Short-term feeding studies have demonstrated that even single meals containing a high proportion of protein can alter post-meal perceptions of hunger [32, 33].

Isocaloric breakfasts with high fat, protein or carbohydrate compositions were provided to subjects in a single-meal study conducted by Stubbs et al. Participants who consumed high-protein breakfasts demonstrated the highest rates of post-meal satiety of all groups [30]. Even snacks differing in protein content, when consumed between meals, impacted subsequent energy-intake [34, 35]. Ortinau and colleagues found high-protein yoghurt snacks, in comparison to nearly isocaloric high-fat cracker and chocolate snacks, delayed the onset of hunger and led to reduced energy intake during a subsequent meal [36]. Several long-term dietary trials have corroborated these results, and have shown that increasing the proportion of protein within the diet can lead to successful long-term weight loss [37, 38]. For example, Leidy et al. placed obese and pre-obese women on two varieties of 750 kcal/d energy-deficit diets, a normal-protein diet (18% protein) and a high-protein diet (30% protein). These diets were maintained for a 12 week period. The high-protein diet aided participants in preserving lean body mass during weight loss. More importantly, this diet was found to induce higher satiety levels throughout the course of the study, leading to consistently higher self-reported satisfaction [39].

While the appetite suppressing effects of high-protein diets have been convincingly demonstrated in animal and human models, the exact mechanism by which these diets induce satiety is currently unknown [22]. Even short-term high-protein diets effect the function of various components of the body, including the gastrointestinal, endocrine and neural system [40]. As a result, there are a number of potential physiological mechanisms by which high-protein diets may induce satiety and reduce energy-intake [41]. Key among these mechanisms are an

increased energy expenditure via diet-induced thermogenesis (DIT), increased levels of gluconeogenesis, increased production of satiety hormones, reduced sensory attractiveness and increased blood plasma amino acid concentrations [22].

1.1.3.1 Dietary-induced thermogenesis

Processing and incorporating food from the diet into the body via digestion, absorption and various metabolic pathways leads to a temporary increase in energy expenditure. This effect, termed dietary-induced thermogenesis, is measured as the percentage increase in energy expenditure above resting metabolic rate [42].

The comparatively more complex structure and components of dietary proteins make them harder to be digested and absorbed than carbohydrates or fats, and thus digesting proteins requires significant energy [43]. Proteins, as a result, have a significantly higher DIT value than either carbohydrates or fats. The DIT values of proteins, carbohydrates and fats have been reported as 20-30%, 5-10% and 0-3% respectively [44].

As such, even when consuming equal portions of isocaloric meals, the macronutrient composition of that meal will directly affect overall energy-intake, by changing the amount of energy required for meal digestion and absorption [45]. High-protein diets have been shown to cause an increase in resting metabolic rates in humans [46]. Mikkelsen et al. demonstrated that the substitution of 18% of dietary carbohydrate with protein caused an measurable increase in 24-hour energy expenditure [47].

The high DIT of protein, in addition to increasing energy expenditure, is argued by some to play a role in the development of satiety [22]. One proposed mechanism by which this could occur is via an increased oxygen demand during protein digestion and absorption. This increased oxygen demand has been suggested to act in a manner resembling appetite suppression observed at high altitude [48]. Another proposed mechanism is through the increased body temperature resulting from DIT translating in some manner to feelings of satiety [42].

1.1.3.2 Gluconeogenesis

The production of glucose via gluconeogenesis has a notable impact on the development of satiety [49]. Increasing glucose levels within the plasma directly suppresses appetite through physiological systems acting to prevent hypoglycaemia [50]. Consuming a high protein diet, especially in combination with low carbohydrate intake, has been shown to promote gluconeogenesis within the liver [51]. Rats fed solely high-protein diets demonstrate increased levels of key enzymes (glucose-6-phosphate and phosphoenolpyruvate carboxykinase) associated with gluconeogenesis [52]. Human subjects fed high-protein diets exhibit increased levels of gluconeogenesis. This was shown in a recent study by Veldhorst et al., which, however, also found heightened gluconeogenesis associated with high-protein diets did not directly impact the development of satiety [53]. Instead this gluconeogenesis lead to increased production of ketones such as beta-hydroxybutyrate which are now suspected to be playing a role in appetite suppression [54].

1.1.3.1 Satiety hormones

There are a many hormones that are synthesised within the gut that play significant roles in digestion and gastric function [55]. These hormones are often synthesised in response specific dietary intake. For example, glucagon-like peptide-1 (GLP-1) is an incretin which induces the release of insulin in response to dietary glucose [56]. GLP-1 also inhibits gastrointestinal secretion and overall motility, playing an important role in the development of satiety and appetite [57]. Cholecystokinin (CCK) is another hormone synthesised within the gastrointestinal tract which impacts digestive activity, largely by inhibiting gastric emptying, inducing gallbladder contraction and stimulating pancreatic enzyme release [58]. CKK also stimulates the vagal afferent pathway that originates from the gastroduodenal mucosa [59]. Studies have shown that humans have an increased peripheral secretion of both GLP-1 and CKK following even a short-term consumption of a high-protein diet [60, 61]. This suggests that some component of high-protein diets induces satiety through increased synthesis of hormones which play a role in delaying the digestive process and decreasing gastric emptying. Furthermore, it has also been shown that the consumption of high-protein diets in contrast to isocaloric high carbohydrate diets leads to a decrease in concentrations of orexigenic hormones, including ghrelin [62].

1.1.3.2 Reduced sensory appeal

A significant drawback to increasing the protein content of the diet is the association between high levels of dietary protein and increased levels of sensory-specific satiety [32]. High-protein food items are perceived as less palatable than high-carbohydrate or high-fat items, leading to a much faster decline in the pleasure of ongoing food consumption [63]. While this accounts for some of the

appetite inhibiting behaviour induced by high-protein diets, it also leads to a lack of diet regimen compliance [64]. This is especially an issue for manufacturers of foods aimed at health-conscious consumers, as a fine balance must be struck between nutritive value and the desirability of the product itself [65]. By reducing the fat and carbohydrate content of food items while increasing protein content important factors associated with food palatability such as scent, taste, appearance and texture are altered unfavourably [66].

Protein-induced decreases in palatability have been shown in food products ranging from sausage meat [67] to rice snacks [68] to yogurt [34]. Even attempts to overcome this effect through modern food technology, such as specialised high-protein frankfurter sausages created by Siversten and colleagues, have produced disappointing outcomes as the food items have been described as less juicy, harder and more granular than their low-protein counterparts [65] This can lead to rejection of the product in favour of less protein-dense counterparts [69]. Siversten et al. predict that, with the application of modern food making techniques, inclusion of vegetable based oils and the addition of flavouring such as MSG, the protein content of meat-based food items similar to frankfurter sausages could be increased to approximately 40% before palatability becomes an unacceptable factor for consumers [65].

1.1.3.3 Component amino acids

It should be emphasized that proteins are extremely complex molecules, differing in the lengths of amino acid chains and the actual content of individual amino acids. Ingested protein is obviously broken down in the digestion process and, therefore, these shorter amino acid chains as well as free amino acids– at the

organism level – facilitate a host of physiological responses following protein consumption and those responses include satiation [40]. In line with that, in 1956 Mellinkoff et al. put forward the aminostatic hypothesis arguing that an increase in individual amino acid concentration in the plasma promotes satiety, whereas a reduction in amino acid levels leads to increased hunger [70]. Surprisingly, relatively little effort has been made in order to identify which specific amino acids (or their select combinations) might play a critical role in activating these satiety mechanisms.

L-tryptophan is one of the candidate amino acids whose involvement in the satiety process has been proposed by several authors [71-74]. The sections below summarise the current status of our knowledge on the link between L-tryptophan and energy homeostasis as well as present basic facts related to the basic functions and biochemical fate of this amino acid in the organism.

1.2 L-tryptophan

L-tryptophan is one of 9 essential amino acids which cannot be synthesized within the human body [75]. Natural L-tryptophan synthesis only occurs within plants and some microorganisms, and hence, L-tryptophan intake is only supplied through dietary sources, namely the consumption of proteins [76]. The daily recommended intake of L-tryptophan is estimated to be 5 mg kg^{-1} day^{-1} [75].

L-tryptophan serves a variety of important functions. In addition to the standard role amino acids play in protein biosynthesis, L-tryptophan acts as a biochemical precursor for a number of substances in both the peripheral and central nervous

systems (CNS). These include serotonin, kynurenine, melatonin and niacin, which have been shown to play a role in a myriad of different physiological processes, including learning, memory, sleep cycles, circadian rhythm, mood, sexual activity, social behaviours, digestive processes and the neurological regulation of appetite.

After consumption and digestion of proteins, L-tryptophan and other neutral amino acids are absorbed through the apical membrane of intestinal enterocytes via the B^0AT1 epithelial amino acid transport system [77]. L-tryptophan has the second lowest affinity for this transport system of all neutral amino acids. The basolateral aromatic amino acid transporter, TAT1, transports L-tryptophan and other neutral amino acids through the basal membrane of enterocytes, into peripheral cells [77]. Ultimately, L-tryptophan enters the circulatory system. However, in contrast to other circulating amino acids, the majority of L-tryptophan is not transported as a free amino acid: McMenamy and Oncley determined that the indole ring on L-tryptophan attaches to a single binding site on serum albumin via Van der Waals' forces [78]. Thus, a large proportion of L-tryptophan within the circulatory system will travel while bound with low affinity to serum albumin, a unique trait among amino acids [79]. As this single albumin binding site is competitively targeted by free fatty acids [80] and a variety of drugs (e.g., salicylates, indomethacin, probenecid and chlorpromazine) [81], a certain proportion of circulatory pool of L-tryptophan remains unbound. Free circulating L-tryptophan can be later stored in the form of membrane lecithins and tissue proteins [82]. These represent metabolic pools of L-tryptophan which can be accessed if sufficient amounts of dietary L-tryptophan are not consumed for a limited time period.

Modifications of dietary L-tryptophan intake have been shown to alter the levels of circulating L-tryptophan [83]. Differences in overall protein intake have a notable effect on the plasma concentrations of all amino acids, including L-tryptophan [40]. For example, Fernstom et al demonstrated significant differences in L-tryptophan plasma levels in people consuming a total of 0 g, 75 g or 150 g of egg-based protein each day. These diets produced pronounced differences within three days of a consumption period, altering diurnal levels of circulating amino acids [84]. Furthermore, episodic (single-event) consumption of specific L-tryptophan-enriched proteins has been shown to drive large transient increases in L-tryptophan plasma levels in people [85]. A long-term feeding study in rats, conducted by Feurté et al., showed that animals fed a diet high in alpha-lactalbumin, a protein rich in L-tryptophan, had a 40% higher ratio of plasma L-tryptophan to other amino acids [86]. Direct supplementation of the diet with free L-tryptophan also proportionally increases the concentration of plasma L-tryptophan. Green et al. drew a direct linear relationship between oral loads of free L-tryptophan and circulating L-tryptophan levels [87]. In a similar manner, the intragastric administration of L-tryptophan to animals has been demonstrated to increase L-tryptophan concentrations in plasma [88, 89].

Overall, this large body of research has led to a consensus that dietary fluctuations in L-tryptophan content have a profound influence on the actual concentration of this amino acid in the plasma [90], and this relationship is apparent regardless of whether L-tryptophan is given as a free amino acid or via consumption of high-tryptophan complex protein meals.

1.2.1 Peripheral utilisation of L-tryptophan

Once taken up by the body, L-tryptophan is subject to a number of metabolic fates, both peripherally and in the CNS. Peripheral utilisation of L-tryptophan accounts for the vast majority of dietary L-tryptophan [91], which enters three key metabolic pathways: protein synthesis, the kynurenine pathway and the serotonin pathway.

1.2.1.1 Protein synthesis

Despite the fact that the dietary sources of L-tryptophan are the only means for this amino acid to become part of protein synthesis in mammals, there is quite a bit of uncertainty within the scientific literature as to what proportion of dietary L-tryptophan is later incorporated into protein synthesis [91]. A comprehensive review of L-tryptophan studies by J. C. Peters held that a vast majority of dietary L-tryptophan (approximately 90%) was utilised in the formation of proteins [92]. More recently, using in vitro enzyme determination to study L-tryptophan's various metabolic pathways in a number of mammalian species, Allegri et al. argued that protein synthesis accounted for approximately 30% of dietary L-tryptophan [93]. While this exact proportion is debated, it is known that if plasma L-tryptophan levels are low, L-tryptophan acts as the rate-limiting amino acid for the synthesis of some proteins [94]. Sidransky and colleagues compared the rate of hepatic protein synthesis in mice fed a balanced mixture of amino acids versus fasting mice given solely dietary L-tryptophan. They found that L-tryptophan consumption enhanced hepatic protein synthesis to a rate similar to mice fed a balanced mixture of amino acids. Other single amino acids, such as isoleucine, threonine and methionine, did not produce this effect [95].

1.2.1.2 Kynurenine

The kynurenine pathway represents another significant metabolic fate for peripheral L-tryptophan [91]. This pathway is likely the largest source of L-tryptophan catabolism [92]. It should be noted that this pathway is the main system by which L-tryptophan plasma concentrations are regulated. The first step of the kynurenine pathway involves the oxidisation of L-tryptophan by tryptophan 2,3-dioxygenase (TDO), an enzyme localised almost entirely within the liver [96]. This initial, rate-limiting step, first discovered by Kotake and Masayama is responsible for removing excess unbound L-tryptophan from the circulation [97]. In fact, within a few hours of a large intake of dietary L-tryptophan, expression and activity of TDO can be increased four- to ten-fold [96]. While hepatic TDO is the only enzyme which acts solely to oxidise L-tryptophan in this manner, it is not the only enzyme to have this function. Another is indoleamine 2,3-dioxygenase (IDO), which is expressed in a range of peripheral tissues, particularly in the small intestine. Yamazaki et al. demonstrated IDO oxidises a wider range of substrates than TDO, including 5-HT, 5-HTP, tryptamine and L-tryptophan [98]. IDO is equally capable of oxidising L-tryptophan during the first step of the kynurenine pathway.

The peripheral kynurenine pathway converts L-tryptophan into a wide variety of metabolites, with a range of functions [99]. Primarily, L-tryptophan is converted into quinolinic acid and nicotinamide [100], while smaller side chains of the pathway generate kynurenic acid and xanthurenic acid [101]. These metabolites are notable in inducing a variety neuroprotective and neurotoxic effects in the

CNS, associated with pathologies such as cataracts, dementia and brain injuries due to ischemia [102]. Nicotinamide and nicotinic acid are both metabolites of the kynurenine pathway in the liver. These substances act as precursors for nicotinamide adenine dinucleotide (NAD^+), a ubiquitous enzyme that plays a critical role in many redox reactions [103]. Another product of the kynurenine pathway is picolonic acid, which acts as a chelating agent, in addition to aiding the absorption of dietary zinc [104].

Kynurenine metabolites are increasingly thought to regulate gastrointestinal functions, and hence impact appetite, although the exact function of most metabolites in this regard is still unknown [91]. Kynurenic acid in particular has been found to regulate gastrointestinal motility by acting as an antagonist of N-methyl-d-aspartate (NMDA) glutamate receptors. This was well demonstrated by Kaszaki et al., who administered mechanical colonic obstructions to dogs before infusing experimental groups with 50 mg kg^{-1} of kynurenic acid. Animals administered kynurenic acid demonstrated a major reduction in the motility response of obstruction-induced colonic contractions [105]. By antagonizing NMDA receptors, kynurenic acid also modulates the mechanosensitivity of both tension and mucosal vagal afferents within the gastrointestinal tract [106].

L-kynurenine is an intermediate for all kynurenine metabolites within the kynurenine pathway. This metabolite of L-tryptophan can itself enter and be transported in circulation. L-kynurenine in circulation can be transported across the BBB by a neutral amino acid carrier [106]. Peripherally formed L-kynurenine

accounts for approximately 60% of the L-kynurenine involved in the kynurenine pathway of the CNS [107].

1.2.1.3 Serotonin

The remaining key metabolic fate for peripheral L-tryptophan is the serotonin pathway, accounting for 1-2% of overall dietary L-tryptophan [104]. L-tryptophan acts as a biochemical precursor for the formation of serotonin. Peripheral serotonin accounts for approximately 95% of total serotonin content within the body, the vast majority of which is located in the gut [108]. Over 90% of this serotonin can be found in the intestinal enterochromaffin cells [109]. Much of the remainder is contained within serotonergic neurons of the enteric nervous system [110].

L-tryptophan is converted into serotonin in two steps. The first, rate-limiting step occurs when L-tryptophan undergoes hydroxylation via tryptophan hydroxylase (TPH). The product of this hydroxylation, 5-hydroxytryptophan, undergoes decarboxylation via aromatic acid decarboxylase, which leads to the formation of serotonin [83]. While the latter enzyme is ubiquitous, expression of TPH is localised to pinealocytes of the pineal gland, neuroendocrine epithelial cells of the lungs, enterochromaffin cells of the intestines, mononuclear leukocytes and neurons [108].

Until recently, it has been thought that TPH was encoded by a single gene. In 2003, Côté et al. discovered that there are in fact two isoforms of tryptophan hydroxylase, TPH1 and TPH2, encoded by two distinct genes [111]. Côté and colleagues produced a tph1 knock-out mouse which at the time was intended to

inhibit all serotonin synthesis. Instead, by knocking out tph1 without affecting the function of the then-unknown tph2 gene, they developed a mouse model displaying typical neuronal serotonin activity while being devoid of peripheral serotonin [111].

Since this discovery, the localisation and functions of TPH1 and TPH2 have been well defined [112, 113]. TPH1 is localised to peripheral tissues, including pinealocytes and neuroendocrine lung cells [111]. Most significantly, TPH1 is strongly expressed in the enterochromaffin cells of the intestine, in which the vast majority of serotonin is synthesised and stored [91]. TPH2 is present solely in neurons. This includes the small proportion of serotonergic neurons in the enteric nervous system, which contribute a much smaller proportion of peripheral serotonin [114].

Enterochromaffin cells are one of 14 kinds of specialised enteroendocrine cells within the intestines, which synthesize serotonin from L-tryptophan, storing it in intracellular vesicles [115]. These cells play a critical role in sensing and signalling both mechanical and chemical stimuli in the intestines [116]. Changes to luminal pressure due to the movement of food boluses through the intestines directly activate enterochromaffin cells [117]. Additionally, enterochromaffin cells are activated by a variety of chemical stimuli including changes in pH, the presence of bile acid and dietary glucose [115]. Activation of enterochromaffin cells induces the extracellular release of serotonin, which has two important physiological effects. Firstly, this serotonin acts to stimulate intrinsic primary afferent neurons belonging to the enteric nervous system [108]. In doing so,

gastrointestinal motor activity and secretory processes critically important to digestion are coordinated, both intrinsically via the enteric nervous system, and extrinsically [118]. Secondly, serotonin originating from enterochromaffin cells directly effects the central nervous system through the activation of vagal afferents projecting to the hindbrain [119].

The serotonergic neurons of the enteric nervous system represent a smaller source of peripheral serotonin synthesis. Small pockets of interneurons within the enteric nervous system not only receive serotonin signal input, but are themselves capable of synthesising and releasing serotonin externally [91].

1.2.2 Uptake of L-tryptophan into the central nervous system

In addition to peripheral L-tryptophan utilisation, L-tryptophan functions as a biochemical precursor for a number of metabolic pathways within the CNS, many of which produce neuroactive substances closely associated with the regulation of food intake. L-tryptophan within the CNS is sourced from blood plasma, and must cross the blood-brain barrier.

Both the plasma concentrations of L-tryptophan and the plasma concentrations of a number of other large, neutral amino acids (LNAAs), such as phenylalanine, leucine, tyrocine, isoleucine and valine, are directly responsible for the rate of uptake of L-tryptophan into the brain [120]. This process is mediated by the transport mechanism which enables these substances to cross the blood-brain barrier (BBB), the large neutral amino acid transporter (LAT1) [121]. LAT1 is a membrane transport protein highly expressed within the capillary endothelia of the BBB [122]. LAT1 transport of LNAAs is a process that is both saturable and

competitive. The affinity of LAT1 is similar across all associated LNAAs, and competition for transport is based largely on the total LNAA plasma concentrations [123]. Thus, the rate of uptake of L-tryptophan into the brain can be increased with the increase of total L-tryptophan plasma concentrations, or increased by reducing plasma concentrations of all other LNAAs competing for transport [121].

Various aspects of dietary intake can alter the rate at which L-tryptophan is transported across the BBB by LAT1 [124]. Simply increasing the proportion of dietary L-tryptophan intake increases L-tryptophan BBB transport [125]. Choi et al. demonstrated that chronic intake of different proteins with various L-tryptophan content, including wheat gluten, soy protein, casein, or α-lactalbumin, significantly altered L-tryptophan levels in the central nervous system of rats [126]. High-carbohydrate diets have also been shown to significantly enhance L-tryptophan BBB transport. Madras et al. demonstrated that the consumption of a carbohydrate rich diet by young rats acted to increase the transport of L-tryptophan into the CNS [127]. This effect has been attributed to an increase in insulin production. Biolo et al. conducted a series of biopsies on fatigued human leg muscles following infusions of insulin [128]. Increased insulin levels were found to promote the movement of amino acids from the circulatory system into the body tissues, especially muscle fibres, for use in protein synthesis. In doing so, insulin significantly reduces the circulatory concentrations of all LNAAs except L-tryptophan, as the large proportion of L-tryptophan bound to serum albumin is not removed from circulation [129]. Lipsett and colleagues established this through a study which carefully measured circulating L-tryptophan levels in

humans following glucose ingestion [130]. Participants consumed various quantities of a ligand competitive for L-tryptophan's albumin binding site, and hence developed differing ratios of albumin-bound to free L-tryptophan. Following the actions of insulin, only the albumin-bound pool of L-tryptophan remained in the plasma [130].

This albumin-bound L-tryptophan can be accessed by LAT1 [131]. A series of in vivo experiments on rat and rabbit brains by Pardridge and Fierer aiming to determine the disassociation constant of albumin binding to free L-tryptophan ultimately showed that L-tryptophan crossing the BBB was drawn not only from free L-tryptophan but also from the larger pool of albumin-bound L-tryptophan via an enhanced disassociation mechanism present at the BBB [132]. The action of insulin following carbohydrate intake increases the ratio of L-tryptophan in circulation to all other LNAAs [133]. This significantly increases the quantity of L-tryptophan transported across the BBB by LAT [127]. In contrast, a diet rich in proteins without high L-tryptophan content can boost plasma concentrations of alternative LNAAs, decreasing the ratio of L-tryptophan to other LNAAs, reducing L-tryptophan BBB transport [85].

After crossing the blood-brain barrier, L-tryptophan enters the extracellular fluid of the CNS, where it is taken up by brain cells [131]. The rate of circulatory L-tryptophan transport across the blood-brain barrier by LAT1 controls the levels of L-tryptophan within the brain [134]. The availability of L-tryptophan in the brain to act as a biochemical precursor has been shown to directly drive brain L-tryptophan metabolite synthesis, such as the synthesis of serotonin [135].

1.2.3 Central nervous system utilization of L-tryptophan

There are a number of metabolic fates for L-tryptophan within the CNS that can impact the regulation of appetite and other important biological functions. As in the periphery, one of the main utilizations of CNS L-tryptophan is for general protein synthesis [83], however, there are three other significant metabolic pathways. These are the kynurenine pathway, the serotonin pathway and the melatonin pathway.

1.2.3.1 Kynurenine

By quantity, the kynurenine metabolic pathway within the CNS represents the largest utilization of CNS L-tryptophan after protein formation, accounting for over 95% of remaining CNS L-tryptophan [124]. The first steps of this pathway are active in most cell types within the CNS, including neurons, astrocytes and microglia, as well as infiltrating macrophages [136]. However, a recent in vivo study of rat brains by Amori et al. in which ^{3}H-kynurenine was traced, demonstrated that the various kynurenine metabolites are only metabolised in specific cell types [137].

The first step of the kynurenine pathway in the CNS, similar to what occurs in the periphery, involves the oxidation of L-tryptophan. Both rate-limiting enzymes responsible for this oxidation (the L-tryptophan specific tryptophan 2,3-dioxygenase and the less specific indoleamine 2,3-dioxygenase) are present within most CNS cells [96]. L-kynurenine is an intermediate in the kynurenine pathway. Only 40% of the L-kynurenine utilised by the CNS is synthesised locally. The

remainder is drawn from circulation via the BBB, hence, peripheral L-kynurenine concentrations can have a direct impact on the activity of CNS kynurenine metabolism [107].

Some significant neuroactive metabolites of the this metabolic pathway include kynurenic acid (an antiexcitotoxic) [138], picolinic acid (a neuroprotectant and immune modulator) [136], and quinolinic acid (a potent neurotoxin) [139]. Many of the functions of these metabolites are still under investigation, and the actions they have on the regulation of appetite and other biological processes are unknown [124]. Currently most research efforts are focused on the role many of these metabolites play in of the pathogenesis of various diseases, including several psychiatric disorders, Huntington's disease, Tourette syndrome as well as peripheral conditions such as cardiovascular disease [140].

1.2.3.2 Serotonin

A second L-tryptophan metabolite within the CNS that has a vital role in many physiological processes is the neurotransmitter serotonin. CNS serotonin is almost exclusively produced in the raphe nuclei, discrete clusters of neurons, forming the medial portion of the reticular formation in the brain stem [141]. The neurons comprising the raphe nuclei are heterogeneous, with a variety of morphologies and characteristics, and these neurons project to various regions of the brain [142]. Many of these neurons are serotogenic, and it is within these nuclei that L-tryptophan is converted into serotonin in a two-step reaction [143]. The raphe nuclei project extensively throughout the CNS. The median and dorsal raphe nuclei project throughout much of the forebrain, including the cortex, amygdala, hippocampus and the hypothalamus [142]. The caudal raphe nuclei project to the

spinal cord and brainstem, including the nucleus of the solitary tract [142]. These regions of the brain, especially the hypothalamus, are essential to many processes involved in the regulation of energy balance and appetite as well as myriad other physiological functions [144]. These brain regions all express a variety of different serotonin receptors, and serotonin input from the raphe nuclei can significantly alter the activity of these structures [145-147]. Furthermore, serotonin appears to functionally interact within wider neuropeptidergic systems involved in feeding control, including those associated (directly or indirectly – via multisynaptic pathways) with the leptin and ghrelin, as well as insulin and orexin [148].

It was recognised as early as the 1970's that dietary manipulations of CNS L-tryptophan concentrations directly alter the rate at which serotonin is synthesised [90, 134, 149, 150]. There is convincing evidence that alteration to serotonin synthesis and concentration within the CNS can have a significant impact on overall food intake and many other ingestive behaviours, by altering the activity of neurological structures associated with the coordination of, and response to, peripheral and metabolic signals [146].

1.2.3.3 Melatonin

Melatonin synthesis is another notable metabolic fate for L-tryptophan. Melatonin is a neurohormone produced primarily within the pineal gland, synthesised from L-tryptophan in a four step reaction using serotonin as an intermediary [151]. Unlike prior CNS metabolic pathways, L-tryptophan involved in the formation of melatonin does not need to cross the BBB, and is instead drawn directly from circulatory L-tryptophan [152].

One of main functions of melatonin is to entrain circadian rhythms in a number of key biological processes, including the sleep-wake cycle and appetite [153]. Melatonin synthesis in the pineal gland is largely dependent on the day-night cycle [151]. Visual detection of blue light, particularly around 460-480 nm, acts to inhibit the function of aralkylamine N-acetyltransferase, a key enzyme within the melatonin metabolic pathway [154]. Melatonin production in the pineal gland, as a result, typically occurs from dusk until dawn.

Dietary manipulations of plasma L-tryptophan levels have been demonstrated to be capable of inducing increased pineal melatonin formation even during the light-phase of pineal function [155]. Broiler chickens orally administered 150 mg^{-1} kg^{-1} of free L-tryptophan during pineal light-phase by Zeman et al. demonstrated enhanced melatonin production three hours following administration, however, this production was limited and did not significantly affect melatonin levels in the following dark-phase [156]. Pineal melatonin can enter into circulation, where it is readily drawn across the BBB and into the CNS [157]. Alternatively, melatonin can be released directly into the third ventricle of the CNS via the pineal recess [158]. CNS melatonin has a wide array of physiological functions [159], but the role melatonin plays in the regulation of appetite is becoming increasingly apparent [151]. Animal models have shown melatonin acts to inhibit food intake. By administering small and large doses of melatonin via water, Piccinetti and colleagues demonstrated that increased melatonin reduced food intake in zebrafish [160]. This result was attributed to stimulation of molecules that act as satiety

signals such as leptin, as well as a notable reduction in orexigenic signals, especially ghrelin [161].

1.3 L-tryptophan as a mediator of satiety

A growing body of research supports the hypothesis that supplementary L-tryptophan causes anorexigenic effects. Supplementing the diet with as little as 5% additional L-tryptophan over the course of several days decreases overall food intake to a degree that promotes body weight reduction [72]. Ng and Anderson demonstrated that direct intragastric infusions of L-tryptophan caused a decrease in episodic chow intake [73]. In line with those findings are the results obtained in a comprehensive study by Jordi et al. who administered individually via intragastric gavage 20 amino acids (including L-tryptophan) in food-deprived rats just prior to re-feeding [162]. They found that L-tryptophan generated a ca. 25% reduction in the amount of consumed chow, which was the fourth largest anorexigenic effect observed for the amino acids used in the experiments. While the effect was statistically significant when compared to the vehicle ($p=0.03$), one of the key problems in that project was testing the effects a very large number of amino acids, which precipitated the need of correcting for multiple comparisons. That additional correction allowed the authors to conclude that there was only a trend suggesting a decrease, however, they did acknowledge the similarity between their result and that of Ng and Anderson [73].

Jordi et al. then went on to decipher neuronal mechanisms underlying the anorexigenic effects of amino acids on food intake by using c-Fos immunoreactivity as a marker of brain regional activation. Their analysis suggested that the vagal input into the brain stem plays a critical role into

stimulating pathways that promote satiety. In fact, they found – by using the amino acids that produced a statistically significant hypophagia after correction for multiple comparisons – that the dorsal vagal complex (DVC, especially the nucleus of the solitary tract (NTS) and area postrema (AP) as parts of the DVC) appears to serve as a common neuroanatomical platform in relaying amino acid-derived satiation [162]. Most likely, these DVC neurons send projections to other brain sites, including the hypothalamus, where they activate specific neuronal populations involved in generating termination of food intake. Unfortunately, very little is known about neuropeptidergic systems mediating satiety that are activated by L-tryptophan.

1.4. Overarching goal and specific aims of the thesis: Closing the knowledge gap on ingested L-tryptophan and hypophagia

Only very initial steps in defining L-tryptophan as a hypophagic molecule have been undertaken: it has been shown that this amino acid decreases intake of standard laboratory chow and this effect is most likely mediated via pathways that are initiated at the brain stem (more precisely, DVC) level. **Therefore, the current project was devised in order to provide further characterisation of L-tryptophan's effects on feeding and to offer a glimpse into neuropeptidergic mechanisms that mediate L-tryptophan-derived hypophagia**. Laboratory mice were used throughout the studies.

The **first aim** of this project focused on defining the effect of increased dietary L-tryptophan levels on deprivation-induced intake of complex milk formula diets that differed in composition (skim versus regular). In order to establish whether amino acid-derived changes in palatability might have been the cause, the effect

of L-tryptophan supplementation on consumption of tryptophan-enhanced water was also examined.

The **second aim** of this thesis defined whether an intragastric preload of L-tryptophan affected intake driven by various stimuli, from energy deprivation to palatability. In this context, it should be noted that research on gastric preloads of L-tryptophan and appetite has revolved thus far exclusively around the issue of energy deprivation. It did not take into account whether this amino acid was capable of reducing consumption evoked by other processes, especially rewarding characteristics of tastants. Thus, I investigated whether L-tryptophan-evoked hypophagia is associated with the intake of “bland” chow or whether it extends upon sweet palatable tastants containing sucrose or a non-carbohydrate sweetener, saccharin.

Finally, in the **third aim**, I sought to determine whether anorexigenic effects of L-tryptophan are mediated by a satiety-related neurohormone, oxytocin. Oxytocin is a nonapeptide synthesised in the hypothalamus and released within the CNS as well as into the periphery (the latter occurs via posterior pituitary). One of the unique neuroanatomical features of oxytocin is that neurons producing it receive powerful input from the brain stem, including from the DVC. In fact, oxytocin is considered the final CNS component of vagally mediated GI signalling. As such, it is released upon gastric distension, an increase in plasma osmolality as well as upon the increase in the concentration of gut hormones in the plasma. Oxytocin decreases food intake driven by energy and it is also effective in reducing consumption for reward; antagonists of the oxytocin receptor cause an opposite effect. Hence, the OT system appears to be a very plausible candidate as a molecular component of anorexigenic pathways activated by L-tryptophan.

Chapter 2: Materials and Methods

2.1 Animals

Adult male C57BL/6 mice (AgResearch, Hamilton, NZ), body weight range 29-33 g, were housed in individually in Plexiglas cages in a temperature controlled room (21-22°C) under a 12:12 light/dark cycle (lights on at 0600 h). Mice had ad libitum access to tap water and standard laboratory chow (Purina) unless stated otherwise.

All the experiments described herein have received prior approval from the institutional animal ethics committee (University of Waikato AEC, Hamilton, NZ).

2.2 Methods

2.2.1 Effect of dietary L-tryptophan enrichment on deprivation-induced intake of palatable and nutritionally adequate liquid diets (milk formulas)

In order to prevent neophobia, several days prior to the beginning of experimental trials, mice were acclimatised to the flavour of two nutritionally adequate and palatable milk formulas (goat WPC+ and goat skim formula (see Table 1.)) via short-term, episodic exposures (at least 2x30 minutes).

Table 1: Composition (%) of goat WPC+ and goat skim milk formulas

	Protein	Lactose	Fat	Ash	Moisture
Goat WPC +	39.5	52.5	1.4	3.6	3
Goat Skim	38.3	48.4	1	9.3	3

On experimental days, the animals were deprived of chow overnight. At 1200h the following day, the mice (n = 7/group) were given 2-hour access to a single bottle containing a goat WPC+ and goat skim formula (DGC, Hamilton, NZ). Chow and water were not present in the cage during the time of milk presentation. The amount of ingested formula was measured (in grams) after 2 hours of consumption. The 2-h consumption time was sufficient for all the animals to finish their deprivation-induced consummatory activity.

The formulas were enriched with L-tryptophan beyond levels provided in the diets to meet basic amino acid needs. Hence, goat WPC+ and goat skim milk formulas contained an additional amount of L-tryptophan in the amount exceeding base line levels by 0 (control), 0.036, 0.12, 0.36 or 1.2 g of L-tryptophan per liter.

The amount of consumed diet was assessed in grams per kg of body weight. Data were averaged per experimental group. The effect of L-tryptophan supplementation on formula intake was determined with one-way ANOVA followed by Fisher's post-hoc analysis (significant when $P<0.05$).

2.2.2 Effect of enrichment of water with L-tryptophan on deprivation-induced drinking behaviour

In order to determine whether adding L-tryptophan to a "neutral" ingestant affects its palatability and whether the effects seen with liquids diets are not driven by

thirst-related processes, we determined whether enrichment of water with L-tryptophan affects drinking. Mice were deprived of water overnight (chow was available). At 1200h the following day, the animals (n = 7) were given L-tryptophan-enriched water. After 2 hours, when all animals finished consumption, the amount of ingested fluid was measured (in grams) and converted to grams per kg of body weight. Tap water contained 0 (control), 0.036, 0.12, 0.36 or 1.2 g of L-tryptophan per liter. The effect of L-tryptophan supplementation on water intake was determined with one-way ANOVA followed by Fisher's post-hoc analysis (significant when $P<0.05$).

2.2.3 Effect of intragastric preload of L-tryptophan on deprivation-induced chow intake

Mice were deprived of chow overnight (water was available at all times). At 1100h the following morning, they received via intragastric gavage an infusion of either 0, 600, 1200 or 1800 mg/kg b. wt. of L-tryptophan (n = 7/group) in 200 μL of water via oral gavage. Chow was returned to cages 20 minutes later and food intake was measured 1, 2, 4 and 24 h after re-feeding. The effect of L-tryptophan preload on chow intake was determined with one-way ANOVA followed by Fisher's post-hoc analysis (significant when $P<0.05$).

2.2.4 Effect of intragastric preload of L-tryptophan on saccharin solution intake in non-deprived mice

In order to prevent neophobia, several days prior to the beginning of experimental trials, mice were acclimatised to the flavour of 0.1% saccharin solution via short-term, episodic exposures (at least 2x30 minutes).

On the experimental day, at 1100h, the animals received via intragastric gavage an infusion of either 0, 300, 600 or 1200 mg/kg b. wt. of L-tryptophan (n = 7/group) in 200 μL of water via oral gavage. Chow and tap water were removed from the cages of treated animals and the formula was given in a single bottle 20 minutes later. Formula intake was measured after 1 h of exposure to the tastant. The effect of L-tryptophan preload on formula intake was determined with one-way ANOVA followed by Fisher's post-hoc analysis (significant when $P<0.05$).

2.2.5 Effect of intragastric preload of L-tryptophan on sucrose solution intake in non-deprived mice

Mice were acclimatised to 10% saccharin solution similarly to what has been described above for sucrose (section 2.2.4). On the experimental day, at 1100h, the animals received via intragastric gavage an infusion of either 0, 1200 or 1800 mg/kg b. wt. of L-tryptophan (n = 7/group) in 200 μL of water via oral gavage. Chow and tap water were removed from the cages of treated animals and 10% sucrose solution was given in a single bottle 20 minutes later. Sucrose solution intake was measured after 1 h of exposure to the tastant. The effect of L-tryptophan preload on sucrose intake was determined with one-way ANOVA followed by Fisher's post-hoc analysis (significant when $P<0.05$).

2.2.6 Effect of intragastric preload of L-tryptophan on palatable and nutritionally adequate milk intake in non-deprived mice

Mice were acclimatised to the milk formula similarly to what has been described above for sucrose (section 2.2.4). On the experimental day, at 1100h, the animals received via intragastric gavage an infusion of either 0, 600 or 1200 mg/kg b. wt. of L-tryptophan (n = 7/group) in 200 µL of water via oral gavage. Chow and tap water were removed from the cages of treated animals and 0.1% saccharin solution was given in a single bottle 20 minutes later. Formula intake was measured after 1 h of exposure to the tastant. The effect of L-tryptophan preload on milk formula intake was determined with one-way ANOVA followed by Fisher's post-hoc analysis (significant when $P<0.05$).

2.2.7 Effect of oxytocin receptor antagonist pretreatment on the ability of intragastric L-tryptophan to suppress deprivation-induced chow intake.

The mice were food-deprived overnight. Fifteen minutes prior to refeeding (at 10:00), they received an IP injection of an OT receptor antagonist, L-368,899 at 0.03, 0.1 and 0.3 mg/kg b. wt. (or saline vehicle). Five minutes later, the 600-mg dose of L-tryptophan (or vehicle) was administered intragastrically (via gavage, in 200µL of water. Chow was returned to the hoppers and consumption was measured 2 hours later. Data were averaged per experimental group (n=8-9/group) and expressed as means +/- SEM. The effect of L-tryptophan on chow

consumption was established using Student's t-test. The effect of the OT receptor antagonist on the ability of L-tryptophan to induce hypophagia was established with one-way ANOVA followed by Fisher's post-hoc test (significant when $P<0.05$).

Chapter 3: Results

3.1 Results

3.1.1 Effect of dietary L-tryptophan enrichment on deprivation-induced intake of palatable and nutritionally adequate liquid diets (milk formulas)

The addition of 0.036, 0.12 or 0.36 g/L of L-tryptophan to palatable liquid diets demonstrated no significant difference in consumption by energy-deprived animals over a 2 hour period. On the other hand, addition of the highest amount of L-tryptophan (1.2 g/L) to the milk formulations caused a modest decrease in consumption regardless which liquid diet was used (Fig. 1 and 2).

3.1.2 Effect of enrichment of water with L-tryptophan on deprivation-induced drinking behaviour

Overnight water-deprived animals given water enriched with L-tryptophan displayed similarly vigorous drinking behaviour regardless of the concentration of the amino acid (Fig. 3).

3.1.3 Effect of intragastric preload of L-tryptophan on deprivation-induced chow intake

While a dose of 600 mg/kg b. wt. was not sufficient to induce an effect, the intragastric preload of 1200 and 1800 mg/kg b. wt. of L-tryptophan in energy-deprived mice was found to cause a moderate decrease in chow consumption.

This effect was observed not only during the first hour of energy-based chow intake, but persisted for multiple feedings over the course of 24 hours (Fig. 4).

3.1.4 Effect of intragastric preload of L-tryptophan on saccharin solution intake in non-deprived mice

The intragastric preload of 600 and 1200 mg/kg b. wt. of L-tryptophan in non-deprived animals was found to moderately decrease the consumption of 0.1% saccharin solution during the first hour of feeding (Fig. 5).

3.1.5 Effect of intragastric preload of L-tryptophan on sucrose solution intake in non-deprived mice

Non-deprived animals administered an intragastric preload of L-tryptophan displayed similarly vigorous consumption of 10% sucrose solution, regardless of L-tryptophan dosage. No effects were seen even at the highest dosage (1800 mg/kg b. wt.) used in these studies (Fig. 6).

3.1.6 Effect of intragastric preload of L-tryptophan on palatable and nutritionally adequate milk intake in non-deprived mice

An intragastric preload of 600 mg/kg b. wt. of L-tryptophan in non-deprived animals was not sufficient to induce a significant effect on the consumption of skim goat milk formula. However, a dosage of 1200 mg/kg b. wt. of L-tryptophan was found to modestly decrease the consumption of this palatable liquid diet during the first hour of feeding (Fig.7).

3.1.7 Effect of oxytocin receptor antagonist pretreatment on the ability of intragastric L-tryptophan to suppress deprivation-induced chow intake.

The intragastric administration of 600 mg/kg b. wt. L-tryptophan was found to significantly decrease the consumption of standard chow. The IP administration of 0.03 mg/kg b. wt. OTA had no significant impact on this effect, however, the IP administration of 0.1 and 0.3 mg/kg b. wt. of OTA had a significant impact on the anorectic effect of intragastric L-tryptophan, returning chow consumption to near baseline levels (Fig. 8).

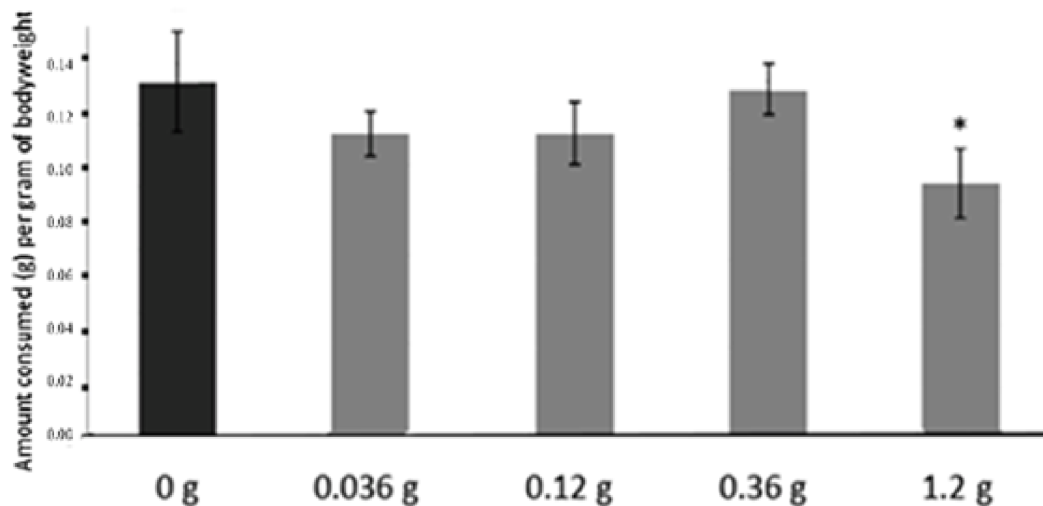

Figure 1: L-tryptophan added to the WBC+ milk formulation in energy-deprived mice decreases milk consumption at 1.2 g/L. Amounts of L-tryptophan are defined on the X axis (per 1 l of fluid). * $P < 0.05$

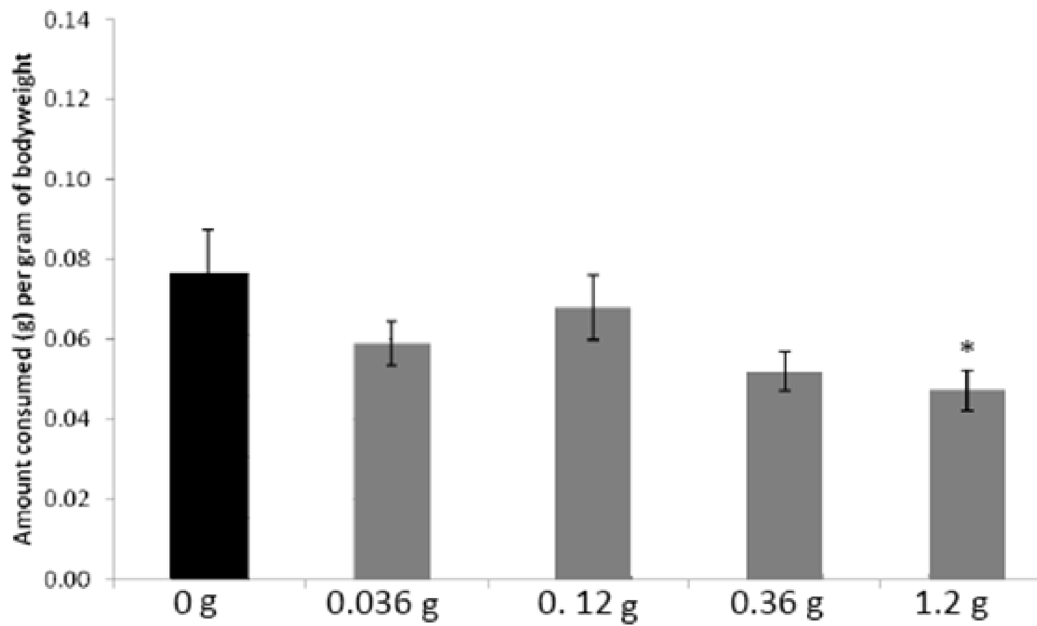

Figure 2: L-tryptophan added to the skim milk formulation in energy-deprived mice decreases milk consumption at 1.2 g/L. Amounts of L-tryptophan are defined on the X axis (per 1 l of fluid). * P < 0.05

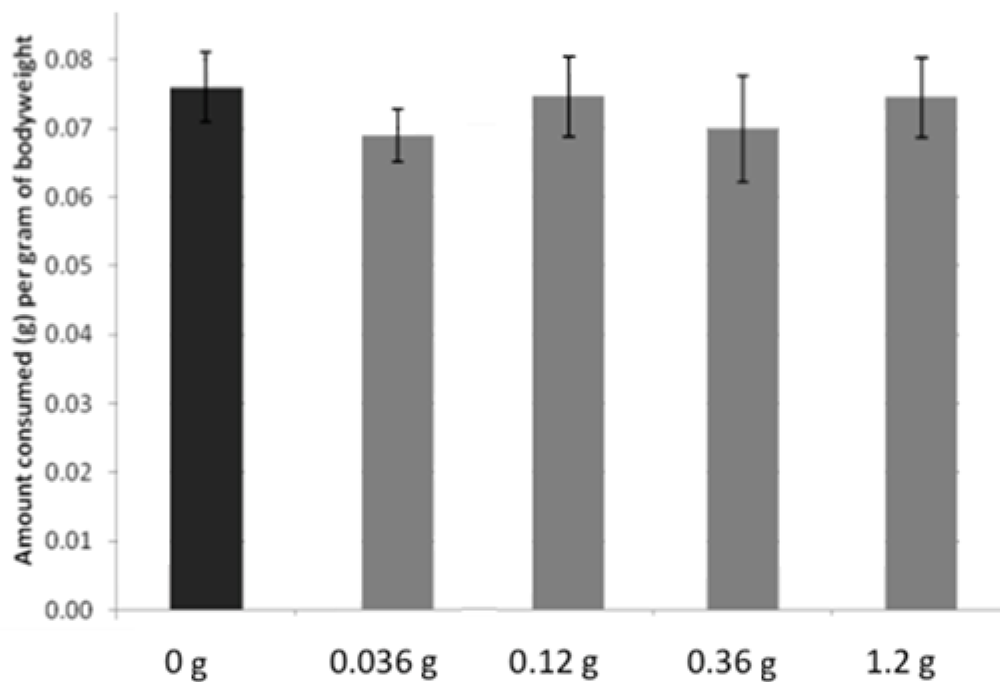

Figure 3: L-tryptophan added to drinking water in water-deprived mice did not affect water consumption. Amounts of L-tryptophan are defined on the X axis (per 1 l of fluid). * P < 0.05

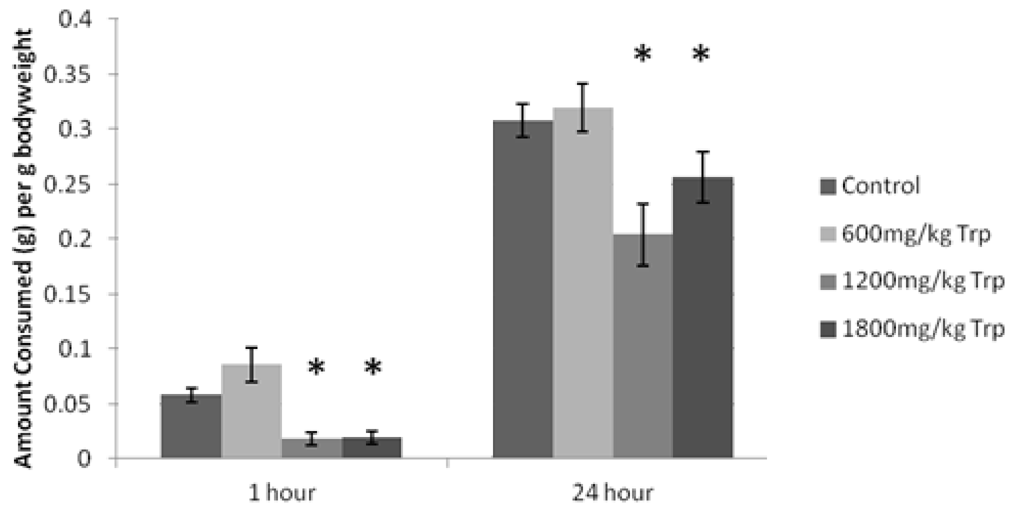

Figure 4: Intragastric preload of L-tryptophan in food-deprived mice reduces chow consumption at 1200 and 1800 mg/kg b. wt., 1 and 24 hours after administration. 24-hour energy deprived mice received infusions of 0, 600, 1200 or 1800 mg/kg b. wt. of L-tryptophan within 200 μL of saline. * $P < 0.05$

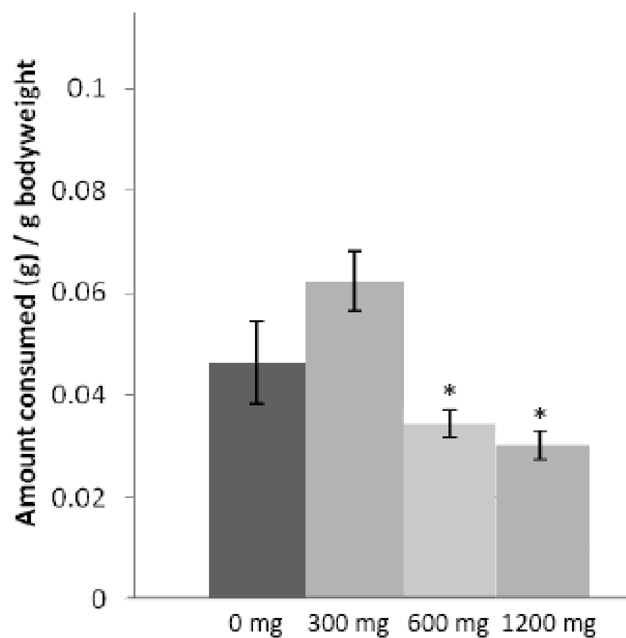

Figure 5: Intragastric preload of L-tryptophan in non-deprived mice reduces 0.1% saccharine consumption at 600 and 1200 mg/kg b. wt. 1 hour after administration. Non-deprived mice received infusions of 0, 300, 600, or 1200 mg/kg b. wt. of L-tryptophan within 200 μL of saline. * $P < 0.05$

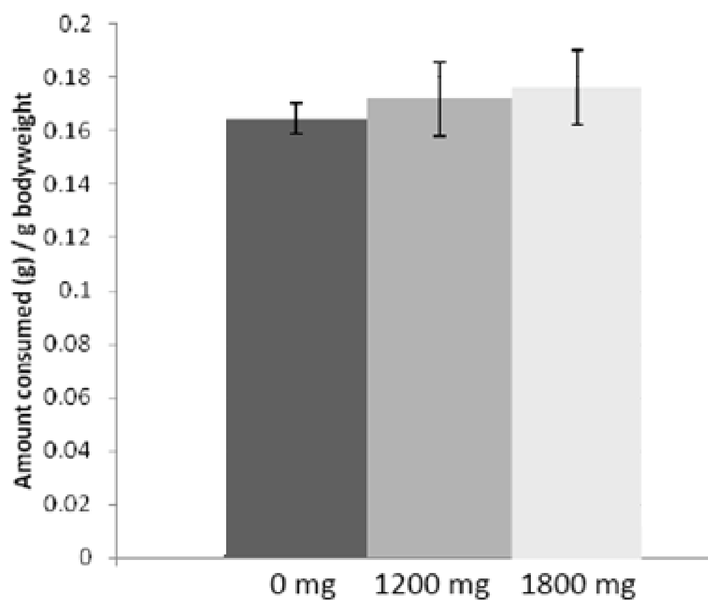

Figure 6: Intragastric preload of L-tryptophan in non-deprived mice did not affect 10% sucrose consumption 1 hour after administration. Non-deprived mice received infusions of 0, 1200 or 1800 mg/kg b. wt. of L-tryptophan within 200 μL of saline. * P < 0.05

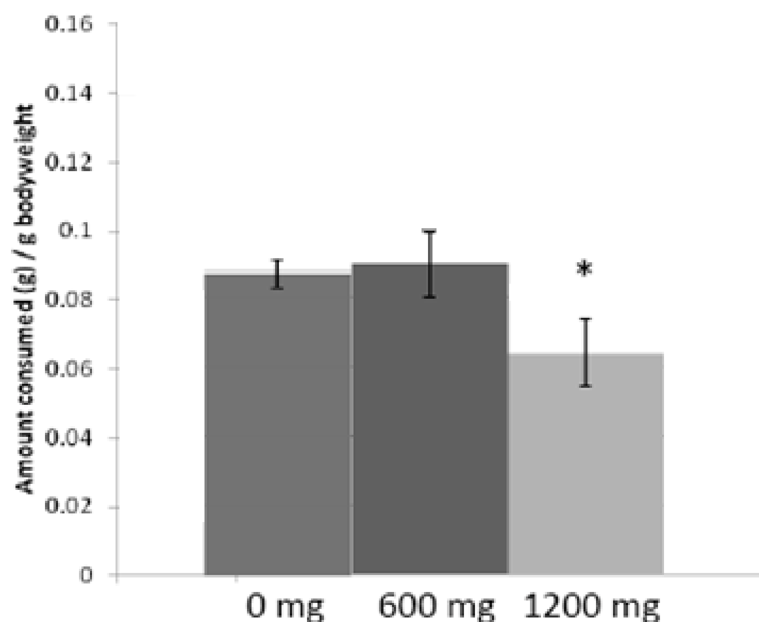

Figure 7: Intragastric preload of L-tryptophan in non-deprived mice reduces palatable milk formula consumption at 1200 mg/kg b. wt. 1 hour after administration. Non-deprived mice received infusions of 0, 600 or 1200 mg/kg b. wt. of L-tryptophan within 200 μL of saline. * P < 0.05

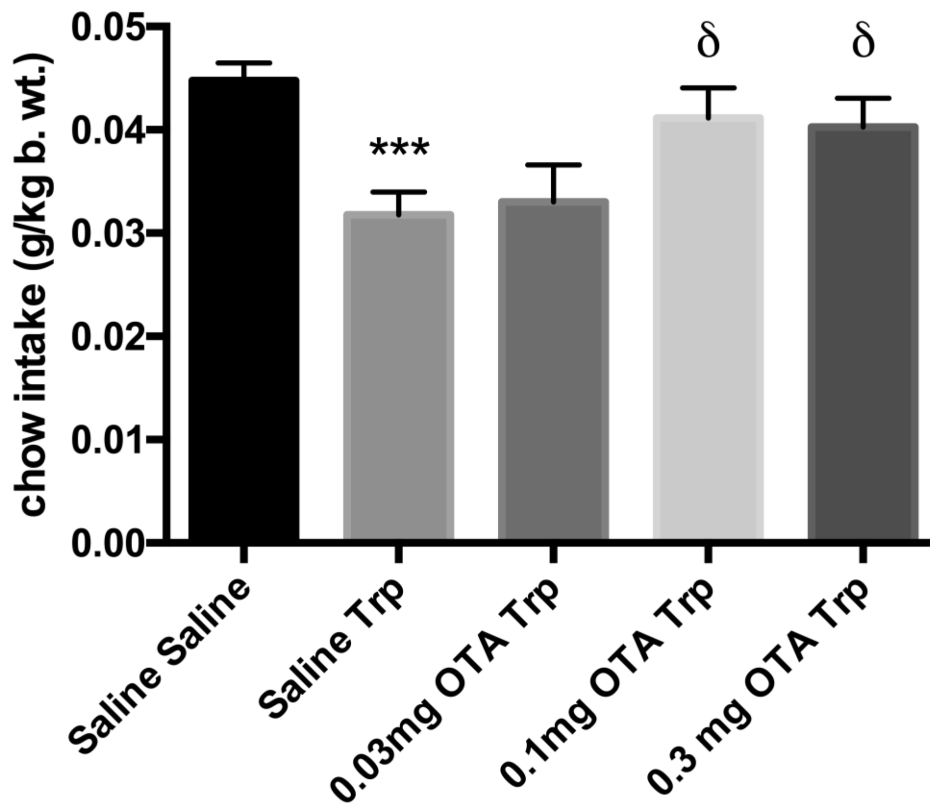

Figure 8: Oxytocin receptor antagonist pretreatment suppresses the anorexigenic effects of intragastric L-tryptophan. Mice received IP injections of oxytocin receptor antagonist L-368,899 at a dose of 0, 0.03, 0.1 or 0.3 mg/kg b. wt. Mice then received an intragastric infusion of 0 or 600 mg of L-tryptophan within 200 μL of water prior to refeeding.

Chapter 4: Discussion

While the neuroendocrine processing of hunger, satiety and reward plays a fundamental role in shaping consumption [163], food characteristics serve as the key factors directly modifying physiological parameters that underlie appetite. Foods differ in, to name a few, appearance (size, shape, colour, consistency), texture, flavour; microbial content, temperature, and macronutrient composition. All these seemingly minute details can have a profound influence on the motivation to seek a given food, how much of this food is eaten, how palatable it is and whether the feeling of "fullness" after a meal persists for a longer or shorter period of time. Even very basic approaches, in which simple tastants are offered or simple modifications to composite tastants are introduced, show surprisingly profound effects. For example, strong preference for a sugar solution is eliminated if a choice between a tasty room-temperature sucrose drink versus cold water is given to a thirsty individual (human or laboratory animal) [164]. The size of fibre molecules affects palatability of standard dog diets, with large sugarcane fibre particles being preferred over the small particles [165]. Presenting a diet in a powdered rather than pelleted texture greatly reduces preference for this diet even though no changes to the composition have been introduced [166]. Despite the myriad of characteristic features that affect acceptability and liking of food items, we are far from understanding the true complexity of the intertwined mechanisms that link food characteristics and food intake.

The current project focused on deciphering the effect of a free amino acid, L-tryptophan, acting intraorally and intragastrically on appetite regulation. The

interest in L-tryptophan (and amino acid control of eating behaviour, in general) as a dietary component stems from the fact that foods contain amino acids in a free form as well bound in proteins, peptides and non-peptide polymers [167]. Therefore, individual amino acids are an essential contributor to food characteristics as they are able to affect appetite from the very early stages of consumption rather than – as presumed earlier – after complex proteins have been reduced to amino acids through the digestion process. In line with that, the assumption that the satiating effect of protein comes from only the complex protein molecule seems premature as some individual amino acids may in fact have the capacity to mediate hypophagia and – thus – they may act as an additional yet individual anorexigenic stimulus accompanying protein intake.

This thesis represents the effort of closing the knowledge gap surrounding the purported anorexigenic effects of ingesting dietary L-tryptophan. This project aimed to do so in three ways. Firstly, through defining the effect of dietary L-tryptophan levels within complex liquid diets on energy deprivation-induced intake, as well as establishing if any observed effect was related to flavour and/or thirst. Secondly, via investigating the effects of intragastric L-tryptophan preload on the consumption of palatable tastants which varied in energy density. Finally, by determining whether any L-tryptophan-induced hypophagia might be mediated at the neuroendocrine level by the anorexigenic neurohormone oxytocin.

Only a select few feeding studies conducted thus far have investigated the impact of adding supplementary L-tryptophan to the diet [72, 168]. These earlier studies have demonstrated that hypophagia ensues after the amino acid supplementation.

However, one of the drawbacks of the initial reports was that they focused on defining the effect of L-tryptophan on energy dense yet “bland” chow. In the current project, instead of studying the effect of this amino acid on standard chow intake, two liquid diets based on the goat milk formula were chosen. While the two complex milk formula diets used in these experiments were calorically and nutritionally adequate, in addition to being more palatable than standard (bland) chow, the two diets differed in composition (Table 1), palatability and energy content. WPC+ goat formula, having a higher fat and lactose content than skim goat formula, was the more energy-dense and palatable of the two. This was reflected in the levels of deprivation-induced formula consumption – animals consumed roughly 50% more WPC+ formula than skim formula when presented with liquid diets unadulterated by L-tryptophan addition (Figs. 1 and 2). Despite the differences in the composition and gustatory quality of those two foods, L-tryptophan supplementation had a very similar impact on the their consumption. Energy-deprived animals fed the milk formula with an additional 1.2 g/L of L-tryptophan content consumed less than those fed unadulterated formula. As animals were deprived of food for 24 hours prior to the experimentation, this consumption can be largely attributed to energy needs as opposed to reward-mediated behaviour [169]. The results of this experiment confirm the anorexigenic effect of dietary L-tryptophan, corroborating the findings by Ayaso et al [72]. These results also demonstrate for the first time that L-tryptophan supplementation decreases consumption of food items that differ in their palatability and energy density characteristics: aside for the “bland” highly caloric

chow, the amino acid reduces intake of two milk formulas differing from each other in palatability and energy density.

While increases to the dietary content of L-tryptophan in the milk formula caused a modest decrease in formula consumption by energy-deprived animals, this anorexigenic effect could be potentially attributed to either a direct impact on the palatability of the diet or alterations in thirst-related physiological processes. A decrease in formula consumption could theoretically occur if, for example, high-level L-tryptophan supplementation simply caused an unpleasant flavour in the liquid diets. One could argue that a decrease in formula consumption would likewise be possible if L-tryptophan induced a lowered drive to drink water rather than because of any impact on appetitive processes. In order to investigate this issue, a “neutral” ingestant, regular drinking water, was supplemented with L-tryptophan in an identical manner to the previous milk formulas. As with the liquid diets, overnight water-deprived animals were given access to water for 2 hours. In contrast to palatable liquid diets, similar levels of water consumption were observed regardless of L-tryptophan concentrations. These data suggest that the anorexigenic effects of dietary L-tryptophan do not stem from an impact on flavour or other components of palatability of the supplemented tastants, nor do they arise from the suppression of thirst mechanisms. Obviously, additional studies are needed to rule out the possibility that L-tryptophan is nonetheless detectable in foods (or, e,g, affects texture, viscosity, etc), but regardless of the outcome of such studies, it is unlikely that its presence in ingestants promotes a significantly adverse gustatory response to high-tryptophan diets.

Ultimately, this and other experiments involving oral intake of L-tryptophan-enriched foods strengthen the notion that ingestion of dietary L-tryptophan leads to a reduction in energy-driven food intake. However, administering this amino acid orally alongside a diet (as this diet's component) introduces certain limitations in our ability to determine the relationship with intricate feeding mechanisms. Most notably, it is difficult to control the rate at which a meal (and, thus, L-tryptophan) is consumed by animals even during a well-defined feeding session, as the speed of consumption varies between meals, days and individuals despite controlled laboratory environment. Another issue with in-food delivery of tryptophan is that its intake (hence, its dose) mirrors consummatory behaviour activity instead of (preferably) preceding it. A caveat of this methodology is that digestion and absorption of the "carrier diet" may alter the rate at which the amino acid becomes available beyond the GI tract, and thus contributes to the fact that the effectiveness of dietary L-tryptophan relies on its cumulative effect which is spread over a meal time rather than occurs in an acute/"bolus" manner. Not surprisingly, therefore, in order to achieve a satisfactory and reliable hypophagic outcome, relatively high concentrations of L-tryptophan have to be used.

In order to bypass the aforementioned problems associated with studying the effects of tryptophan by modifying its presence in a diet, the anorexigenic action of L-tryptophan was examined in an experimental paradigm that excludes the oral stage of consumption, namely in the intragastric delivery of L-tryptophan preloads. We chose to administer via gavage a single preload of L-tryptophan shortly before

mealtime, thereby ensuring that the onset of tryptophan's action can coincide with the beginning of consummatory activity. The intragastric route of delivery, though it minimises the impact of oral mechanisms (provided they might play a role in the case of this particular amino acid), focuses on the influence of digestive, absorptive and post-absorptive processes.

The intragastric infusion of L-tryptophan in energy-deprived mice given chow during the re-feeding phase confirmed the anorexigenic effects of L-tryptophan. Ng and Anderson found that a gastric preload of 600 mg/kg b. wt. decreased chow consumption [73]. In our set of studies, the 1200 mg/kg b. wt. dose of L-tryptophan was sufficient to cause a moderate decrease in deprivation-induced chow consumption (Fig. 4). Not only did this gastric preload decrease immediate energy-based chow consumption, but hypophagia persisted for a period of over 24 hours. The persistence of this anorexigenic effect and the lack of the compensatory intake during the subsequent night-time feeding bout suggests that L-tryptophan impacts long-term mechanisms driving energy balance. This is somewhat similar to the action of other anorexigenic amino acids. For example, Jordi et al. measured chow consumption for 48 hours after intragastric infusion of L-arginine or L-lysine [162]. Similar to L-tryptophan, these two other amino acids caused a long-term decrease in chow consumption, persisting for over 48 hours. While additional experiments are needed to determine the precise length of hypophagia after L-tryptophan administration, the fact that a bolus delivery of this amino acid has a long-term anorexigenic effect makes L-tryptophan an excellent

candidate for pharmacological as well as functional foods-based strategies aimed at curbing excessive food intake.

In the context of applicability of any method or compound in anti-obesity appetite suppressant strategies, once has to consider the fact that the primary reason for overeating in the industrialised world is not hunger, but rather the pleasant/rewarding aspect of consumption [170]. Therefore, successful approaches should be able to diminish the drive to consume palatable foods irrespective of tastants' energy density. In most experimental scenarios, even at the very basic laboratory animal level, paradigms are included that target intake for reward. Hence, a part of this project was also dedicated to investigating whether L-tryptophan impacts hedonic aspects of food intake, in particular those driven by pleasant (usually, sweet) taste.

In our experiments, non-deprived animals (thus, animals that do not have a negative energy balance and do not initiate consumption in order to replenish lacking calories) were presented with one of three palatable tastants. These tastants, 0.1% saccharin solution, 10% sucrose solution and skim goat milk formula, shared a common denominator of a pleasant sweet taste (albeit derived from different sources: non-carbohydrate non-caloric sweetener; disaccharide; complex formula) and they were characterised by a range of energy densities (none in the case of saccharin and low/moderate with the sugar solution and skim milk). Due to the hedonic value of these ingestants, the animals consumed them readily despite being non-deprived. An intragastric preload of L-tryptophan

decreased the intake of 0.1% saccharin solution and it was effective already at a 600 mg/kg b. wt. dose, which was lower than the one needed to reduce deprivation-induced chow intake (Fig. 5). A moderate decrease in the consumption of skim milk formula was also achieved, however, the 1200 mg/kg b. wt. dose had to be used (Fig. 7). Surprisingly, administration of intragastric preloads of L-tryptophan had no impact on the intake of 10% sucrose solution (Fig. 6), even at very high doses (1800 mg/kg b. wt.).

As mentioned before, the animals given palatable tastants had not been food-deprived (also, saccharin does not provide calories). Consequently, differences in tastant consumption likely arose due to an effect on reward-mediated food intake. These results imply that gastric preloads of L-tryptophan – via yet undefined mechanisms - decrease the intake for palatability (with the exception of sucrose). That the consumption of 0.1% saccharin solution (which has no energy content but a palatable sweet flavour) and skim milk formula (which has a moderate energy content and a composition rich in sweet tasting carbohydrate lactose) should decrease following a preload of L-tryptophan, but not 10% sucrose solution (which has a low energy content and a sweet flavour) suggests that sucrose may interact differently than other tastants with reward processes affected by L-tryptophan. Indeed, recent studies have demonstrated that, irrespective of energy content, there is variation in preference for sweet carbohydrates, and that sucrose appears to be a more reinforcing carbohydrate than glucose or fructose [171]. It suggests that the breadth and type of mechanisms underlying sucrose overeating may be at least partially different from those affected by other

carbohydrates and sweet tastants, thereby contributing to the inefficiency of select anorexigenic agents (including L-tryptophan) as sucrose consumption suppressants. While this fact does not negate the fact that L-tryptophan reduces feeding for reward, certain caution is warranted in defining this amino acid's action as an anorexigen. L-tryptophan's hypophagic role is conditional and it shows "functional interaction" with select food components (and sucrose hampers effectiveness of this amino acid in consumption scenarios).

The data obtained in this project and by other authors [72-74, 162, 168] strongly indicate that L-tryptophan induces hypophagia in a much broader sense than previously understood. It appears that L-tryptophan decreases food intake via both energy-balance and reward-mediated pathways. Thus, the question arises as to whether neuroendocrine mechanisms known to reduce both feeding for energy and feeding for reward mediate this anorexigenic effect. That neural circuits involved in such a combined suppression of energy homeostasis- and reward-dependent feeding are involved is in fact very much plausible. For example, the analysis of brain activity (using an immediate-early gene product, c-Fos, as a neuronal activation marker) has revealed that anorexigenic free amino acids activate the brainstem pathways, including the nucleus of the solitary tract (NTS) and the area postrema (AP). A number of metabolites of L-tryptophan are known to stimulate vagal afferents projecting to the AP and the NTS. Importantly, the AP and the NTS innervate the hypothalamus [172] as well as the ventral tegmental area (VTA) and nucleus accumbens (Nacc), two key reward sites [173]. Moreover, changes in hypothalamic c-Fos levels are affected by administration of intragastric

L-tryptophan [174]. One of the key hypothalamic neuropeptides that reduces consumption for energy and for reward is nonapeptide oxytocin, synthesised mainly in the paraventricular and supraoptic nuclei [175]. Oxytocin promotes early satiation by acting via several routes that incorporate the vagal afferent input as well as hypothalamic and extrahypothalamic forebrain regions: on the one hand it affects gastric motility/emptying and limits the volume of ingested food (an action also attributed to L-tryptophan supplementation [176]); on the other, it is particularly effective at decreasing the intake of rewarding (palatable) tastants. The oxytocin system is closely intertwined via reciprocal connections with the reward areas, including the VTA and Nacc. Its communication with the dorsal vagal complex areas is also reciprocal.

The results obtained in this project suggest that there is a functional link between the anorexigenic action of L-tryptophan and oxytocin. Not only do the actions of those two compounds parallel each other, but also – as shown in this thesis - the disturbance in oxytocin signalling prevents L-tryptophan from inducing an anorexigenic response. Peripheral administration of a blood-brain barrier penetrant oxytocin receptor antagonist (OTA), L-368,899 [177], abolished L-tryptophan-induced hypophagia. This was achieved by injecting OTA at low doses (0.1 mg/kg b. wt.) – at 0.1 mg/kg, this antagonist has been shown to be unable to increase deprivation-induced feeding [178]. Thus, the observed effect of the antagonist on chow intake in hungry mice cannot be attributed to a hypothetical orexigenic outcome of the OTA's action. While further studies are needed to expand our understanding of the functional relationship between L-

tryptophan and oxytocin, data indicate oxytocin pathways are at least to some degree part of the anorexigenic neuroendocrine circuit affected by tryptophan.

While the evidence base supporting the hypothesis that the anorexigenic effects of L-tryptophan are mediated by OT is significant, one point of contention is the interaction between L-tryptophan and sucrose. OT has a notable effect on sucrose intake which was not demonstrated by gastric preloads of L-tryptophan. This suggests that L-tryptophan may mediate food intake by activating a slightly different subset of neuronal pathways than that of OT.

In sum, this project has identified L-tryptophan delivered orally or intragastrically as an amino acid that promotes hypophagia. It decreases consumption for energy as well as for reward, however appetite for sucrose seems to be resistant to tryptophan's action. Anorexigenic neurohormone oxytocin appears to act as a central mediator of feeding termination processes triggered by L-tryptophan. Though the current status of research on the link between tryptophan and appetite is still very preliminary, it is apparent that L-tryptophan can be regarded as a candidate amino acid for pharmacological and functional food strategies aimed at curbing excessive food consumption.

Chapter 5: Conclusions

- Dietary supplementation of L-tryptophan decreases energy-driven intake of palatable and nutritionally adequate goat milk-based liquid diets that serve as a source of calories.

- A gastric preload of L-tryptophan induces a long-term (>24 h) reduction in deprivation-induced chow intake.

- Gastric preloads of L-tryptophan reduce the reward-driven intake of palatable non-caloric saccharin solution and palatable goat milk formula, but not sucrose.

- Very low doses of a BBB penetrant oxytocin receptor antagonist, L-368,899, blocks L-tryptophan-dependent hypophagia, suggesting that oxytocin circuits mediate anorexigenic effects of tryptophan.

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