Effects of naltrexone on energy- and palatability-driven consumption and neuronal activation in feeding-related forebrain areas: relationship with age

A thesis

submitted in partial fulfilment

of the requirements for the degree

of

Master of Science (Research)

at

The University of Waikato

by

LAURA MCCOLL

THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2017

Abstract:

Intake of energy as well as interest in palatable foods diminish during the ageing process. One of the likely reasons underlying this phenomenon is an age-related decrease in the opioid tone in the brain. To further substantiate this hypothesis, the current project was aimed to elucidate the effects of an opioid receptor antagonist, naltrexone, on energy- and palatability-driven consumption in 6-, 16- and 22-month old mice and on the activation of feeding-related forebrain circuitry (hypothalamic and accumbal) in adult vs old male mice. The findings indicate that old (22-month old) mice exhibit diminished responsiveness to anorexigenic properties of naltrexone in deprivation-induced intake of “bland” chow and eating for reward (consumption of low-/non-calorie solutions containing sucrose and saccharin). Interestingly, in the pilot studies performed in rats, sensitivity to anorexigenic action of naltrexone was similar at two different phases of adult lifespan prior to reaching the old age (2 months and 18months). The c-Fos immunoreactivity analysis in mice indicated that unlike in adult animals at a younger age, in old animals, naltrexone fails to activate the nucleus accumbens shell, the ventromedial and hypothalamic nuclei, and it activates the lateral hypothalamus. These c-Fos data provide an insight into neural responsiveness changes that might underscore differential feeding regulatory outcomes seen after naltrexone administration in old animals compared to their adult yet younger counterparts.

Acknowledgements:

Firstly I would like to thank my supervisor, Pawel Olszewski and postdoctoral fellow, Anica Klockars for their continual support and guidance through this research project. I have appreciated everything that you have taught me.

Secondly I would like to thank my fellow lab students for being such encouraging people to work with.

And finally to my family and friends and the endless support and inspiration to keep moving forward.

Table of contents:

List of figures and tables:

the parasympathetic system with preganglionic neurones for the efferent portion of the vagus nerve located in the dorsal motor nucleus of the vagus (DMX). The efferent autonomic innervation regulates, e.g. glucose homeostasis via actions in liver and skeletal muscle. From Brain regulation of food intake and appetite: molecules and networks (1).

Figure 3: Sensory factors that make food attractive may over-ride existing satiety signals. Schematic diagram to show how sensory factors interact in the orbitofrontal cortex with satiety signals to produce the hedonic, rewarding value of food, which leads to appetite and eating. Cognitive factors directly modulate this system in the brain. Figure from Taste, olfactory and food texture reward processing in the brain and the control of appetite (51).

Figure 4: Schematic representation of neural circuits that regulate feeding. Dopaminergic neurons originating in the VTA project to neurons within the nucleus accumbens of the ventral striatum. The lateral hypothalamus receives input from GABAergic projections from the nucleus accumbens as well as melanocortinergic neurons from the Arc of the hypothalamus. In addition, melanocortin receptors are also found on neurons in the VTA and the nucleus accumbens. Figure from Homeostatic and hedonic signals interact in the regulation of food intake (76).

Figure 5: The effect of saline, 0.1, 0.3, 1.0 and 3.0 mg/kg of NTX on deprivation induced chow intake consumption in male 6 month old mice. NTX was injected IP following overnight deprivation of regular chow, food intake was then measured

after an hour. The data is recorded as mg of food consumed per kg of body weight. * ($p = <0.05$), ** ($p = <0.01$) significantly different from saline.

Figure 6: The effect of saline, 0.1, 0.3 and 1.0 mg/kg of NTX on deprivation induced chow intake consumption in male 16 month old mice. NTX was injected IP following overnight deprivation of regular chow, food intake was then measured after an hour. The data is recorded as mg of food consumed per kg of body weight. * ($p = <0.05$) significantly different from saline.

Figure 7: The effect of saline, 1.0, 3.0 and 10 mg/kg of NLX on deprivation induced chow intake consumption in male 22 month old mice. NTX was injected IP following overnight deprivation of regular chow, food intake was then measured after an hour. The data is recorded as mg of food consumed per kg of body weight. * ($p = <0.05$) significantly different from saline.

Figure 8: The effect of saline (control), 0.1, 0.3mg/kg of NTX on sucrose intake in 6 month old male mice. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * ($p = <0.05$), ** ($p = <0.01$) significantly different from saline.

Figure 9: The effect of saline (control), 0.1, 0.3mg/kg of NTX on sucrose intake in 16 month male mice. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * ($p = <0.05$), ** ($p = <0.01$) significantly different from saline.

Figure 10: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on sucrose intake in 22 month old male mice. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * ($p = <0.05$), ** ($p = <0.01$) significantly different from saline

Figure 11: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on saccharin intake in 6 month old male mice. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured after 1 hour. The data is recorded as ml of saccharin consumed per kg of body weight.

Figure 12: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on saccharin intake in 16 month old male mice. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured after 1 hour. The data is recorded as ml of saccharin consumed per kg of body weight.

Figure 13: The effect of saline (control), 1.0, 3.0 and 10.0 mg/kg of NTX on saccharin intake in 22 month old male mice. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured after 1 hour. The data is recorded as ml of saccharin consumed per kg of body weight

Figure 14: The effect of Saline and 1.0 mg/kg of NTX on neuronal activity in specific brain sites in 6 and 16 month old male mice. NTX was injected IP, after one hour mice were perfused with brains extracted for C-Fos activity. The data is recorded as the number of Fos positive neurons per mm2. * ($p = <0.05$), ** ($p= <0.01$), *** ($p= <0.001$) significantly different from saline.

Figure 17: The effect of saline, 0.1, 0.3 and 1.0 mg/kg of NTX on deprivation induced food intake consumption in 18 month old rats. NTX was injected IP following overnight deprivation of regular chow, food intake was then measured. The data is recorded as mg of food consumed per kg of body weight. * (p = <0.05), ** (p= <0.01) significantly different from saline.

Figure 18: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on sucrose intake in 2 month old male rats. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * (p = <0.05), ** (p= <0.01), *** (p = <0.001) significantly different from saline.

Figure 19: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on sucrose intake in 18 month old male rats. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * (p = <0.05), ** (p= <0.01) significantly different from saline.

Figure 20: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on saccharin intake in 2 month male rats. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured. The data is recorded as ml of saccharin consumed per kg of body weight.

Figure 21: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on saccharin intake in 18 month old male rats. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured after 1,

2 and 4 hours. The data is recorded as ml of saccharin consumed per kg of body weight.

Figure 22: The effect of saline (control), 0.03 and 0.1 butorphanol on ad libitum chow intake in 18 month old male rats. Butorphanol was injected IP with regular chow being present ad libitum. Intake was then measured at 1, 2 and 4 hours. The data is recorded as mg of chow consumed per kg of body weight. * ($p = <0.05$), ** ($p= <0.01$), *** ($p= <0.001$) significantly different from saline.

List of abbreviations:

ABC – Avidin-biotin complex

AMY- Amygdala

AgRP – Agouti related neuropeptide

ARC – Arcuate nucleus

CART - Cocaine- and amphetamine-regulated transcript

CeA – Central nucleus of the amygdala

CNS – Central nervous system

DAB – Dibenzylidene

DVC- Dorsal-vagal-complex

DMH- Dorsomedial hypothalamus

GI – Gastrointestinal

IP – intraperitoneally

LH – Lateral hypothalamus

NAcc- Nucleus accumbens

NLX - Naloxone

NTS - Nucleus tractus solitarius

NPY – Neuropeptide Y

PFA – Paraformaldehyde

POMC - Pro-opiomelanocortin

PVN – Paraventricular nucleus

SON – Supraoptic nucleus

TBS – Tris-buffered saline

VMH - Ventromedial nucleus of the hypothalamus

VTA – Ventral tegmental area

1. Introduction – Literature review

1.1. Homeostatic regulation of food intake:

Mechanisms which govern food intake are conserved throughout the animal kingdom, including in humans. Food is consumed for a number of reasons, the main one being to replenish lacking energy. This is the primary drive underlying our motivation to eat. Eating helps us to meet our energy demands and maintain homeostasis.

Feeding activity consists of three key components. The first is the initiation phase; this is when the energy needs of the organism in combination with external factors, such as availability of food, palatability of food and social interactions, to name a few, facilitate a search for food. The second phase, the procurement, is when an animal seeks out the food through intense foraging and finally the third one is the actual consumption. As eating continues the mechanisms which drive satiation and termination of a meal gradually ensue (1).

During these stages of food intake both peripheral and central mechanisms are "interacting" with each other to keep the body within homeostasis to prevent both starvation and extreme overeating. The brain coordinates with the periphery to control the intake of food. This control is due to changes in energy levels which arise in the periphery and signal back to the brain (2). These mechanisms can be short- (e.g., neuropeptide Y release upon presentation of a food cue) and long-term (e.g. leptin release from the adipose tissue) in order to

initiate feeding during calorie depletion and terminate feeding at the time of satiety (3).

Consumption for energy is controlled at the central nervous system (CNS) level by a complex network of brain sites that are interconnected by intricate molecular mechanisms. The majority of these sites are localised within two key anatomical subregions of the central nervous system (CNS), the brain stem and the hypothalamus. Both the hypothalamus and the brain stem are capable of receiving neurohormonal and nutrient-derived signals arising in the periphery. The brain stem also “communicates” with the peripheral organs via a direct afferent-efferent vagal neural pathway (4).

Several decades of research have led to the identification of discrete hypothalamic and brainstem sites that play a particularly critical role in the control of eating for energy. Already in 1981, Leibowtz et al. found that lesioning the paraventricular nucleus of the hypothalamus in rodents promotes extreme overeating and eventually leads to obesity (5). Knife cut experiments determined that severing connections between the brain stem and the PVN greatly hampers the ability of the animal to develop satiation and it prolongs a meal (6). Furthermore, since the PVN sends the projections to the pituitary as well as to multiple CNS targets, developmental abnormalities pertaining to this area result in aberrant feeding behaviour (7). In this context, the SIM-1 gene mutation, which underlies improper neuropeptidergic cytoarchitecture of the PVN, is associated with obesity in mice (8, 9). The impaired satiety response in animals with a PVN dysfunction does not come as a surprise considering that PVN

neurons typically exhibit an increased level of activity upon meal termination (10). Thus, anomalous neuronal network development at the PVN level serves as a neuropathology underlying a vast and abnormal processing of feeding-related signalling throughout the brain stem-hypothalamic pathways (and beyond).

While the initial research on central regulation of appetite focused on the PVN, mounting evidence pointed to additional hypothalamic sites as co-players involved in this process. The current literature signifies the importance of the brainstem hypothalamic network that encompasses in the hypothalamus the PVN, SON, DMH, VMH, ARC and LH in the hypothalamus (11).

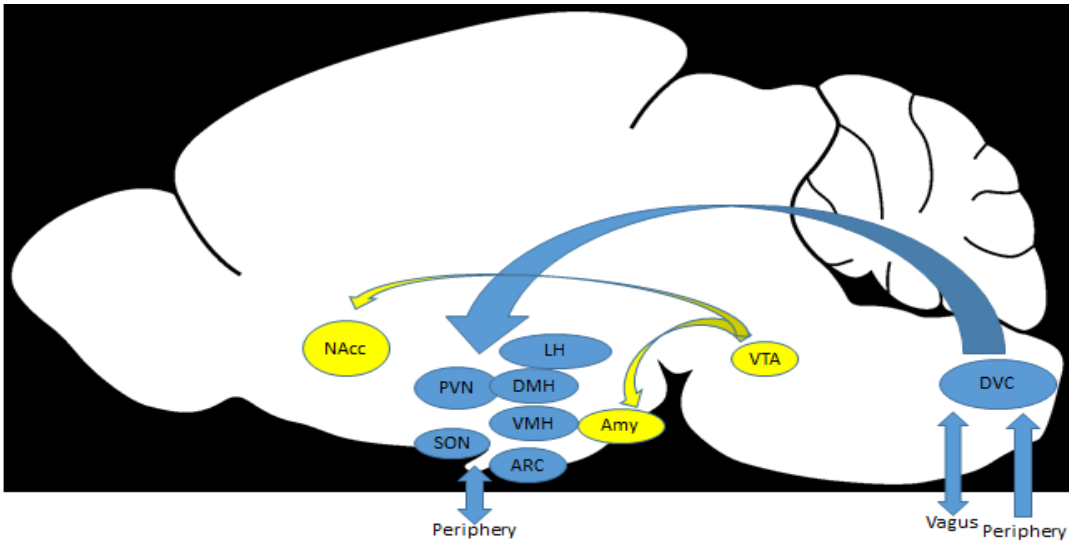


Figure 1: Schematic representation of the brainstem-hypothalamic network involved in homeostatic regulation of food intake (i.e., hunger-reward; blue colour). Reward pathways that control consumption for pleasure are depicted in yellow. Details related to the functioning of the two circuits are described in the text.

Each of these sites contributes to shaping the final outcome of the orexigenic/anorexigenic responses observed under specific circumstances, yet it is clear that the combined activity of the entire feeding-related central network

plays a decisive role in generating a dynamic change in appetite induced by transient and long-term environmental and physiological challenges. For example, the supraoptic nucleus (SON) contains oxytocin and vasopressin (12) neurons, which are activated upon excessive stomach distension and increase in plasma osmolality (those parameters accompany meal termination). (13, 14). DMH ablation in rats leads to hypophagia and reduces body weight (15) DMH-lesioned rats do not show sensitivity to anorexigenic properties of an opioid receptor antagonist, naloxone, which is known to diminish feeding induced by pleasant taste (16). On the other hand, while ablation of the VMH causes hyperphagia (17), the general neuronal activity of this site in rats is dependent on these animals' body weight (18). Several populations of LH neurons, including those that synthesise orexin A and melanin concentrating hormone, promote consumption, (19) however, it should be noted that their influence on food intake (especially in the case of orexin cells) is closely tied with the regulation of sleep-wake (and locomotor activity) cycle (20).

Finally, the arcuate nucleus (ARC) plays a special role in this network of hypothalamic sites as is crucial in how the peripheral and central signals which regulate food intake are integrated (21). The ability of the ARC to be responsive to peripheral signals stems from the weak blood-brain barrier in this area. Hence, hormones, such as leptin or cholecystokinin, are able to directly affect ARC neurons containing relevant receptors (22). The neurons within the ARC are stimulated by a large range of hormones and changes in nutrients. It is thought to act as a conveyor of information from all these different signals involved in homeostasis to other areas of the brain (23).

It should be noted that this brainstem-hypothalamic network of sites is able to receive peripheral information associated with the feeding/energy status of the animal. This ability of this circuitry to respond to peripheral signals stems from, among others, the fact that some hormones released by the GI tract, adipose tissue, liver and pancreas can penetrate through the blood-brain barrier and induce a change in neuronal activation (24). In addition, some of those peripheral signals – even though they are not BBB-penetrant – are able to reach their receptors in the brain in those central areas where the BBB is very weak (for example, the ARC and – in the brain stem - the dorsal vagal complex, especially the AP) (25).

From this point of view, significant research effort has been dedicated to the understanding the feeding effects arising from the functional link between GI hormones such as ghrelin, PYY, and insulin, and the CNS. One of the key hormones which relay information about the body's metabolic stores is ghrelin (26). An orexigenic hormone, ghrelin, is secreted by the stomach during energy deprivation and – particularly in an abundant manner – before a scheduled meal (27). When ghrelin is peripherally injected into Siberian hamsters they increase all behaviours shown in there feeding repertoire such as, foraging, intake and hoarding (28). Other studies show that when a ghrelin receptor antagonist is injected, food intake is decreased in lean mice, mice with an obesity inducing diet (high fat & high sugar) and in ob/ob knockout obese mice (29). Importantly, ghrelin signals to the brain via receptors located in the brain stem and the hypothalamic arcuate nucleus, but the communication within the larger network leads to the integration of the signal within pathways that converge at the

hypothalamus (30). Other key hormones include peptide YY (PYY) also from the GI tract, this has the opposite effect of ghrelin and many studies have shown the peripheral administration of PYY in rats and mice not only decreases food intake and weight gain (including in deprivation-induced intake) but also increases neuronal activity (measured by c-Fos immunoreactivity) in the arcuate nucleus (31, 32). In human studies, PPY infusion decreases food intake in healthy and obese subjects (33). Pancreatic hormone, insulin, is released after a spike in glucose levels during refeeding, but it also works to stop glucose levels getting too low during fasting by releasing glucose stores from the liver (1). In rodent studies, central injections of insulin decrease intake of both chow (34). When rats are chronically injected with insulin they show a suppression of food intake paired with weight loss and this change is returned to normal after infusions cease (35).

Importantly, the CNS sites that regulate food intake utilize a number of molecules as a means of synaptic transmission between neurons belonging to this vast network. The hypothalamic network of sites mentioned above utilises a vast array of neuropeptides and neurotransmitters to execute the host of proper neuroendocrine and behavioural responses to energy balance/feeding-related challenges. Orexigenic molecules include NPY, Agouti-related protein, melanin concentrating hormone and orexin; whereas oxytocin, corticotropin releasing hormone, CART and vasopressin serve as anorexigens. These molecules are dispersed throughout the hypothalamus allowing for the flow of neutrally mediated information to occur. For example, at the “entry level” into the CNS, adipocyte hormone leptin stimulates the release of anorexigenic peptides by

activating pro-opiomelanocortin (POMC) and cocaine and amphetamine related transcript (CART) when energy levels have been adequately met and also works to inhibit the release of orexigenic peptides neuropeptide-Y (NPY) and agouti-related peptide (AgRP) (26). During times of energy insufficiency ghrelin stimulates both NPY and AgRP and these peptides work to inhibit the stimulation of the anorexigenic peptides (36). Orexigenic peptides such as NPY act to increase food intake and the intake of calories where as anorexigenic peptides such as melanocortins have an opposite effect where they work to decrease food intake and body weight (37). Arcuate POMC neurons (one splice product of POMC is an anorexigenic melanocortin ligand, alpha-melanocyte stimulating hormone) stimulated by leptin send projections to the hypothalamic PVN, where they terminate on anorexigenic oxytocin, vasopressin and CRH cells, leading to an increase in the activity of this hypophagic network (38, 39), On the other hand, orexigenic beta-endorphin (another POMC splice product) release from ARC terminals at the PVN level, stimulated by ghrelin's action, leads to a decrease in oxytocin signalling arising from the PVN, likely hampering meal-suppressing activation of PVN OT neuronal population.

Furthermore, activity of the aforementioned hypothalamic network of sites is also regulated by input from the brain stem. This is important from the point of food intake regulation as the brain stem receives vagal afferents which carry information directly from the gut. The nucleus of the solitary tract (NTS) and dorsal motor nucleus of the vagus, the two brainstem nuclei directly involved in this process (1), send projections to forebrain areas (1), thereby affecting not merely a drive to eat (or the need to discontinue consumption), but also

memory, motivation and motor patterns that ensure having energy requirements adequately met (40).

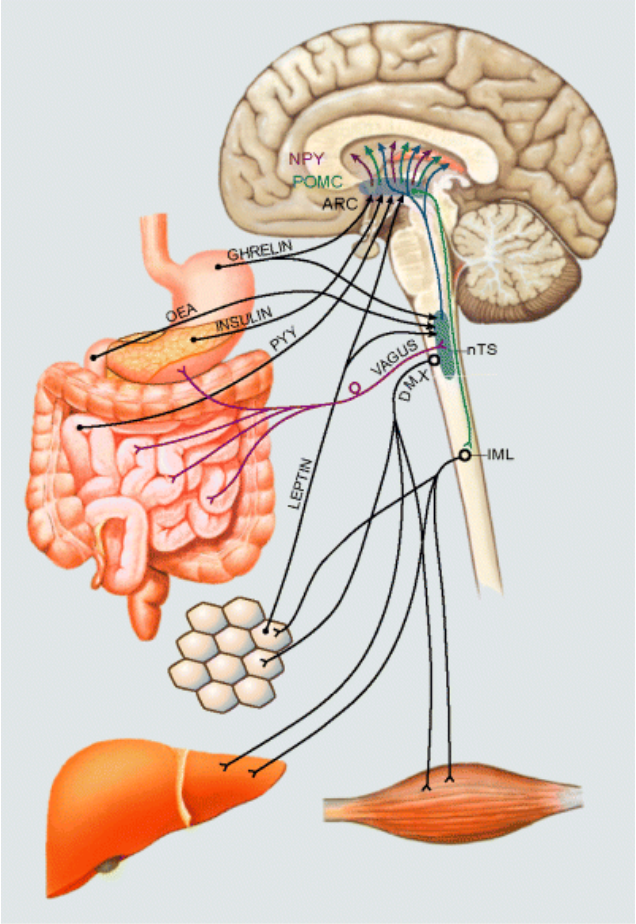


Figure 2: The central metabolic circuitry is regulated by numerous endocrine and neural inputs. Schematic illustration of how brain networks regulating ingestive behaviour communicate with peripheral organs. Hormones supplying information about the peripheral metabolic state to the brain include the gastrointestinal peptides ghrelin and PYY(3-36), insulin from the pancreas and leptin from adipose tissue. Ghrelin and leptin act both on the hypothalamus (Arc) and the brainstem (nTS). The afferent portion of the vagus nerve innervates most of the gastrointestinal tract where it collects information about the immediate alimentary state, and terminates in the nTS. The lipid mediator OEA is produced in the duodenum and activates the brainstem, possibly via the vagus nerve. Both the Arc (via antagonistic NPY- and POMC-expressing cells) and the nTS project further into the brain in parallel pathways to engage higher brain regions into ingestive behaviour. Outputs from the brain regulating energy expenditure include both branches of the autonomic nervous system; the sympathetic system whose preganglionic neurones are located in the intermediolateral cell column (IML), which is directly innervated by POMC neurones from the Arc, as well as the parasympathetic system with preganglionic neurones for the efferent portion of the vagus nerve located in the dorsal motor nucleus of the vagus (DMX). The efferent autonomic innervation regulates, e.g. glucose homeostasis via actions in liver and skeletal muscle. From *Brain regulation of food intake and appetite: molecules and networks* (1).

1.1.1. The reward system:

Aside from eating for energy, there is another critical factor that propels animals to consume food. This factor is palatability. Typically, in humans and in animal models that are used to study food intake regulation, sweet and/or fatty foods are considered to be particularly attractive. Palatable foods are overeaten in no-choice and choice paradigms. It has been well established that offering animals a high-sugar or high-fat diet as the only source of calories will lead to excessive consumption of such diet over standard chow (41, 42). Furthermore, if a palatable and bland diets are offered simultaneously, animals will mainly eat the palatable (thus, preferred) food (43, 44). Interestingly, in choice scenarios, consumption of the "bland" chow never really ceases even if the other diet option is highly palatable (adding to the multidimensionality of feeding reward that encompasses variety of food rather than only relative palatability as a singular factor) (45). When two palatable diets are given (for example, HF and HS), some animals show inherent preference for one of the tastes. (46, 47). It should be noted that solid foods are not always the only source of energy and/or flavour. Importantly, liquid diets/solutions/etc also serve as an important source of calories and thus should be considered as "food" (48).

Palatability is just one of the factors which mediate reward, as intake can be stimulated by cognitive cues such as the smell or a visual image. Cognitive cues are enough to motivate consumption of the palatable tastant due to the orbitofrontal cortex processing (49). Overall, even a completely satiated (understood as energy-nondeprived) animals will continue to consume palatable

food in excessive amounts simply because its tastes pleasant. The reward component of food can be powerful enough to override our homeostatic systems in place and seek out foods whose consumption is not necessary to fulfil our energy needs or – in fact – foods that might potentially jeopardise our homeostasis because of, e.g., high salt content (50).

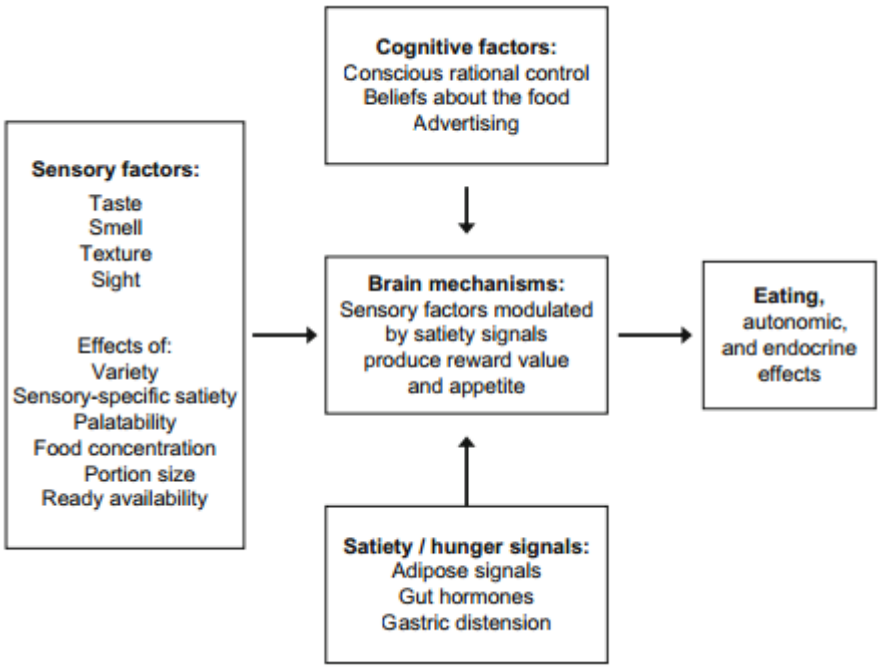


Figure 3: Sensory factors that make food attractive may over-ride existing satiety signals. Schematic diagram to show how sensory factors interact in the orbitofrontal cortex with satiety signals to produce the hedonic, rewarding value of food, which leads to appetite and eating. Cognitive factors directly modulate this system in the brain. Figure from *Taste, olfactory and food texture reward processing in the brain and the control of appetite* (51).

The question arises here as to what central mechanisms underlie this liking and wanting of palatable foods which motivate animals to eat in excess. The key component of the reward system is the ventral tegmental area-nucleus accumbens pathway, which is a part of the corticolimbic system (51). The presence of, anticipation and consumption of palatable food has been shown to

increase activation of neurons within this pathway (52, 53). Individuals that due to underlying mental health pathology, such as e.g. depression, are anhedonic, or that due to a temporary inability to process external stimuli (e.g., extreme stress), experience anhedonia, show a diminished activation of the NAcc to natural rewards, including food (54). Lesions to the shell of the NAcc in rodents have been shown to impair the learning of how the reward source was located and received as they were unable to relocate the reward in a maze trial after the lesion (55). In VTA lesion studies, palatable sucrose solution consumption is significantly decreased, giving evidence to the VTAs role in reward and promoting intake of ingestants characterised by pleasant taste (56).The NAcc and VTA mediate the rewarding components of natural reinforcers such as food (especially high sugar and high fat diets), but also substances which can be abused such as drugs (57); consequently, these findings paved the way to concluding that intake of highly palatable foods has addictive-like properties (58).

The molecular systems that underlie the activity of the reward pathways comprise of dopamine, GABA and opioids. Dopamine is synthesised in the VTA and released from VTA-derived projections into the NAcc. Importantly, this release is triggered by presentation of various rewards, most notably, food. The greater the attractiveness of the food, the more robust activation of this pathway occurs (59). In the presence of palatable food, there is a release of dopaminergic neurons in the NAcc and this release of dopamine is thought to increase future motivation to seek out this palatable food through increasing arousal, motor activities and a coordinated learning response to the substance

(26). Dopamine neurons primary function is to reinforce the behaviours which lead to the exposure of the rewarding substance and is associated with the learning and motor functions which are required for behaviour for motivation to seek out the food which is a positive reinforcer (2). The release of dopamine into the NAcc is triggered by foods and other rewards but also to their predictive cues when a reward is about to occur (60). Studies have shown that dopamine antagonist's block the learning of behaviours that lead to the reward that dopamine helps to reinforce (61). Geary and Smith in 1985, showed that sham-fed sucrose intake in the rat is decreased when injected with dopamine antagonist, pimozide, by removing the positive reinforcing effect of this sweet drink (62).

Early GABA studies have implicated its role in food intake and reward through increasing consumption. The ICV administration of a GABA receptor agonist increases food intake in already sated pigs and when paired simultaneously with the receptor antagonist this response is abolished (63). Furthermore when AgRP cells known to stimulate food intake in mice are deleted in mice and unable to release GABA, mice become lean and resistant to obesity (64). When GABA agonists are injected directly into the VTA there is an increase in food intake (65). Furthering this evidence, a blockade of GABA receptors promotes weight loss in mice (66).

Opioids and opioid antagonists also play a key role in influencing food intake for reward. Opioid receptors located in numerous brain sites, including the hypothalamic PVN, LH, ARC, VMH and DMH and the amygdala (including the

central and basolateral nuclei) and mediate palatable food intake (67). Many studies have implicated opioids and their role in taste preferences and thus reward. Opioid antagonists such as naltrexone and naloxone (NLX) have long been shown to decrease the consumption of palatable substances (68). Levine et al, in 1995 gave evidence to the role opioids have in reward by measuring the effect of NLX on rats given access to either standard or sweetened chow. They found NLX to be more potent in those rats fed the sweetened chow as NLX blocked the rewarding aspect of the sweet taste (69). A blockade of the opioid receptors in the PVN and ACe using naltrexone also decreases both palatable and non-preferred food consumption (67). Administration of NLX decreased normal taste preferences in binge and control eaters and this decrease is more potent in sweet high fat foods considered highly palatable (70).Conversely, opioids drive the intake of preferred food choices such as high sugar and high fat (71). Importantly, all classes of opioids (dynorphins, endorphins and enkephalins) increase food intake in human and laboratory animal studies (72, 73).

Homeostatic mechanisms and the reward system are intertwined in how they affect consumption. For example, ghrelin - mentioned above for its role in hunger signalling - is also involved in the reward system. It signals to the VTA during palatable meal consumption, so intake of the food is increased. This is due to ghrelin causing an increase in the number of dopamine neurons firing within the VTA (74). Glucagon-like peptide-1 and oxytocin injections in the nucleus accumbens decrease food intake. On the other hand, opioid peptides suppress satiety signalling derived from oxytocin neuronal activation even in circumstances under which potential food toxicity may endanger the internal

milieu (14). In essence, the mechanisms which drive palatable food intake have extremely high efficiency precisely because they do not require any hunger signals or actual energy needs to occur. Instead, any cue that drives us to seek out feeding reward may trigger consumption of energy. This means large amounts of food can be consumed even when the stomach is distended and nearly at full capacity, despite ensuing toxicity, hyperosmolality and general lack of energy needs (75).

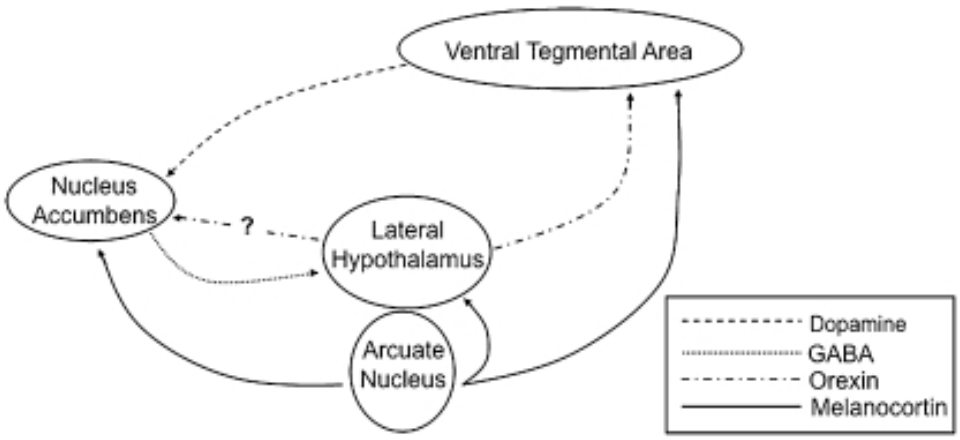


Figure 4: Schematic representation of neural circuits that regulate feeding. Dopaminergic neurons originating in the VTA project to neurons within the nucleus accumbens of the ventral striatum. The lateral hypothalamus receives input from GABAergic projections from the nucleus accumbens as well as melanocortinergic neurons from the Arc of the hypothalamus. In addition, melanocortin receptors are also found on neurons in the VTA and the nucleus accumbens. Figure from *Homeostatic and hedonic signals interact in the regulation of food intake (76).*

1.1.2. Aging studies; human and animal studies:

Aging has long been associated with a decrease in appetite. This phenomenon, also referred to as 'anorexia of aging', is poorly understood (76). At a very young age preferences for food are just beginning to form especially when a change in

the diet occurs during the gradual switch from an exclusive milk diet to an extremely varied diet available in a typical environment rich in foods and dietary choices (77). There is an innate preference for the taste of sweet and salty flavours and the rejection of foods that are sour and bitter, and this is combined with the capacity to learn and develop new preferences (78). At an old age, disturbances in appetite occur. The most prevalent one is eating anhedonia, defined according to the DSM-IV (1994) as the loss of ability to feel pleasure that one would normally receive from preferable foods (79). Donini et al (2003) reported a decline in food intake and a lack of motivation to consume food in this older age (80). Although aging has been linked to a decline in food intake strictly for the purpose of energy consumption (due to decreased energy expenditure) (81), one should note that this decline arises from multiple other factors. One of the main issues is a decreased ability to perceive taste. Age has been found, for example, to be associated with decreased responsiveness to salty and sweet solutions (82). It stems from several reasons. One of them is poor oral health, which affects – among others – salivary output and contributes to poor initial phases of food digestion in the oral cavity, thereby diminishing availability of molecules capable of activating taste receptors (83). The other is the reduction in density of taste buds correlated with aging (84). There are changes in the olfactory system: for example, participants in a study aged from 65- 80, over half showed olfactory deterioration, and this decline in the sense of smell progressed with age; 75% of participants who were 80 and over were affected by this decline (85). One study has suggested that reduced food consumption is caused by an increase in satiety signalling from the periphery

including an increase in the amount of circulating leptin, paired with a decline in motivation to seek and consume food (86). Currently, virtually no studies exist which examine the effect that aging has on the brain in food intake-related sites.

As the population is aging is important to look into the mechanisms which cause these appetite disturbances as we are still far from understanding. It is agreed among studies that there is a loss of appetite found consistently in humans aged over 65, but how this loss is mediated is currently unknown. This is where animal studies hold relevance.

These differences in food intake have been analysed in several animal models such as work done by Frutus et al (2012) with young and old rats looking at the changes in the hedonic value of food that tends to occur with aging and the motivation to obtain this food (87). They found that old rats had a decreased motivation to get food as there was a significant decline in the amount of effort exerted on the incentive runway compared to the younger cohort of rats. The ability to develop associations has also been assessed by Renteria et al (2008) in rats at 24 months of age. They found that rats which were given both sweetened and unsweetened solutions showed no preference for either and consumed very similar amounts of both solutions even though the more palatable option should have shown a higher consumption (88). This effect of aging has also been shown in mice with both young and old cohorts (89). In this study it was found that the old mice consumed significantly less 1% sucrose solution and with the 5% sucrose solution which also involved a larger amount of effort and novel exploration the old mice again showed reduced consumption and effort.

Research has also shown that fasting in both young and old mice leads to an increase in the motivation to feed in the young cohort but not in the old, however the old mice showed higher levels of acyl ghrelin present which is known to stimulate food intake. Old mice injected with acyl ghrelin also showed no increase in food intake compared to young mice where feeding significantly increased (90). A similar study of old rodents deprived of chow for 72 hrs showed they were unable to gain back the weight lost from fasting or eat enough to cover the lost calories during the refeeding period (91).

Importantly, the few studies that have been performed thus far suggest that aging animals show a diminished sensitivity to opioid receptor ligands in terms of the effectiveness of the injectants to change appetite. For example, mice aged 1-2 months and 24-30 months were injected with either agonist ketocyclacine and morphine or the antagonist NLX. The young mice increased feeding when injected with the agonists and decreased intake after NLX. However, the old mice were insensitive to opioid receptor ligands (92). Similarly, Gosnell et al. found that 24-month old rats did not change food intake even after an injection of a high dose of an opioid receptor agonist, butorphanol, or an antagonist naloxone. To date, there have been no studies on the effectiveness of another opioid receptor antagonist, naltrexone in relation to aging and appetite (93). In light of these findings as well as of clear gaps in knowledge, continuation of studies exploring altered responsiveness of aging animals to both feeding reward as well as of drugs that affect feeding reward is essential.

1.2. Overarching goals and specific aims:

The overarching goal of this project was to determine central mechanisms which underlie changes in food intake regulation at an old age. Specifically, the effect of an opioid receptor antagonist, naltrexone, on intake of tastants that differ in palatability and on the activation of feeding-related forebrain circuitry (hypothalamic and accumbal) was established. Animal models were employed in the studies. The project consisted of the following specific aims:

1. Determine the minimum dose of naltrexone required to reduce the consumption of “bland” chow, and palatable sucrose and saccharin solutions in 6-, 16- and 22-month old mice.

2. Determine naltrexone-induced patterns of brain activity (defined by mapping c-Fos immunoreactivity) in feeding-related forebrain in the hypothalamus and accumbal complex (shell and core) sites in adult and old male mice.

3. Determine the effects of naltrexone and butorphanol in adult rats that differ in age (2 and 18 months old).

It should be noted that while the mouse study focused on determining the effects of age that approaches the end of a typical lifespan of a laboratory mouse, rat studies looked at animals considered to be “adult”, however, still a significant time away from reaching the final stages of life. Because of this discrepancy in age, in rats, both agonist and antagonist were used to substantiate the findings, whereas in the old mice, only the antagonist was employed as a sufficient means to assess the outcome.

2. Materials and Methods

Animals:

All experiments had prior approval of the Animal Ethics Committee at the University of Waikato. 6-month old, 16-month old and 22-month old C57BL male mice were placed into a single-housed environment in a standard macrolon cage. 16, 2 month old and 19, 18 month old Sprague Dawley rats were also placed into a single housed environment in standard rat cages. The animal facility was temperature controlled at 22^{o}C with a 12:12 hour light:dark cycle where the lights turn on at 0700. Food (Sharpes feed) and water were available ad libitum unless it has been otherwise stated.

2.1. Experiment 1: The effect of naltrexone on deprivation induced chow intake in 6-, 16 -and 22-month old male mice.

The 16-month old mice were placed into four treatment groups (n=8). The 6 month old mice were placed into five treatment groups (n=10). The treatment groups for both 6- and 16-month old animals are: control (saline), 0.1, 0.3 and 1.0 mg/kg of naltrexone. The 6-month old mice were injected with 3.0 mg/kg as the fifth treatment group. The 22 month old mice were injected with saline, 1.0, 3.0 or 10.0 mg/kg of naltrexone (n=9/group). Prior to injection, animals were deprived overnight of chow, water available ad libitum. On experimental day all animals were then injected intraperitoneally with saline or naltrexone at one of the varying doses. Post injection they were given access too regular chow, intake is measured for 1 h.

2.2. Experiment 2: The effect of naltrexone on sucrose intake in 6-, 16- and 22-month old male mice.

Six- and 16-month old mice were treated with saline (control), 0.1 and 0.3 mg/kg of naltrexone (6 m.o., n=10; 16 m.o., n=8 per group). The 22 month old mice received saline, 1.0, 3.0 or 10mg/kg of naltrexone (n=9/group). On the experimental day all animals were injected intraperitoneally with either saline or naltrexone at one of the varying doses as stated above. Just prior to the doses being administered, chow and water were removed. Once injected, animals were given access to a 10% sucrose solution and consumption was measured after 2 h.

2.3. Experiment 3: The effect of naltrexone on saccharin intake in 6-, 16- and 22-month old male mice.

Six- and 16-month old mice were treated with saline (control), 0.1, 0.3 and 1.0 mg/kg of naltrexone (6 m.o., n=10; 16 m.o., n=8 per group). The 22 month old mice received saline, 1.0, 3.0 or 10mg/kg of naltrexone (n=9/group). On the experimental day all animals were injected intraperitoneally with either saline or naltrexone at one of the varying doses as. Just prior to the doses being administered, chow and water were removed. Once injected, animals were given access to a 0.1% saccharin solution and consumption was measured after 1 h.

2.4. Experiment 4: The effect of naltrexone on neuronal activity in 6 month old and 16 month old male mice.

Animals were split into two groups (n=5 (aged), n=6 (6 m.o.)), which were saline (control) and 1.0 mg/kg of naltrexone (treatment). On the experimental day, group 1 from each age cohort received an injection of saline and the group 2 from each age received 1.0 mg/kg of naltrexone. Prior to the injection food and water were removed. After 60 minutes animals were euthanized (see description below).

Euthanasia and Perfusions:

Sixty minutes post-injection, the mice were deeply anaesthetised with 0.6 mL of 35% urethane and were intracardially perfused with 50 mL of a 4% paraformaldehyde (PFA) solution in a 0.1 mol L-1 phosphate buffer with a pH of 7.4. Once perfused their brains are dissected out and placed in the PFA solution for a further 48 hours in a refrigerator set at 4 degrees.

Immunohistochemistry:

The brains were postfixed in PFA for 48 hours and they were transferred to Tris-buffered saline (TBS). A vibratome (Leica) was used to cut 60-µm coronal brain sections. Brain sections were treated for 10 minutes in a 3% H_2O_2 in 10% methanol (in TBS) solution, washed in TBS incubated at 4oC overnight with goat anti-rabbit anti-c-Fos antibody (1:15000; SYSY) in a supermix solution (0.25% gelatin and 0.5% Triton x-100 in TBS). Sections were washed again in TBS and incubated for one hour in anti-rabbit antibody in supermix (1:400; Vector Laboratories). After another set of washes with TBS, the sections are incubated for another hour in avidin-biotin complex (ABC) that is diluted also in supermix (1:800; Vector Laboratories). This all occurs at room temperature on an agitator.

After the final incubation in ABC the sections are rinsed again and placed in a 0.05% diaminobenzidene (DAB) (Millipore), 0.01% H_2O_2 and 0.2% Nickel sulphate solution in TBS for 10 minutes allowing staining to develop.

Mounting:

The sections were mounted onto gelatinised microscope slides and left to dry. They were then dehydrated in increasing concentrations of ethanol (70%, 90%, 100%) for 10 minutes at a time, immersed in xylene for 20 minutes, then cover slipped using Entallan (Merck, Switzerland).

Analysis:

The sections were examined using a light microscope and images were gathered using a camera (Olympus DP70) and analysed using the software imageJ. The areas of interest were located using the neuroanatomical atlas by Paxinos and Watson. The forebrain areas were analysed using imageJ to count the Fos-positive nuclei bilaterally per brain site. Data were analysed by taking the number of active neurons per sq mm of tissue and then averaged per animal and per group.

2.5. Experiment 5: The effect of naltrexone on deprivation-induced food intake in 2-month old and 18-month old rats

The 2 month and 18 month old rats were injected with saline, 0.1, 0.3 and 1.0 mg/kg of naltrexone (18 m.o., n=10, 2 m.o., n =8). Animals were deprived overnight of chow, water still available ad libitum prior to injection. On the experimental day animals were injected intraperitoneally with either saline or

naltrexone at one of the varying doses. Once injected they were given access to regular chow, intake was measured at 1 h.

2.6. Experiment 6: The effect of naltrexone on the intake of a 10% sucrose solution in 2- and 18-month old male rats.

Two- and 18-month old rats were treated with saline (control), 0.1, 0.3 and 1.0 mg/kg of naltrexone (2 m.o., n=10; 18 m.o., n=8 per group). On the experimental day all animals were injected intraperitoneally with either saline or naltrexone at one of the varying doses as stated above (Exp. 5). Just prior to the doses being administered, chow and water were removed. Once injected, animals were given access to a 10% sucrose solution and consumption was measured at 1 h.

2.7. Experiment 7: The effect of naltrexone on 0.1% saccharin intake in 2- and 18- month old male rats.

Two- and 18-month old rats were treated with saline (control), 0.1, 0.3 and 1.0 mg/kg of naltrexone (2 m.o., n=10; 18 m.o., n=8 per group). On the experimental day all animals were injected intraperitoneally with either saline or naltrexone at one of the varying doses as stated above (Exp. 5). Just prior to the doses being administered, chow and water were removed. Once injected, animals were given access to a 0.1% saccharin solution and consumption was measured at 1 h.

2.8. Experiment 8: The effect of butorphanol on ad libitum food intake consumption in 18 month old male rats.

18 month old rats are placed into three treatment groups (n=9) and injected with saline, 0.03 and 0.1 mg/kg of butorphanol. Food is available ad libitum prior to and on the experimental day. On the experimental day all animals had their chow removed just prior to being injected intraperitoneally saline or butorphanol at one of the doses as stated in Exp. 8. Once injected, animals were re-given access too regular chow, and their food intake was measured over 4 hours.

Statistics:

Results are presented as means ± standard error (SEM). Data were analysed using one-way ANOVA followed by Fisher's post-hoc test with the exception of two-group comparisons where the Student's t-test was used. Values were considered significantly different when $p<0.05$.

3. Results:

3.1. Experiment 1: Different doses of naltrexone are required to decrease deprivation-induced chow intake in 6- 16- and 22-month old mice.

Naltrexone significantly reduced deprivation-induced chow intake at 3.0 mg/kg in 6 month old mice (Figure 4). In 16 month old mice, the 1.0 mg/kg dose of the drug was effective (Figure 6). Finally, in the 22 month old mice a dose as high as 10.0 mg/kg of naltrexone was necessary to induce an anorexigenic effect (Figure 7).

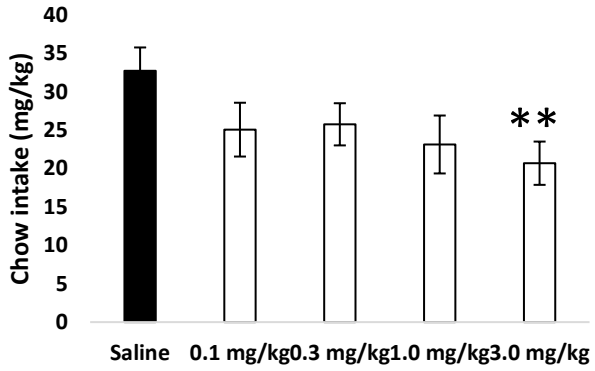


Figure 5: The effect of saline, 0.1, 0.3, 1.0 and 3.0 mg/kg of NTX on deprivation induced chow intake consumption in male 6 month old mice. NTX was injected IP following overnight deprivation of regular chow, food intake was then measured after an hour. The data is recorded as mg of food consumed per kg of body weight. * ($p = <0.05$), ** ($p = <0.01$) significantly different from saline.

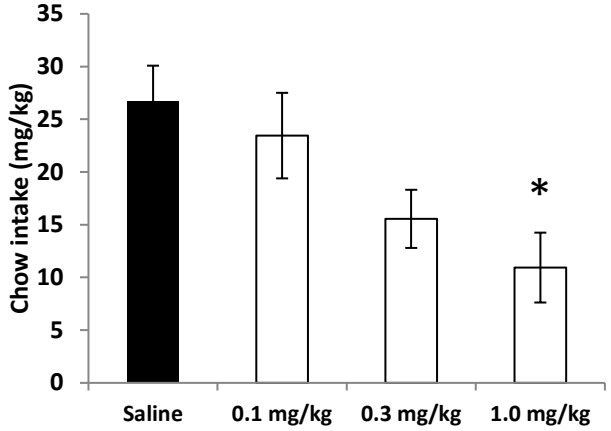


Figure 6: The effect of saline, 0.1, 0.3 and 1.0 mg/kg of NTX on deprivation induced chow intake consumption in male 16 month old mice. NTX was injected IP following overnight deprivation of regular chow, food intake was then measured after an hour. The data is recorded as mg of food consumed per kg of body weight. * (p = <0.05) significantly different from saline.

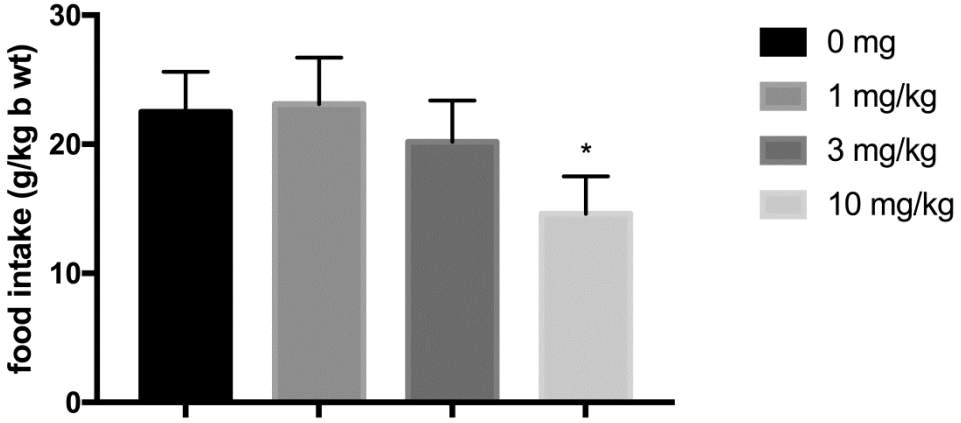


Figure 7: **The effect of saline, 1.0, 3.0 and 10 mg/kg of NLX on deprivation induced chow intake consumption in male 22 month old mice.** NTX was injected IP following overnight deprivation of regular chow, food intake was then measured after an hour. The data is recorded as mg of food consumed per kg of body weight. * (p = <0.05) significantly different from saline.

3.2. Experiment 2: Different doses of naltrexone are required to decrease sucrose intake in 6- 16 and 22-month old mice.

In 6- and 16-month old animals that were episodically exposed to the 10% sucrose solution, 0.3 mg/kg of naltrexone suppressed the 2-h consumption of the palatable tastant (Figures 8, 9). On the other hand, the oldest cohort of mice showed a decrease in sucrose intake only after a 10 mg/kg dose (Figure 10).

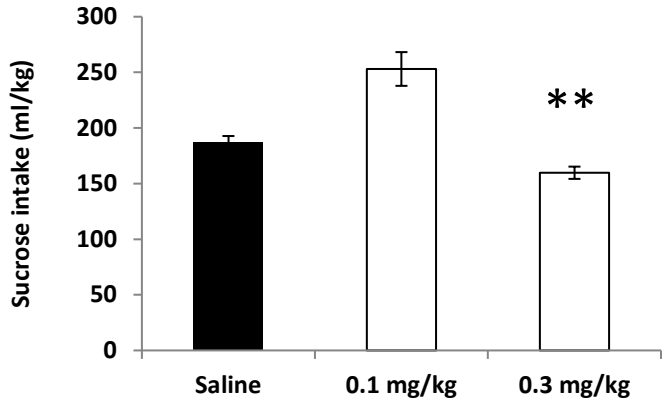


Figure 8: The effect of saline (control), 0.1, 0.3mg/kg of NTX on sucrose intake in 6 month old male mice. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * (p = <0.05), ** (p = <0.01) significantly different from saline.

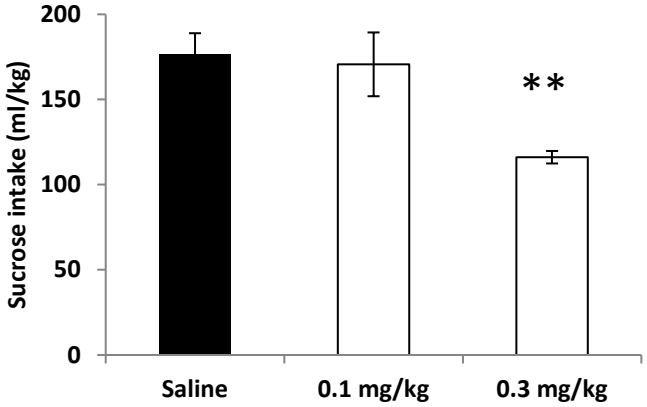


Figure 9: The effect of saline (control), 0.1, 0.3mg/kg of NTX on sucrose intake in 16 month male mice. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * (p = <0.05), ** (p = <0.01) significantly different from saline.

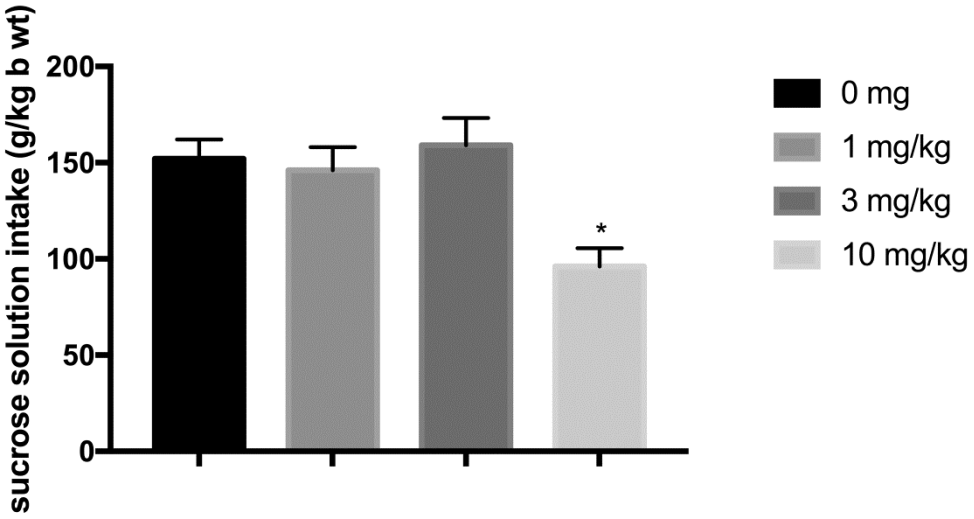


Figure 10: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on sucrose intake in 22 month old male mice. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * (p = <0.05), ** (p = <0.01) significantly different from saline.

3.3. Experiment 3: Naltrexone has no effect on saccharin intake in 16- and 22-month old mice, but it decreases consumption in the youngest cohort.

Episodic saccharin intake was decreased by ca. 30% in 6 month old mice injected with 1.0mg/kg of naltrexone (Figure 11). The same dose of the drug had no effect in the older cohort of 16-month old mice (Figure 12). Even 10 mg/kg of the compound was ineffective in the 22-month old animals (Figure 13).

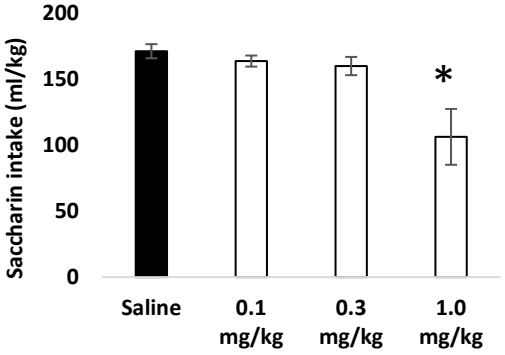


Figure 11: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on saccharin intake in 6 month old male mice. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured after 1 hour. The data is recorded as ml of saccharin consumed per kg of body weight.

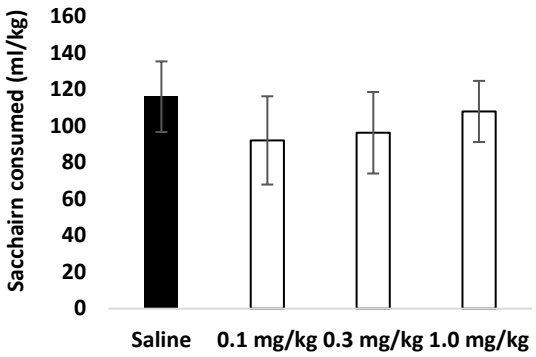


Figure 12: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on saccharin intake in 16 month old male mice. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured after 1 hour. The data is recorded as ml of saccharin consumed per kg of body weight.

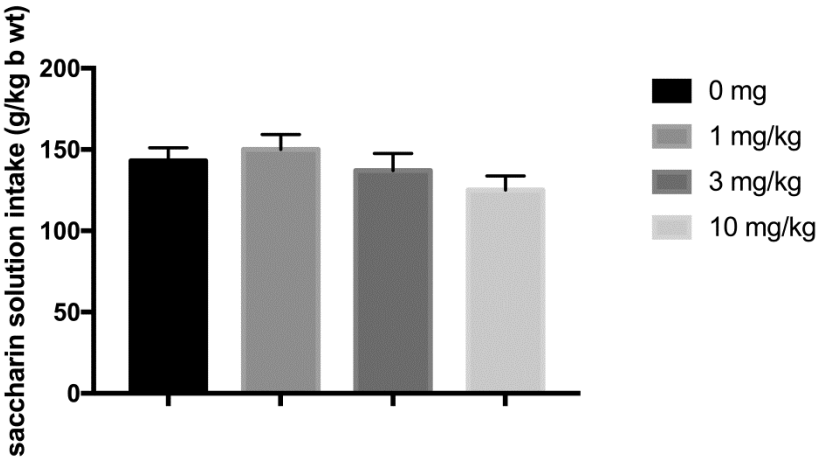
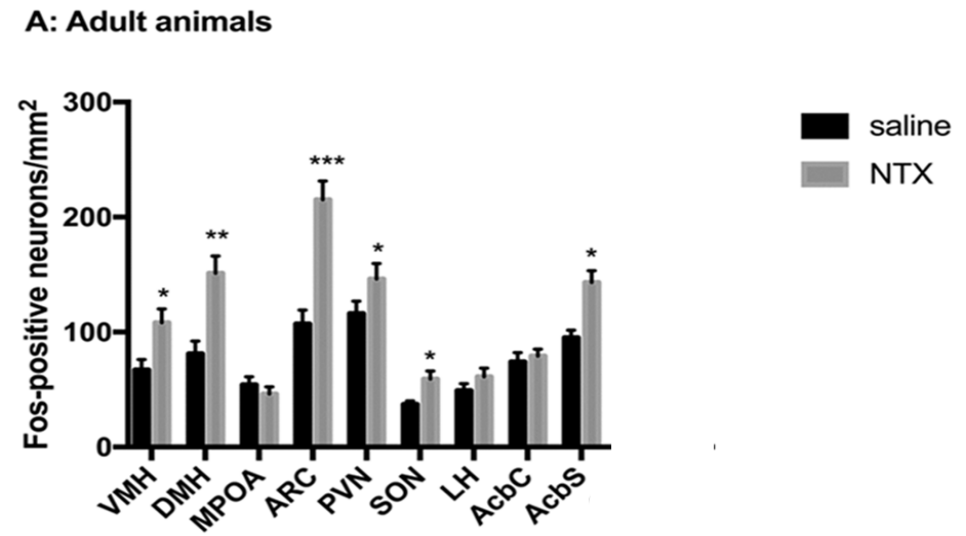


Figure 13: The effect of saline (control), 1.0, 3.0 and 10.0 mg/kg of NTX on saccharin intake in 22 month old male mice. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured after 1 hour. The data is recorded as ml of saccharin consumed per kg of body weight

3.4. Experiment 4: Naltrexone activates a different subset of feeding-related forebrain sites in adult vs old mice.

Among the seven hypothalamic areas analysed in this study, in the younger cohort, the VMH, DMH, ARC, PVN and SON show a significantly increased neuronal activation after the naltrexone treatment. In the older animals, the DMH, ARC and PVN exhibit higher Fos immunoreactivity, too, whereas the activation of the VMH and SON is unchanged in the naltrexone- vs saline-injected animals. On the other hand, significantly more LH neurons are Fos-positive in this cohort. Among the two reward-related sites, the nucleus accumbens shell is activated by naltrexone in the younger group, but the activation is the same in the older animals. (Figures 14 & 15).



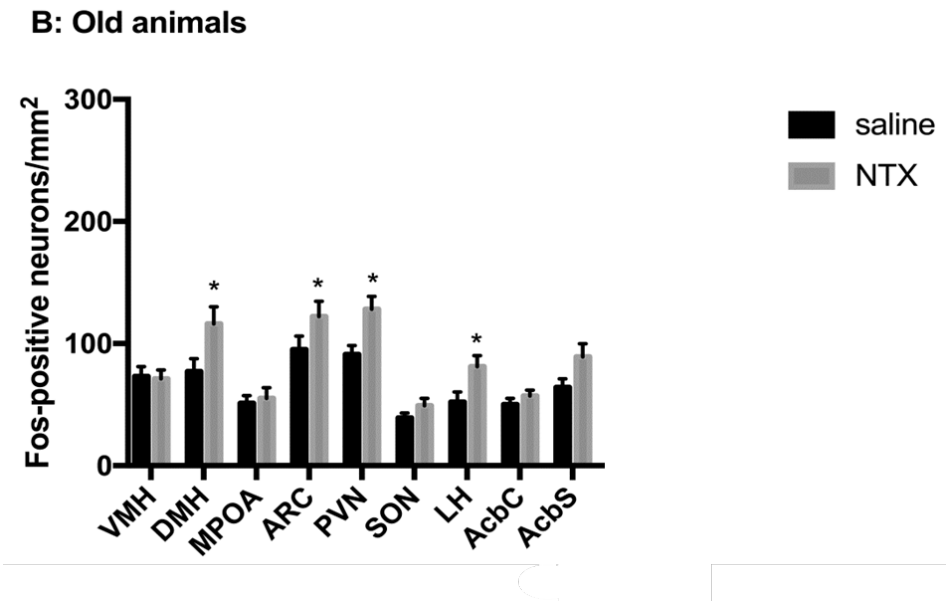


Figure 14: The effect of Saline and 1.0 mg/kg of NTX on neuronal activity in specific brain sites in 6 and 16 month old male mice. NTX was injected IP, after one hour mice were perfused with brains extracted for C-Fos activity. The data is recorded as the number of Fos positive neurons per mm^2. * (p = <0.05), ** (p= <0.01), *** (p= <0.001) significantly different from saline.

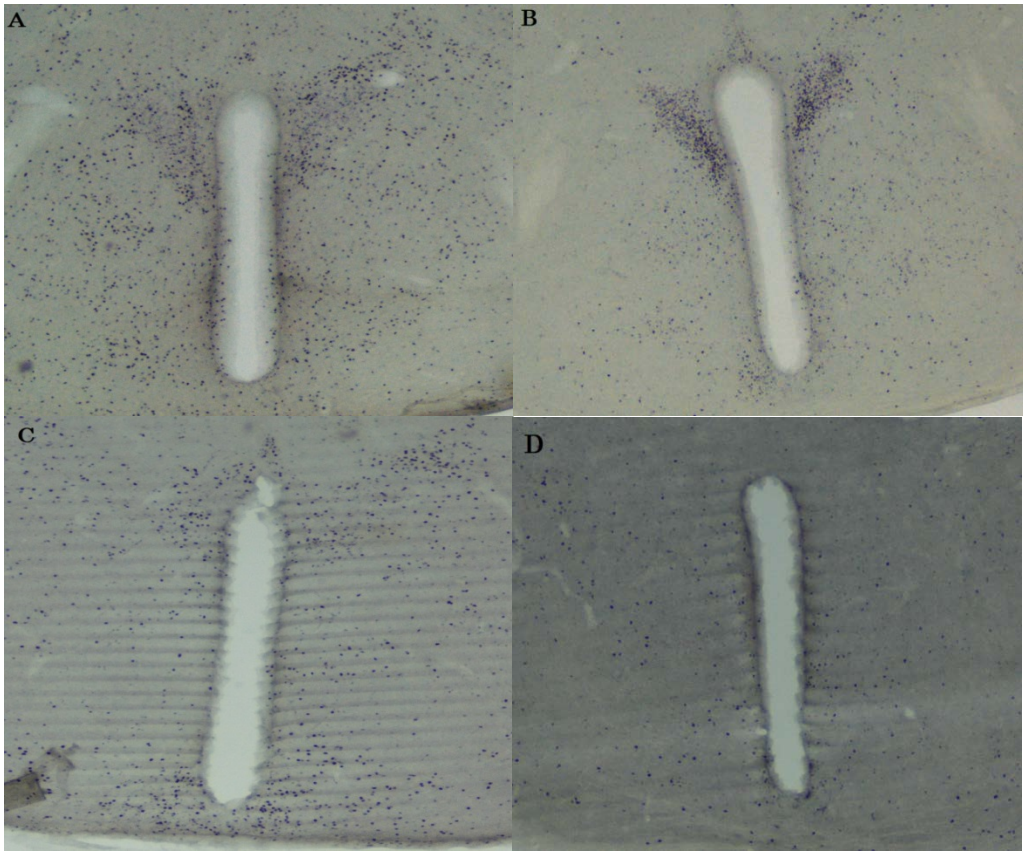


Figure15: Photomicrographs depicting c-Fos immunoreactivity within the paraventricular nucleus (PVN) of the hypothalamus of 6 and 16 month old male mice injected with saline or

naltrexone. **(A),** Saline and 6 m.o. mice **(B),** NTX and 6 m.o. mice **(C)** Saline and 16 m.o. mice**,** and **(D)** NTX and 16 m.o. mice.

3.5. Experiment 5: Naltrexone reduces deprivation-induced chow intake in 2 and 18 month old rats.

The two month old rats had a significant decline in deprivation induced intake at 0.3mg/kg of naltrexone (Figure 16) The same dose of the drug was required in 18 month old rats (Figure 17).

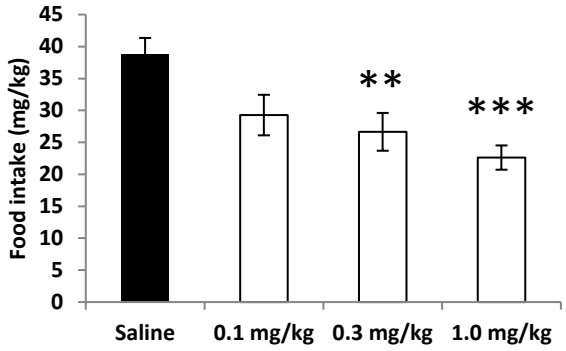


Figure 16: The effect of saline, 0.1, 0.3 and 1.0 mg/kg of NTX on deprivation induced food intake consumption in 2 month old rats. NTX was injected IP following overnight deprivation of regular chow, food intake was then measured. The data is recorded as mg of food consumed per kg of body weight. * (p = <0.05), ** (p= <0.01) significantly different from saline.

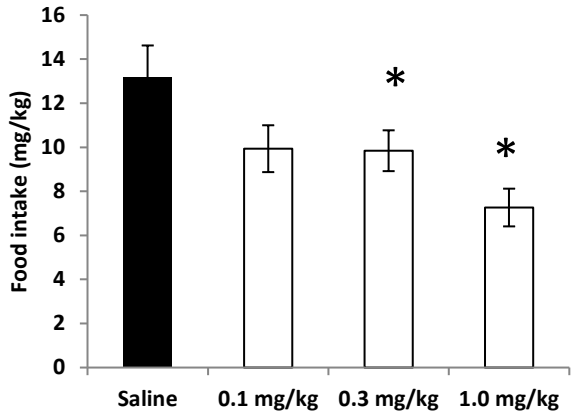


Figure 17: The effect of saline, 0.1, 0.3 and 1.0 mg/kg of NTX on deprivation induced food intake consumption in 18 month old rats. NTX was injected IP following overnight deprivation of regular chow,

food intake was then measured. The data is recorded as mg of food consumed per kg of body weight. * (p = <0.05), ** (p= <0.01) significantly different from saline.

3.5. Experiment 5: Naltrexone reduces sucrose intake in all age groups

A dose of 0.3mg/kg of naltrexone is needed to significantly reduce sucrose intake in 2 month old rats (Figure 18). A similar result is seen in the 18 month old rats as 0.3mg/kg of naltrexone is required also (Figure 19).

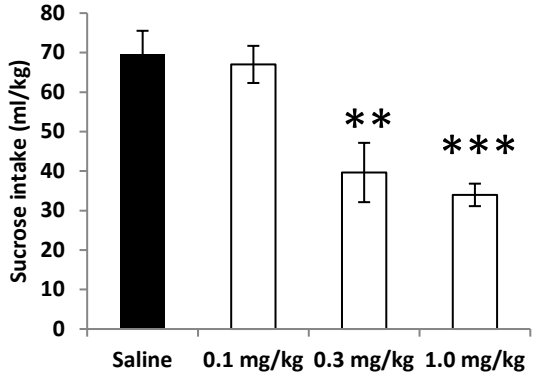


Figure 18: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on sucrose intake in 2 month old male rats. NTX was injected IP with sucrose being presented immediately after. Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * (p = <0.05), ** (p= <0.01), *** (p = <0.001) significantly different from saline.

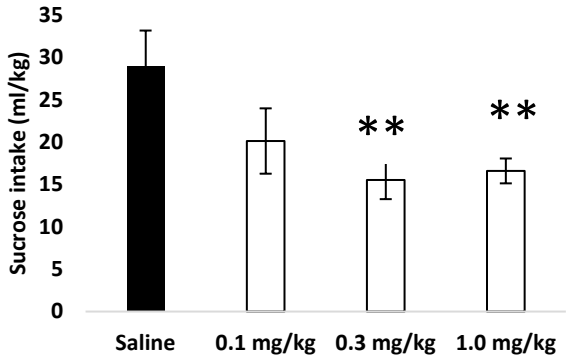


Figure 19: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on sucrose intake in 18 month old male rats. NTX was injected IP with sucrose being presented immediately after.

Sucrose intake was then measured. The data is recorded as ml of sucrose consumed per kg of body weight. * (p = <0.05), ** (p= <0.01) significantly different from saline.

3.6. Experiment 6: Naltrexone does not reduce consumption of saccharin in either age group.

Saccharin seems to be non-preferred in our Sprague Dawley strain of rats. Naltrexone administered at 1.0 mg/kg shows no significant suppression in 2- and 18- month old rats (Figures 20, 21).

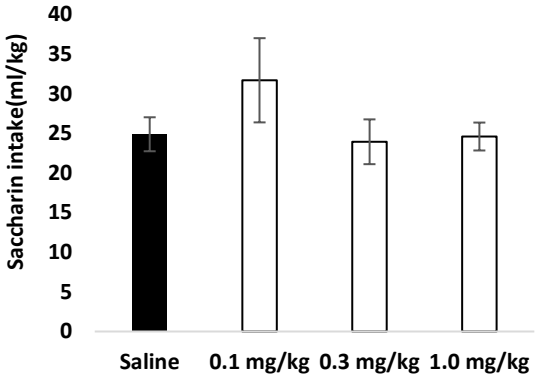


Figure 20: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on saccharin intake in 2 month male rats. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured. The data is recorded as ml of saccharin consumed per kg of body weight.

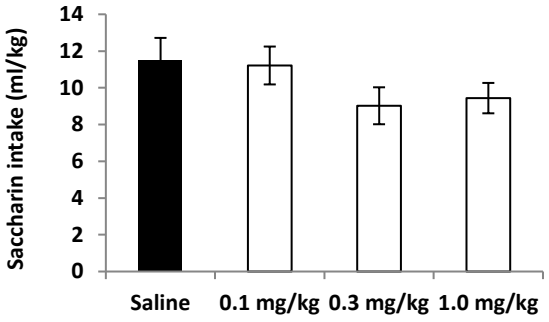


Figure 21: The effect of saline (control), 0.1, 0.3 and 1.0 mg/kg of NTX on saccharin intake in 18 month old male rats. NTX was injected IP with saccharin being presented immediately after. Saccharin intake was then measured after 1, 2 and 4 hours. The data is recorded as ml of saccharin consumed per kg of body weight.

3.7. Experiment 7: Butorphanol increases the consumption of ad libitum chow intake in 18 month old rats

Butorphanol injected at 1.0 mg/kg trends towards significance shown at 4 hours (Figure 22).

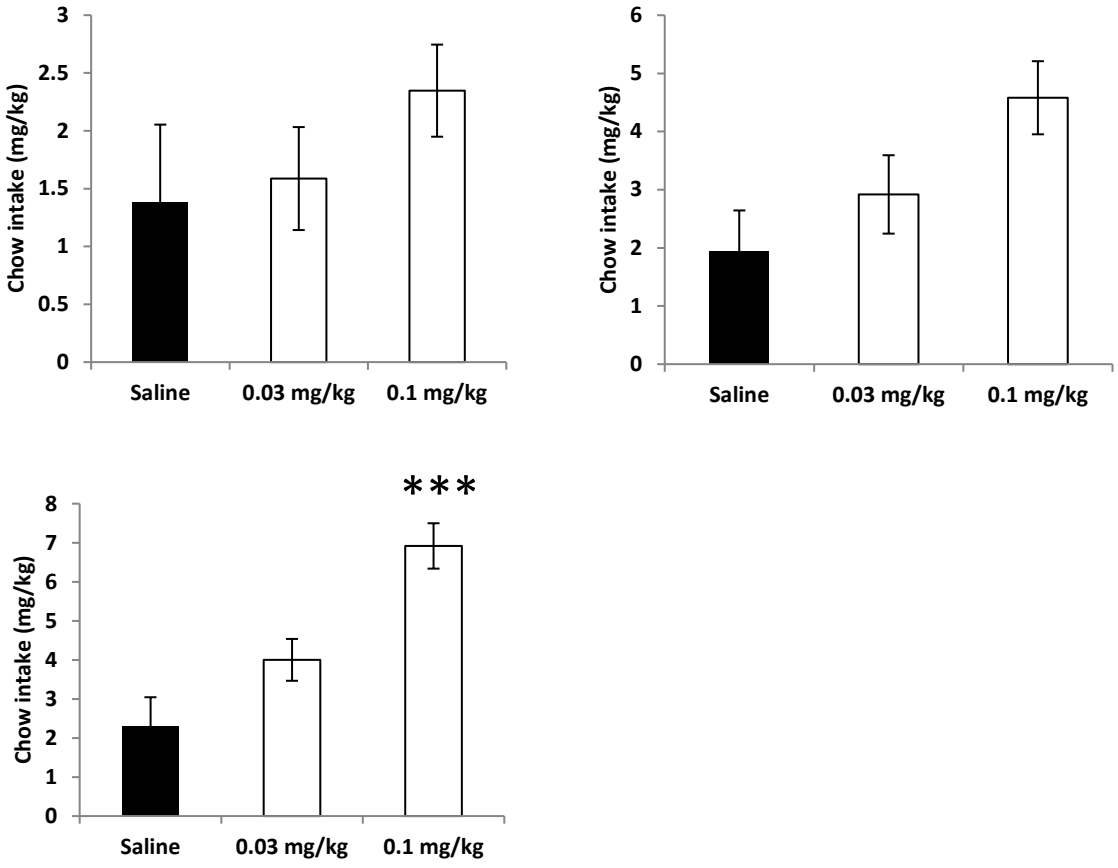


Figure 22: The effect of saline (control), 0.03 and 0.1 butorphanol on ad libitum chow intake in 18 month old male rats. Butorphanol was injected IP with regular chow being present ad libitum. Intake was then measured at 1, 2 and 4 hours. The data is recorded as mg of chow consumed per kg of body weight. * (p = <0.05), ** (p= <0.01), *** (p= <0.001) significantly different from saline.

4. Discussion:

As individuals age and reach the final stages of their lifespan, their risk of developing disturbances in food intake increase. One of the most typically seen problems is age-related anorexia in which individuals do not ingest sufficient amount of food to maintain their energy stores (94). This gradually leads to a decline in body weight, widely reported for humans and laboratory animal species (80,95). The second problem is associated with a decreased interest in palatable tastants (96). In this context, even when foods that are (and have earlier been) considered as highly attractive, are presented at an old age, they do not appear to convey the same motivating and rewarding value as during the earlier life stages (97).

The issue of a decreased motivational value of palatable foods at an old age is evident in the very few animal model studies available thus far, which have consistently shown that old rats and mice consume smaller amounts of palatable solid foods and palatable solutions in choice and no-choice scenarios (87). To further support the notion of diminished sensitivity to food-driven reward at an old age, initial experiments suggested that laboratory rodents injected with opioid receptor ligands display a shift in their responsiveness to orexigenic or anorexigenic properties of select doses of such compounds (93).

The current set of studies involving mouse and rat models confirms the previously reported phenomenon of diminished consumption of food at an older age. My experiments show that at an old age, mice display a diminished sensitivity to anorexigenic properties of an opioid receptor antagonist,

naltrexone; whereas adult rats that have not yet reached the age that would mirror a more advanced life stage (≤18 months), do not differ in their sensitivity to the drug. The c-Fos studies define a differential pattern of neuronal activation after naltrexone injection at a younger versus older age as a possible underlying reason for the changed feeding response at an older age to the same dose of the drug.

A decreased drive to obtain food associated with advanced age is confirmed by the outcome of the experiments presented in this thesis. Although not formally compared in the course of the study, it is evident that in all paradigms used herein, intakes of palatable tastants and "bland" chow were lower in more age-advanced cohorts of animals. For example, in mice, in deprivation-induced chow intake, the 22-month old mice eat on approximately 10g less of chow per kg/b. wt. compared to 6 month old mice. In sucrose-presented animals the 22 moth old consume approximately 40g less of sucrose per kg/ b. wt. compared to 6 month old mice. In the saccharin paradigm 18 month old mice consumed 40g less of saccharin per kg/b. wt. compared to 6 month old mice. This trend indicating a decrease in consumption is apparent even in two adult cohorts of rats – the 2-month old rats eat on average 40g of chow per kg/b. wt. and 70g of sucrose per kg of b. wt., whereas 18-month old rats eat 13g and 30g, respectively.

As suggested by previously done research, the current studies involving naltrexone injections in mice show that while the younger animals (6- and 16-months old) require 0.3-3 mg/kg b. wt. naltrexone to decrease any aspect of

food intake (palatability- and energy-driven), 22-month old mice need as much as 10 mg/kg b. wt. naltrexone to show hypophagia. In fact, in the saccharin experiment, even a 10-mg/kg dose was ineffective in the 22-month old group.

On the one hand, the data confirm the effect of opioid ligand administration in adult (not yet old) animals. In those studies, antagonists, such as naltrexone, typically cause a reduction in deprivation-induced chow intake at doses (in the case of naltrexone and naloxone) around 0.3-3 mg/kg b. wt., whereas consumption of palatable ingestants in such animals is generally suppressed by somewhat lower doses (0.1 mg/kg is oftentimes sufficient). This is in line with the concept of opioids affecting primarily the aspect of feeding driven by pleasure and – only to some extent – the component driven by energy needs (98). Conversely, opioid receptor agonists increase consumption of palatable foods and fluids more avidly than of “bland” tastants. For example, a mu opioid receptor agonist DAMGO injected directly into the Nacc, a key site of reward, increases intake of saccharin (99). An opiate, morphine, is particularly effective in increasing consumption of preferred tastants, but it also elevates intake of regular chow in sated animals (100). Kappa opioid receptor agonist, dynorphin A, also increases consumption of palatable tastants and it elevates operant responding for palatable sugar pellets (101). In line with that, the expression of kappa and mu opioid receptor changes upon exposure to palatability as well as energy deprivation (102, 71).

As opioid agonists and antagonists show differential effects on aging animals, it is crucial to note there is little information on why these differential effects occur

and the effect that aging has on neuronal activity in feeding-related sites. This gap in in knowledge had led me to ask the question as to whether animals at an old age differ from younger adult cohorts in the magnitude of anorexigenic responses to an opioid receptor antagonist naltrexone and whether feeding-related hypothalamic and accumbal areas display a different responsiveness to opioid antagonism in animals at an adult versus more advanced age. To begin this project, a naltrexone dose response curve to deprivation-induced chow intake, and sucrose and saccharin consumption without deprivation, was established in aging cohorts of mice. A lower dose of naltrexone (0.3mg/kg) is required to reduce deprivation-induced chow intake in 6- and 16-month old mice. The 22 month old mice were insensitive to lower doses; they required 10.0 mg/kg of naltrexone to cause a significant response. In line with these current results were the data obtained by Gosnell et al who studied the effects of naloxone in aging Fisher rats. Naloxone in the 2- and 12-month old rats supressed intake at all doses (0.1, 1.0 and 10.0mg/kg), whereas the rats aged 22 and 28 months were insensitive to naloxone's effects at all doses (93). Another study confirmed the suppressive effects of naltrexone in Syrian hamsters under the deprivation-induced feeding paradigm with decreased food intake and body weight (103).

In the episodic sucrose intake paradigm in mice in the current set of experiments, the same lower dose of 0.3mg/kg was required to decrease consumption in 6-and 16-month old animals. The 22-month old mice were insensitive to the effects of naltrexone at lower doses. This is an insensitivity not seen in the younger age groups. Many studies confirm the suppressive effect of

naltrexone on sucrose consumption. For example, Beczkowska et al in 1992, in a similar paradigm to this current study, found that naltrexone administration decreased sucrose consumption in a dose dependant manner in rodents (104). In rhesus monkeys, behaviour maintained by access to sucrose as a reward is reduced by central injections of naltrexone (105). Although there is abundant evidence of the suppressive effects of naltrexone on sucrose intake in many different experimental scenarios, studies on the effects of aging on sucrose-motivated eating behaviour remain limited.

The next step in the experimental phase was to establish the effect of naltrexone on 0.1% saccharin intake. Six-month old mice showed a decrease in consumption of saccharin at a lower dose (1.0 mg/kg). At 16 and 22 months old, the mice drank very little saccharin and naltrexone failed to suppress this further. Many studies have been able to reliably reduce saccharin intake following opioid antagonist administration. Beczkowska et al showed significant decreases of saccharin consumption following an ICV injection of naltrexone in rats (106). Conversely a study using an opioid receptor agonist DAMGO found an increase in saccharin intake (99).

There is little data on age-driven differences in brain circuitry's responsiveness to foods or eating-related cues in humans. One of the few fMRI studies published thus far reported that taste-derived activity of secondary and higher order taste processing and reward regions such as the orbitofrontal cortex, amygdala, hippocampus, thalamus, and caudate nucleus occurs in both older and younger individuals, but – surprisingly – additional brain areas are activated in the older

cohort (107). In another fMRI study in older adults with the metabolic syndrome, differences in the hypothalamic activity were noted upon sweet taste sensory stimulation (108). Animal data support the notion of differential brain responsiveness to food-related cues. For example, Kotz et al. showed that Fos immunoreactivity response to central injection of an orexigenic peptide, orexin A, is diminished in aged rats (109). Age-induced alterations in brain distribution of critical neural modulators of appetite, such as nitric oxide, dynorphin, and NPY, have also been observed (110).

Numerous studies have shown usability of c-Fos immunoreactivity mapping in defining the CNS sites of action of specific drugs as well as in defining brain areas associated with particular behavioural and physiological phenomena, including food consumption. Previous experiments have pointed that opioid receptor antagonists, such as naloxone and naltrexone, induce activation of specific neuronal populations that are involved in, among other phenomena, appetite control. For example, in animals subjected to a scheduled access to palatable food, naltrexone increases Fos immunoreactivity in the VTA, NAC shell, and central nucleus of the amygdala as well as the bed nucleus of the stria terminalis. In food restricted adult rats, naltrexone induced Fos expression in the LH, DMH and ARC (111). Finally, in sated rats, central naltrexone infusion elevates Fos in the LH (112). In line with those findings, morphine withdrawal increases Fos immunoreactivity in the PVN (113). Another opioid receptor antagonist, naloxone, causes an increase in c-Fos expression in the ARC of lactating dams. Funabashi et al reported that naloxone has a stimulatory effect on Fos expression in the mediobasal hypothalamus (114). Finally, pretreatment with

naloxone blocks the pheromone-induced changes in Fos patterns in the PVN (115).

Considering a differential responsiveness of the brain circuitry in aged vs younger individuals to food-related cues (both actual food-related sensory stimuli as well as molecules that promote changes in appetite, such as orexin), it is not surprising that in the current c-Fos experiments utilising naltrexone, a somewhat different set of brain sites was activated by the very same dose of the antagonist in each of the two groups. Differences were noted in the VMH and the SON, these two sites being activated in the 6-month old mice. Conversely, the LH was activated only in the older cohort. At the same time, while there was a clear difference in how the three aforementioned brain sites responded to naltrexone, there was a "core" of hypothalamic areas which exhibited elevated c-Fos immunoreactivity regardless of the animals' age. Those included the PVN, ARC, DMH and CeA. These sites may remain unchanged in response to naltrexone due to the fundamental roles they have in food intake regulation. The PVN, ARC and DMH relate to homeostatic feeding, their separate roles work together to form a coordinated feeding response. In line with that, the PVN is a key sit of integration from signals received from the CNS and the ARC, and it serves as a key source of neural and neurohormonal input to the pituitary (116). Injections of orexigenic hormone ghrelin, or conversely the anorexigenic hormone leptin into the PVN alters appetite accordingly (117, 118). The ARC also integrates information received from circulating hormones due to its weak BBB (119), information projects to relevant brain sites including the PVN (50). The ARC is the primary site for orexingenic neurons synthesising NPY and AgRP (37), and it projects to the

DMH (120). It should be particularly emphasised that the lack of increase in the nucleus accumbens shell defines a likely neuroanatomical component of the reward system whose diminished responsiveness to naltrexone might play a significant role to the diminished anorexigenic effect of this opioid receptor ligand in aged animals. It is very much in line with data showing that opioid receptor ligands increase food consumption particularly well when injected directly in the shell subdivision of the accumbens complex (121).

The activation of the LH in the old mice is interesting due to the combined role it plays in food intake regulation and the sleep-wake cycle. The LH is a key area for expression of orexin neurons. Opioid systems within the LH regulate feeding via orexin neurons. They cause orexigenic effects and increased arousal (122, 123). Studies have shown that orexin injected directly into the LH, increases ethanol drinking in rats (124). Other studies show that downregulation of opioid receptors within the LH decrease food intake via inhibition of orexin neurons. (60). Injecting naltrexone directly into the LH significantly reduces deprivation-induced feeding in mice (125). The LH and orexin neurons also play a significant role in sleep regulation involved in the sleep wake cycle. This is important as aging is not only associated with appetite disturbances, as sleep problems are common among the elderly (126). Orexins are found in high levels during the waking period and low levels during sleep (127). Orexin receptor knockout mice show a sleep disturbance behaviour known as narcolepsy in humans (128). In fact, orexin deficient narcolepsy is primarily defined by a difficulty to stay awake during the day (129). Sleep in the elderly is characterised by an earlier wake time compared to younger adults. There is also a tendency for older people to be

disrupted during sleep and therefore is aging is associated with a decrease in the duration and the consolidation of the sleep (127). As the older old mice in this current study showed activity in the LH, it would be of interest to look into this area further and assess the mechanisms that contribute to dysregulated sleep and appetite processes on neurochemistry and transcriptional activity at an advanced age.

As an additional pilot study, I also wished to examine by using the rat model whether adult animals that are less advanced in age show a differential responsiveness to naltrexone. I therefore compared two cohorts – one whose age was 2 months at the beginning of the experimental trials (thus, they can be considered as entering the adult phase of their lifespan), and the other that was 18 months, thus the animals that still do not show the full repertoire and behavioural and physiological changes associated with an old age. I found that the same 0.3mg/kg of b. wt. dose of naltrexone was necessary to decrease deprivation-induced chow intake and episodic sucrose intake in rats at both ages. Surprisingly, I did not detect a naltrexone-induced reduction in saccharin consumption in any of the age cohorts. It should be noted, however, that our rats seem to consume quite low quantities of saccharin compared to sucrose, which suggests that they may be non-preferrers of saccharin. That rats can differ greatly in their inherent preference for saccharin has been well documented in previous studies, in which preference for this tastant could be achieved even by selective breeding for this phenotype (130, 131). Consequently, intake of a non-preferred saccharin may not be sensitive to opioid antagonism.

In order to provide additional evidence that there is no difference in sensitivity between the varying ages of adult rats in my project, aside from testing these animals in naltrexone injection studies, I also assessed whether they would differ in sensitivity to an opioid receptor agonist, butorphanol. older group of rats. The results showed an increase in chow intake available ad libitum in both age groups. Notably, Gosnell et al, who also used burtorphanol in found that the two older groups of rats aged 22 and 28 months old were much less responsive to the opioid agonist than their younger counterparts (93). It indicates that 18 months of age is not yet the threshold age in rats to display different sensitivity to orexigenic or anorexigenic properties of ligands that modify activity of the opioid system.

5. Conclusions:

The findings indicate that old (22-month old) mice exhibit diminished responsiveness to anorexigenic properties of naltrexone in feeding paradigms that involve eating for energy (deprivation-induced intake of “bland” chow) and eating for reward (consumption of low-/non-calorie solutions containing sucrose and saccharin). The c-Fos immunoreactivity analysis indicates that unlike in adult mice at a younger age, in old animals, naltrexone fails to activate the nucleus accumbens shell, the ventromedial and hypothalamic nuclei, and it activates the lateral hypothalamus. These c-Fos data provide an insight into neural responsiveness changes that might underscore a differential feeding regulatory outcomes seen after naltrexone administration. Finally, naltrexone is similarly effective in reducing food intake in 2-month old rats as in the 18-month old animals (i.e., in the latter case, the adult rats that have not yet reached the old age).

6. References:

1) Berthoud, H. (2002). Multiple neurosystems controlling food intake and body weight. *Neuroscience and Biobehavioural Reviews,* 26(4), 393-428.

2) Broberger, C. (2005). Brain regulation of food intake and appetite: molecules and networks. *Journal of Internal Medicine,* 258(4), 301-327.

3) Havel, P. J. (2001).Peripheral signals conveying metabolic information to the brain: short-term and long-term regulation of food intake and energy homeostasis. *Experimental Biology and Medicine*,226, (11), 963 – 977.

4) Blevins, J. E., & Baskin, D. G. (2009). Hypothalamic-brainstem circuits controlling eating. In *Frontiers in eating and weight regulation* (Vol. 63, pp. 133-140). Karger Publishers.

5) Leibowitz, Hammer, & Chang. (1981). Hypothalamic paraventricular nucleus lesions produce overeating and obesity in the rat. *Physiology & Behavior*, 27(6), 1031-1040.

6) Lang, Heil, Ganten, Hermann, Rascher, & Unger. (1983). Effects of lesions in the paraventricular nucleus of the hypothalamus on vasopressin and oxytocin contents in brainstem and spinal cord of rat. *Brain Research,* 260(2), 326-329.

7) Arnett, M. G., Muglia, L. M., Laryea, G. & Muglia, L. J. (2015). Genetic Approaches to Hypothalamic-Pituitary-Adrenal Axis Regulation. *Neuropsychopharmacology,* 41(1), 245-260.

8) Holder, Butte & Zinn (2000). Profound obesity associated with a balanced translocation that disrupts the SIM1 gene. *Human Molecular Genetics,* 9(1), 101-108.

9) Sim1 haploinsufficiency causes hyperphagia, obesity and reduction of the paraventricular nucleus of the hypothalamus. (2001). *Human Molecular Genetics*, 10(14), 1465-1473.

10) Uchoa, Da Silva, De Castro, Antunes-Rodrigues, & Elias. (2010). Corticotrophin-releasing factor mediates hypophagia after adrenalectomy, increasing meal-related satiety responses. *Hormones and Behavior,* 58(5), 714-719.

11) Olszewski, Cedernaes, Olsson, Levine, & Schiöth. (2008). Analysis of the network of feeding neuroregulators using the Allen Brain Atlas. *Neuroscience and Biobehavioral Reviews,* 32(5), 945-956.

12) Song, Levin, Stevens, & Sladek, (2014). Supraoptic oxytocin and vasopressin neurons function as glucose and metabolic sensors. *American Journal of Physiology*, 306 (7), 447-456.

13) Stricker, & Hoffmann. (2007). Presystemic signals in the control of thirst, salt appetite, and vasopressin secretion. *Physiology & Behavior,* 91(4), 404-412.

14) Olszewski, Klockars, Schiöth, & Levine. (2010). Oxytocin as feeding inhibitor: Maintaining homeostasis in consummatory behavior. *Pharmacology, Biochemistry and Behavior*, 97(1), 47-54.

15) Bellinger, & Bernardis. (1999). Effect of dorsomedial hypothalamic nuclei knife cuts on lesioned behaviour. *American Journal of Physiology,* 276(6), 1772-1779.

16) Bellinger, Bernardis, & Williams. (1983). Naloxone suppression of food and water intake and cholecystokinin reduction of feeding is attentuated in weanling rats with dorsomedial hypothalamic lesions. *Physiology & Behavior,* 31(6), 839-846.

17) Shimizu, Oomura, Plata-Salamán, & Morimoto. (1987). Hyperphagia and obesity in rats with bilateral ibotenic acid-induced lesions of the ventromedial hypothalamic nucleus. *Brain Research*, 416(1), 153-156.

18) Rao, & Prabhakar. (1992). Effects of body weight loss and taste on VMH-LH electrical activity of rats. *Physiology & Behavior*, 52(6), 1187-1192.

19) Broberger, Lecea, Sutcliffe, & Hokfelt. (1998). Hypocretin/Orexin- and melanin-concentrating hormone-expressing cells form distinct populations in the rodent lateral hypothalamus: Relationship to the neuropeptide Y and agouti gene-related protein systems. *Journal of comparative neurology,* 402(4), 460-474.

20) Sakurai, T. (2013). Roles of orexin in modulating arousal, feeding and motivation. *Frontiers in Behavioral Neuroscience*, 7(NA), NA.

21) Bellinger, & Bernardis. (2002). The dorsomedial hypothalamic nucleus and its role in ingestive behavior and body weight regulation: Lessons learned from lesioning studies. Physiology & Behavior, 76(3), 431-442

22) Honda, Wada, Battey, & Wank. (1993). Differential Gene Expression of CCK A and CCK B Receptors in the Rat Brain. *Molecular and Cellular Neuroscience, 4*(2), 143-154.

23) Cone, Cowley, Butler, Fan, Marks, & Low. (2002). The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *International Journal of Obesity*, 25(5), 63.

24) Banks, W. (2008). The blood-brain barrier: Connecting the gut and the brain. *Regulatory Peptides*, 149(1), 11-14.

25) Broadwell, & Brightman. (1976). Entry of peroxidase into neurons of the central and peripheral nervous systems from extracerebral and cerebral blood. *Journal of Comparative Neurology,* 166(3), 257-283.

26) Lutter, M., & Nestler, E. (2009). Homeostatic and Hedonic Signals Interact in the Regulation of Food Intake1-3. *The Journal of Nutrition, 139*(3), 629-32.

27) Drazen, Vahl, D'Alessio, Seeley, & Woods. (2006). Effects of a fixed meal pattern on ghrelin secretion: evidence for a learned response independent of nutrient status. *Endocrinology,* 147(1), 23-30.

28) Keen-Rhinehart, & Bartness. (2005). Peripheral ghrelin injections stimulate food intake, foraging, and food hoarding in Siberian hamsters. *America Journal of Physiology,* 288(3), 716-722.

29) Asakawa, Inui, Kaga, Katsuura, Fujimiya, Fujino, & Kasuga. (2003). Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut*, 52(7), 947.

30) Klok, M., Jakobsdottir, S., & Drent, M. (2007). The role of leptin and ghrelin in the regulation of food intake and body weight in humans: A review. *Obesity Reviews*, 8(1), 21-34.

31) Batterham, Cowley, Small, Herzog, Cohen, Dakin, . . . Bloom. (2002). Gut hormone PYY3-36 physiologically inhibits food intake. *Nature,* 418(6898), 650.

32) Challis, Pinnock, Coll, Carter, Dickson, & O'rahilly. (2003). Acute effects of PYY 3–36 on food intake and hypothalamic neuropeptide expression in the mouse. *Biochemical and Biophysical Research Communications,* 311(4), 915-919.

33) Batterham, R., Cohen, M., Ellis, S., Le Roux, C., Withers, D., Frost, G., . . . Bloom, S. (2003). Inhibition of food intake in obese subjects by peptide YY^sub 3-36^. *The New England Journal of Medicine*, 349(10), 941-8.

34) Air, Benoit, Blake Smith, Clegg, & Woods. (2002). Acute third ventricular administration of insulin decreases food intake in two paradigms. *Pharmacology, Biochemistry and Behavior*, 72(1), 423-429.

35) Brief, & Davis. (1984). Reduction of food intake and body weight by chronic intraventricular insulin infusion. *Brain Research Bulletin,* 12(5), 571-575.

36) Zigman, J.M., & Elmquist, J.K. (2003). Minireview: From anorexia to obesity-The yin and yang of body weight. *Endocrinology,* 144(9), 3749-3756.

37) Hahn, T.M., Breininger, J.F., Baskin, D.G., & Schwart, M.W. (1998). Coexpression of Agrp and NPY in fasting-activated hypothalamic neurons. *Nature Neuroscience*, 1(4), 271.

38) Schwartz, M., Seeley, R., Woods, S., & Weigle, D. (1997). Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the Rostral arcuate nucleus. *Diabetes*, 46(12), 2119-2.

39) Cowley, Smart, Rubinstein, Cerdán, Diano, Horvath, . . . Low. (2001). Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature*, 411(6836), 480.

40) Parker, & Bloom. (2012). Hypothalamic neuropeptides and the regulation of appetite. *Neuropharmacology,* 63(1), 18-30.

41) Olszewski, & Levine. (2007). Central opioids and consumption of sweet tastants: When reward outweighs homeostasis. *Physiology & Behavior,* 91(5), 506-512.

42) Sclafani, A. (1987). Carbohydrate-induced hyperphagia and obesity in the rat: Effects of saccharide type, form, and taste. *Neuroscience and Biobehavioral Reviews,* 11(2), 155-162.

43) Spector, & Smith. (1984). A detailed analysis of sucrose drinking in the rat. *Physiology & Behavior,* 33(1), 127-136.

44) Tordoff, M. G. (2002). Obesity by choice: the powerful influence of nutrient availability on nutrient intake. *American Journal of Physiology,* 1536-1539.

45) Jurdak, Lichtenstein, & Kanarek. (2008). Diet-induced obesity and spatial cognition in young male rats. *Nutritional neuroscience,* 11(2), 48-54.

46) Gosnell, Krahn, & Majchrzak. (1990). The effects of morphine on diet selection are dependent upon baseline diet preferences. Pharmacology, Biochemistry and Behavior, 37(2), 207-212.

47) Weldon, O'Hare, Cleary, Billington, & Levine. (1996). Effect of naloxone on intake of cornstarch, sucrose, and polycose diets in restricted and nonrestricted rats. *American Journal of Physiology,* 270(6), 1183-1188.

48) Dimeglio, & Mattes. (2000). Liquid versus solid carbohydrate: Effects on food intake and body weight. *International Journal of Obesity,* 24(6), 794.

49) Rolls, E. (2012). Taste, olfactory and food texture reward processing in the brain and the control of appetite. 71(4), 488-501.

50) Stanley, S., Wynne, K., McGowan, B., & Bloom, S. (2005). Hormonal regulation of food intake. *Physiological Reviews,* 85(4), 1131-1158.

51) Berthoud, H. (2004). Mind versus metabolism in the control of food intake and energy balance. *Physiology & Behavior,* 81(5), 781-793.

52) Adcock, Thangavel, Whitfield-Gabrieli, Knutson, & Gabrieli. (2006). Reward-Motivated Learning: Mesolimbic Activation Precedes Memory Formation. *Neuron*, 50(3), 507-517.

53) Zellner, & Ranaldi. (2010). How conditioned stimuli acquire the ability to activate VTA dopamine cells: A proposed neurobiological component of reward-related learning. *Neuroscience and Biobehavioral Reviews*, 34(5), 769-780.

54) Shirayama, Y., & Chaki, S. (2006). Neurochemistry of the Nucleus Accumbens and its Relevance to Depression and Antidepressant Action in Rodents. *Current Neuropharmacology*, 4(4), 277-291.

55) Albertin, Mulder, Tabuchi, Zugaro, & Wiener. (2000). Lesions of the medial shell of the nucleus accumbens impair rats in finding larger rewards, but spare reward-seeking behavior. *Behavioural Brain Research*, 117(1), 173-183.

56) Shimura, Kamada, & Yamamoto. (2002). Ventral tegmental lesions reduce overconsumption of normally preferred taste fluid in rats. *Behavioural Brain Research*, 134(1), 123-130.

57) Carelli, R. (2002). Nucleus accumbens cell firing during goal-directed behaviors for cocaine vs. 'natural' reinforcement. *Physiology & Behavior*, 76(3), 379-387.

58) Avena, N., & Mello, Nancy K. (2007). Examining the Addictive-Like Properties of Binge Eating Using an Animal Model of Sugar Dependence. *Experimental and Clinical Psychopharmacology*, 15(5), 481-491.

59) Lammel, S., Lim, B.K., Ran, C., Huang, K.W., Betley, M.J., Tye, K.M., . . . Malenka, R.C. (2012). Input-specific control of reward and aversion in the ventral tegmental area. *Nature,* 491(7423), 212.

60) Berridge, K. (2009). 'Liking' and 'wanting' food rewards: Brain substrates and roles in eating disorders. *Physiology & Behavior*,97(5), 537-550.

61) Wise, & Schwartz. (1981). Pimozide attenuates acquisition of lever-pressing for food in rats. *Pharmacology, Biochemistry and Behavior,* 15(4), 655-656.

62) Geary, & Smith. (1985). Pimozide decreases the positive reinforcing effect of sham fed sucrose in the rat. *Pharmacology, Biochemistry and Behavior*, 22(5), 787-790.

63) Baldwin, Ebenezer, & De La Riva. (1990). Effects of intracerebroventricular injection of muscimol or GABA on operant feeding in pigs. *Physiology & Behavior,* 48(3), 417-421.

64) Tong, Ye, Jones, Elmquist, & Lowell. (2008). Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance. *Nature Neuroscience,* 11(9), 998.

65) Arnt, & Scheel-Krüger. (1979). GABA in the ventral tegmental area: Differential regional effects on locomotion, aggression and food intake after microinjection of GABA agonists and antagonists. *Life Sciences*, 25(15), 1351-1360.

66) Wu, Boyle, & Palmiter. (2009). Loss of GABAergic Signaling by AgRP Neurons to the Parabrachial Nucleus Leads to Starvation. *Cell,* 137(7), 1225-1234.

67) Glass, M.J., Billington, C.J., & Levine, A.S. (2000). Naltrexone administered to central nucleus of amygdala or PVN: neural dissociation of diet and energy. *American Journal of Physiology*, 279(1), 86-92.

68) Cooper, S. (1983). Effects of opiate agonists and antagonists on fluid intake and saccharin choice in the rat. *Neuropharmacology*, 22(3), 323-328.

69) Levine, Weldon, Grace, Cleary, & Billington. (1995). Naloxone blocks that portion of feeding driven by sweet taste in food-restricted rats. *American Journal of Physiology,* 268(1), 248-252.

70) Drewnowski, Krahn, Demitrack, Nairn, & Gosnell. (1992). Taste responses and preferences for sweet high-fat foods: Evidence for opioid involvement. *Physiology & Behavior,* 51(2), 371-379.

71) Olszewski, Alsiö, Schiöth, & Levine. (2011). Opioids as facilitators of feeding: Can any food be rewarding? *Physiology & Behavior,* 104(1), 105-110.

72) Morley, & Levine. (1983). Involvement of dynorphin and the kappa opioid receptor in feeding. *Peptides,* 4(6), 797-800.

73) Mckay, Kenney, Edens, Williams, & Woods. (1981). Intracerebroventricular beta-endorphin increases food intake of rats. *Life Sciences,* 29(14), 1429-1434.

74) Meye, & Adan. (2014). Feelings about food: The ventral tegmental area in food reward and emotional eating. *Trends in Pharmacological Sciences,* 35(1), 31-40.

75) Levine, A., & Billington, C. (1989). Opioids Are They Regulators of Feeding?. *Annals of the New York Academy of Sciences,* 575(1), 209-220.

76) Macintosh, Morley, & Chapman. (2000). The anorexia of aging. *Nutrition,* 16(10), 983-995.

77) Birch, L. L., & Fisher, J. O. (1998). Development of eating behaviors among children and adolescents. *Pediatrics,* 101(3), 539-549.

78) Scaglioni, S., Arrizza, C., Vecchi, F., & Tedeschi, S. (2011). Determinants of children's eating behavior. The American Journal of Clinical Nutrition, 94.

79) American Psychiatric Association. Task Force on DSM-IV. (1994). Diagnostic and statistical manual of mental disorders: DSM-IV (4th ed.). Washington, DC: American Psychiatric Association.

80) Donini, L. M., Savina, C., & Cannella, C. (2003). Eating habits and appetite control in the elderly: The anorexia of aging. *International Psychogeriatrics*, 15(1), 73-87.

81) Hetherington, M. H. (1998). Taste and appetite regulation in the elderly. *Proceedings of the Nutrition Society,* 57, 625-631.

82) Guido, Perna, Carrai, Barale, Grassi, & Rondanelli. (2016). Multidimensional evaluation of endogenous and health factors affecting food preferences, taste and smell perception. *The Journal of Nutrition, Health & Aging,* 20(10), 971-981.

83) Matsuo R. (2000). Role of saliva in the maintenance of taste sensitivity. *Critical Reviews in Oral Biology & Medicine,* 11, 216-29.

84) Feng, P., Huang, L., & Wang, H. (2014). Taste Bud Homeostasis in Health, Disease, and Aging. *Chemical Senses,* 39(1), 3-16.

85) Doty, R., Shaman, P., Applebaum, S., Giberson, L., Siksorski, & Rosenberg. (1984). Smell identification ability: Changes with age. *Science,* 226(4681), 1441-1443.

86) Morley, J.E. (2001). Decreased food intake with aging. *The Journals of Gerontology*, 56a, 81-8.

87) Frutos, Pistell, Ingram, & Berthoud. (2012). Feed efficiency, food choice, and food reward behaviors in young and old Fischer rats. *Neurobiology of Aging,* 33(1), 41-53.

88) Renteria, Silbaugh, Tolentino, & Gilbert. (2008). Age-related changes in conditioned flavor preference in rats. *Behavioural Brain Research,* 188(1), 56-61.

89) Malatynska, Steinbusch, Redkozubova, Bolkunov, Kubatiev, Yeritsyan, . . . Strekalova. (2012). Anhedonic-like traits and lack of affective deficits in 18-month-old C57BL/6 mice: Implications for modeling elderly depression. *Experimental Gerontology,* 47(8), 552-564.

90) Akimoto, Yosuke, Kanai, Setsuko, Ohta, Minoru, Akimoto, Saeko, Uematsu, Hiroshi, & Miyasaka, Kyoko. (2012). Age-associated reduction of stimulatory effect of ghrelin on food intake in mice. *Archives of Gerontology and Geriatrics,* 55(2), 238-243.

91) Wolden-Hanson, T. (2006). Mechanisms of the anorexia of aging in the Brown Norway rat. *Physiology & Behavior*, 88(3), 267-276.

92) Kavaliers, & Hirst. (1985). The influence of opiate agonists on day-night feeding rhythms in young and old mice. *Brain Research*, 326(1), 160-167.

93) Gosnell, Levine, & Morley. (1983).The effects of aging on opioid modulation of feeding in rats. *Life Sciences,* 32(24), 2793-2799.

94) Wysokiński, Sobów, Kłoszewska, & Kostka. (2015). Mechanisms of the anorexia of aging—a review. *AGE*, 37(4), 1-14.

95) Wolden-Hanson, T. (2006). Mechanisms of the anorexia of aging in the Brown Norway rat. *Physiology & Behavior*, 88(3), 267-276.

96) Nieuwenhuizen, Weenen, Rigby, & Hetherington. (2010). Older adults and patients in need of nutritional support: Review of current treatment options and factors influencing nutritional intake. *Clinical Nutrition,* 29(2), 160-169.

97) De Boer, Ter Horst, & Lorist. (2013). Physiological and psychosocial age-related changes associated with reduced food intake in older persons. *Ageing Research Reviews,* 12(1), 316-328.

98) Marks-Kaufman, Balmagiya, & Gross. (1984). Modifications in food intake and energy metabolism in rats as a function of chronic naltrexone infusions. *Pharmacology, Biochemistry and Behavior,* 20(6), 911-916.

99) Zhang, M., & Kelley, A. (2002). Intake of saccharin, salt, and ethanol solutions is increased by infusion of a mu opioid agonist into the nucleus accumbens. *Psychopharmacology,* 159(4), 415-423.

100) Glass, Grace, Cleary, Billington, Levine. (1996). Potency of naloxone's anorectic effect in rats is dependent on diet preference. *American Journal of Physiology,* 271, 217–221..

101) Cooper, Jackson, & Kirkham. (1985). Endorphins and food intake: Kappa opioid receptor agonists and hyperphagia. *Pharmacology, Biochemistry and Behavior,* 23(5), 889-901.

102) Olszewski, Shaw, Grace, Höglund, Fredriksson, Schiöth, & Levine. (2009). Complexity of neural mechanisms underlying overconsumption of sugar in scheduled feeding: Involvement of opioids, orexin, oxytocin and NPY. *Peptides,* 30(2), 226-233.

103) Jones, & Corp. (2003). Effect of naltrexone on food intake and body weight in Syrian hamsters depends on metabolic status. *Physiology & Behavior,* 78(1), 67-72.

104) Beczkowska, Bowen, & Bodnar. (1992). Central opioid receptor subtype antagonists differentially alter sucrose and deprivation-induced water intake in rats. *Brain Research,* 589(2), 291-301.

105) Williams, K., Winger, G., Pakarinen, E., & Woods, J. (1998). Naltrexone reduces ethanol- and sucrose-reinforced responding in rhesus monkeys. *Psychopharmacology*, 139(1), 53-61.

106) Beczkowska, Koch, Bostock, Leibowitz, & Bodnar. (1993). Central opioid receptor subtype antagonists differentially reduce intake of saccharin and maltose dextrin solutions in rats. *Brain Research*, 618(2), 261-270.

107) Jacobson, Green, & Murphy. (2010). Age-related functional changes in gustatory and reward processing regions: An fMRI study. *Neuroimage,* 53(2), 602-610.

108) Jacobson, Green, Haase, Szajer, & Murphy. (2017). Age-related changes in gustatory, homeostatic, reward, and memory processing of sweet taste in the metabolic syndrome: An fMRI study. *Perception*.

109) Kotz, Mullet, & Wang. (2005) Diminished feeding responsiveness to orexin A (hypocretin 1) in aged rats is accompanied by decreased neuronal activation. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, 289(2), 359-366.

110) Morley. (1997). Anorexia of aging: physiologic and pathologic mechanisms. *American Journal of Clinical Nutrition*, (66), 760–773.

111) Carr, Park, Zhang, & Stone. (1998). Neuroanatomical patterns of Fos-like immunoreactivity induced by naltrexone in food-restricted and ad libitum fed rats. *Brain Research*, 779(1), 26-32.

112) Li, Olszewski, Shi, Grace, Billington, Kotz, & Levine. (2006). Effect of opioid receptor ligands injected into the rostral lateral hypothalamus on c- fos and feeding behavior. *Brain Research,* 1096(1), 120-124.

113) Johnstone, Brown, Meeran, Vuijst, Brooks, Leng, & Russell. (2000). Local morphine withdrawal increases c-fos gene, Fos protein, and oxytocin gene expression in hypothalamic magnocellular neurosecretory cells. *Journal of Neuroscience*, 20(3), 1272-1280.

114) Funabashi, T., Jinnai, K., & Kimura, F. (1997). Fos Expression by Naloxone in LHRH Neurons ofthe Mediobasal Hypothalamus and Effects of Pentobarbital Sodium in the Proestrous Rat. *Journal of Neuroendocrinology*, 9(2), 87-92.

115) Kobayashi, Kiyokawa, Takeuchi, & Mori. (2005). Neural correlates underlying naloxone-induced amelioration of sexual behavior deterioration due to an alarm pheromone. Frontiers in *Neuroscience* , 9.

116) Sawchenko & Swanson. (1983). The organization and biochemical specificity of afferent projections to the paraventricular and supraoptic nuclei. *Progress in Brain Research*, 60, 19-29.

117) Elmquist, Ahima, Maratos-Flier, Flier, & Saper. (1997). Leptin activates neurons in ventrobasal hypothalamus and brainstem. *Endocrinology*, 138(2), 839-842.

118) Lawrence, Snape, Baudon, & Luckman. (2002). Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology*, 134(1), 155-162.

119) Rodríguez, Blázquez, & Guerra. (2010). The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: The former opens to the portal blood and the latter to the cerebrospinal fluid. *Peptides,* 31(4), 757-776.

120) Kalra, Dube, Pu, Xu, Hovath, & Kalra. (1999). Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocrine Reviews,* 20(1), 68-100.

121) Stratford, T. (2007). The Nucleus Accumbens Shell as a Model of Integrative Subcortical Forebrain Systems Regulating Food Intake-Chapter 3. In *Appetite and Body Weight* (pp. 27-65).

122) Sakurai, Amemiya, Ishii, Matsuzaki, Chemelli, Tanaka, . . . Yanagisawa. (1998). Orexins and Orexin Receptors: A Family of Hypothalamic Neuropeptides

and G Protein-Coupled Receptors that Regulate Feeding Behavior. *Cell*, 92(4), 573-585.

123) Chen, Yu-Wei, Barson, Jessica R., Chen, Aimee, Hoebel, Bartley G., & Leibowitz, Sarah F. (2013). Glutamatergic Input to the Lateral Hypothalamus Stimulates Ethanol Intake: Role of Orexin and Melanin-Concentrating Hormone. *Alcoholism: Clinical and Experimental Research*, 37(1), 123-131.

124) Schneider, E., Rada, P., Darby, R., Leibowitz, S., & Hoebel, B. (2007). Orexigenic Peptides and Alcohol Intake: Differential Effects of Orexin, Galanin, and Ghrelin. *Alcoholism: Clinical and Experimental Research*, 31(11), 1858-1865.

125) Ardianto, Yonemochi, Yamamoto, Yang, Takenoya, Shioda, . . . Kamei. (2016). Opioid systems in the lateral hypothalamus regulate feeding behavior through orexin and GABA neurons. *Neuroscience,* 320, 183-193.

126) Bonanni, Tognoni, Maestri, Salvati, Fabbrini, Borghetti, . . . Murri. (2010). Sleep disturbances in elderly subjects: An epidemiological survey in an Italian district. *Acta Neurologica Scandinavica*, 122(6), 389-397.

127) Yoshida, Fujiki, Nakajima, Ripley, Matsumura, Yoneda, . . . Nishino. (2001). Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light–dark cycle and sleep–wake activities. *European Journal of Neuroscience,* 14(7), 1075-1081.

128) Hoyer, & Jacobson. (2013). Orexin in sleep, addiction and more: Is the perfect insomnia drug at hand? *Neuropeptides,* 47(6), 477-488.

129) Tahara, Y., Takatsu, Y., Shiraishi, T., Kikuchi, Y., Yamazaki, M., Motohashi, H., . . . Shibata, S. (2017). Age-related circadian disorganization caused by sympathetic dysfunction in peripheral clock regulation. *NPJ Aging and Mechanisms of Disease*, 3.

130) Regier, Carroll, & Meisel. (2012). Cocaine-induced c-Fos expression in rats selectively bred for high or low saccharin intake and in rats selected for high or low impulsivity. *Behavioural Brain Research*, 233(2), 271-279.

131) Radke, Zlebnik, & Carroll. (2015). Cocaine withdrawal in rats selectively bred for low (LoS) versus high (HiS) saccharin intake. *Pharmacology, Biochemistry and Behavior*, 129, 51-55.