Connexin 36 as a Regulator of Consummatory Behaviour

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Abstract

Gap junctions enable metabolic and electrical coupling of adjacent cells. Connexin 36 (Cx36) is a gap junction protein found predominantly in mammalian neurons. Because Cx36 is expressed in many areas involved in the regulation of food intake, its role in this was explored. I therefore investigated whether genetic knockout (KO) of Cx36 affects the intake of various sweet tastants during long-term concurrent feeding with bland chow, and affects intake of sweet tastants alone during short-term feeding. In addition, I investigated whether a conditioned taste aversion (CTA) would be altered by KO of Cx36. LiCl was injected intraperitoneally (IP) following exposure to a sweet novel tastant. 48 hours later mice were given a two-bottle preference test of tastant vs. water to determine aversive response. Cx36 KO animals consumed less sweet palatable tastants and consumed more bland chow during long-term intake. Sweet tastant consumption was similarly increased during short-term intake. This is suggestive of Cx36 being implicated in both reward mediated and homeostatic regulation. A CTA was enhanced by the KO of Cx36, potentially due to its role in the reward system, and/or an effect of hypothalamic endocrine nuclei implicated in the acquisition of a CTA. In summary, Cx36 may be involved in reward system response to sweet palatable food consumption, and the magnitude of aversive response. Cx36 may also be implicated in post-ingestive endocrine food intake and aversion regulation.

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Contents

List of figures

List of abbreviations

GJ	Gap junction
IP	Intraperitoneal
KO	Knockout
LiCl	Lithium chloride
OT	Oxytocin
PVN	Paraventricular nucleus of the hypothalamus
SON	Supraoptic nucleus
VTA	Ventral tegmental area
WT	Wild type
CNS	Central nervous system
NAc	Nucleus Accumbens
ER	Endoplamic reticulum
Cx	Connexin
CaM	Calmodulin
DA	Dopamine
MAPK	Mitogen-activated protein kinase
PKC	Protein kinase C
PKA	cAMP-dependent protein kinase
CaMKII	Ca2+/calmodulin-dependent protein kinase II
NPY	Neuropeptide Y
a-MSH	Alpha-melanocyte stimulating hormone
AGRP	Agouti-related protein
CCK	Cholescystokinin

1

1. Introduction

1.1 Gap Junctions

Gap junctions are permeable channels established between adjacent cells that enable metabolic and electrical coupling. They are created when a hexameric connexon structure of connexin proteins on one cell docks to another connexon hemichannel on a neighbouring cell[1,2]. The formed channel allows exchange of small hydrophilic molecules up to 1kD such as ions and secondary messengers[2]. Gap junctions are crucial for cells located distantly from blood vessels, the shared cytoplasm means cell nourishment can be facilitated cell to adjacent cell. Conversely, gap junctions can uncouple to protect healthy cells by isolating them from dysfunctional cells[3]. The ionic permeability of gap junctions allows propagation of electrical signals to gap junction connected cells. Gap junctions are found throughout the vertebrate body in most tissues such as the CNS, reproductive organs, retina, liver, kidney, and even in bone[1,4]. A specialized role for each connexin is suspected as they show tissue specific expression, despite most tissues expressing at least two connexins[5].

There are a number of tissues such as the heart and brain that require much faster electrical signal transmission than allowed by synaptic chemical communication. This requirement is met by membrane-bound gap junctions allowing for ultra-fast intercellular transmission between cells in immediate proximity to each other,

bypassing the need to send chemicals extracellularly[6]. In addition to increased speed of signal transmission, gap junctions give the ability for the synchronization and co-ordination of groups of cells[7,8].

Evidence of this is shown by the heart which requires a coordinated rhythmic contraction. Cardiomyocytes require action potential synchrony and coordinated contraction to maintain the correct function of the heart[9]. This propagation is possible because sodium ions can travel cell to cell through gap junctions located at intercalated discs[10]. There is evidence that a loss of synchrony or decreased gap junction conduction is implicated in arrhythmias and ventrical fibrillation[11,12,13]. Another example of an electrical synapse requirement is the role of gap junctions during parturition. During pregnancy, gap junctions are not present in uterine myocardium smooth muscle; which may be a requirement for the maintenance of gestation[14]. Immediately prior to parturition, there is rapid formation of gap junction plaques in the myocardium. These plaques remain only throughout partruition and for a short time following[15]. As a result of this production of gap junctions, internal resistance of myometrial cells decreases, enhancing cell coupling which may be implicated in synchrony of uterine smooth muscle contractions during labour. Further, the formation of myometrial gap junctions may be hormonally controlled both locally and systemically. This has been shown to be modulated by estrogen and prostaglandin, and progesterone induced depression of gap junctions[15,16,17]. These examples illustrate the wide range of functions and variety of tissues gap junctions are an integral part of.

1.2 Connexins

Connexins are one of three gap junction protein families, and are found in chordates, whereas gap junction proteins from invertebrates are called innexins

and evolved independently as evidenced by the lack of amino acid homology[18]. Interestingly, all three families share strong structural similarities regardless of their evolutionary history[19]. There are however vertebrate proteins with amino acid homology to innexins, called panexins, though these are thought to rarely create gap junctions with other cells[20]. Pannexins may instead remain as hemi-channels and mediate cell activity by releasing chemicals into the extracellular space between cells[21]. In mammals, genes have been found for 20-21 different connexins[22,23]. The nomenclature of each connexin is determined by its molecular size in kilodaltons (kD), and the abbreviation "Cx" for the name "connexin", eg. Cx36 is 36 kD[23].

1.3 Structure

These connexin proteins combine with 5 other connexins to construct hexamers called connexons. These connexon hemichannels then pair with another connexon on another cell, linking the two cytoplasms, with the gap junction bridging a 2nm space separating the cells[22] (Fig. 1.1). This extracellular space was first visualized with the introduction of lanthanum which penetrated and differentiated the space in contrast to tight junctions which lanthanum could not pass through[24,25,26].

All connexins proteins are constructed of four a-helical transmembrane domains, a cytoplasmic loop, two extra-cellular loops, an N-terminal and C-terminal. The extracellular loops between M1 & M2 and M3 & M4 each contain three cystein residues which are important in creating the alignment of connexons when forming a gap junction[2,27] (Fig. 1.2). The four transmembrane domains and the extracellular loops are well conserved allowing the formation of connexons constructed of differing connexins[22]. In contrast to the remainder of the connexin

protein; both the intracellular loop and primarily C-terminal have a variable sequence and length[28], and these domains are targeted by a number of different proteins for post-translational modification[29]. It is these differences that predominantly determine the molecular differences between connexins and the differences in functionality and activity between connexons of various connexin compositions[30].

Connexons can be constructed with one type of connexin; called a homomeric connexon or with multiple connexin types; called a heteromeric connexon. This ability to combine different connexins is carried through to the entire gap junction itself, where two identical homomeric connexons from adjoining cells can dock to form a homotypic gap junction, or two differing connexons can dock to form heterotypic gap junctions[22]. There are only specific combinations of heterotypic junctions that can form, and the composition of gap junctions from various connexins subsequently alters their function by changing the permeability, ion selectivity, conductivity and metabolite selectivity[30,31].

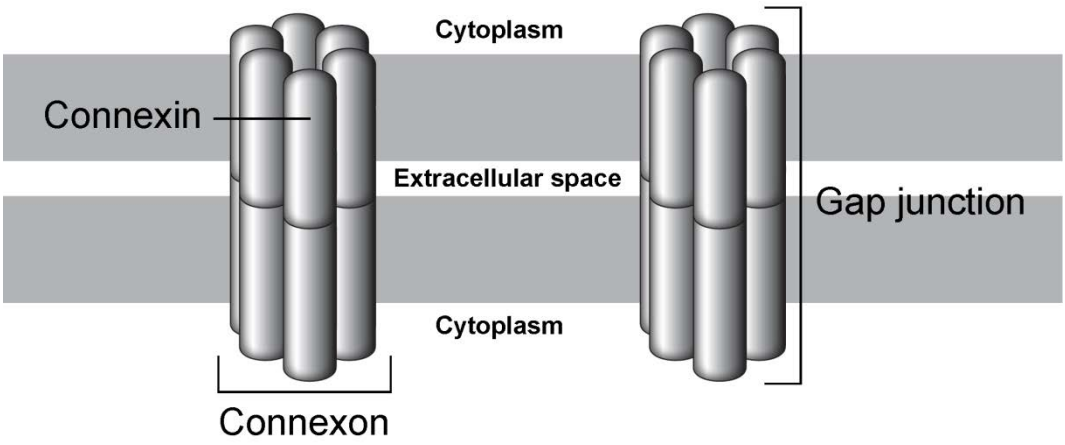

Figure.1.1 A gap junction is an intercellular channel connecting cytoplasms of two cells. Each gap junction bridges a 2mm extracellular space and is constructed of two opposing connexon hemichannels; each a six connexin heaxamer.

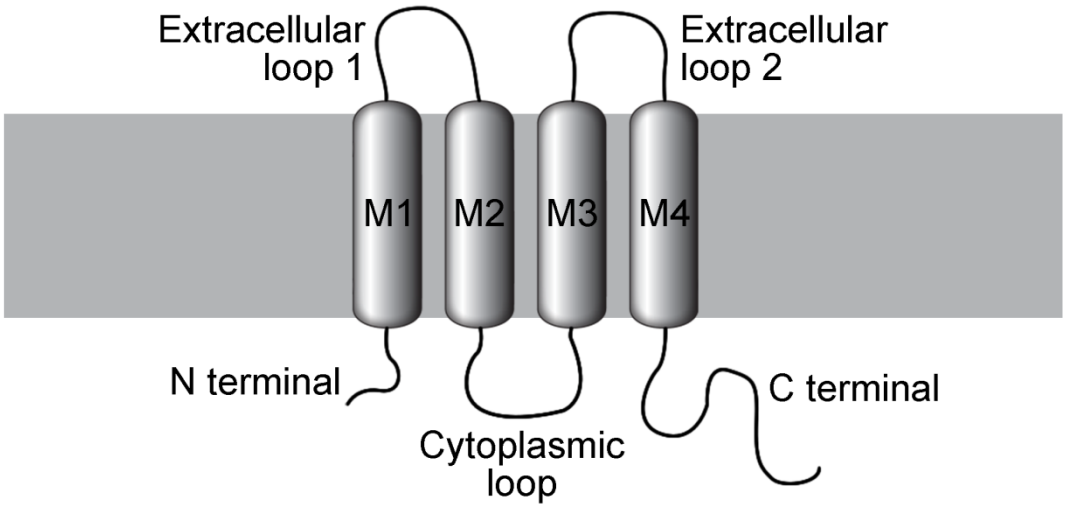

Figure.1.2 Connexins have four transmembrane domains and two extracellular loops which are well conserved. The C terminal and cytoplasmic loop domains are targets for post-translational modification.

1.4 Gap Junction Lifecycle

Connexin proteins are synthesized and trafficked by the secretory pathway: they are first synthesized by ribosomes on the rough endoplamic reticulum (ER) before being co-translationally inserted into the ER membrane, then trafficked in vesicles to the golgi apparatus via the trans-golgi network[32]. The particular connexin isoform determines where they oligomerize with other connexins to form connexons; some are formed in the ER and others in the golgi apparatus. Following oligomerization, connexons are transported to the cellular membrane in transport vesicles assisted by microtubules where they become membrane-bound, and are available as hemi-channels to form gap junctions with adjoining cells[33]. Once inserted into the cell membrane, gap junctions congregate into groups of hundreds or thousands of channels by migrating laterally into clusters called plaques[34]. Plaques are maintained beyond the lifecycle of single connexons by the introduction of new connexons to the outside edge the plaque. Conversely,

the recycling of older gap junctions occurs from within the middle of the plaque. The addition of new connexons to the periphery and removal from the centre enables gap junction stability. Removal of gap junctions occurs by endocytosis of entire sections of the gap junction plaques by one of the two connected cells[35,32]. Degradation of these connexosomes is by both proteosome and lysosome pathways.[36]

1.5 Regulation

A number of factor influence the regulation of gap junction channel gating; transjunctional voltage (i.e., the difference in voltage between two connected cells), increases in Ca2+ levels, pH, and phosphorylation[3,37]

1.5.1 Voltage

Large voltages will induce the closure of gap junction channels, although the level required is variable depending on the isoform of the connexins involved. Gap junctions are maximally open when the transjunctional voltage is at 0mV, and begin to close as the transjunctional voltage changes either negatively or positively[38]. An example of this is Cx36 found in the brain, which at 100mV, has ten times the half-maximal inactivation voltage than the most sensitive connexin; Cx45, found in Purkinje fibres of the heart, at 10mV[39'40].

The sensor responsible for detecting these changes in connexin transjunctional voltage has been found to be located at the N- terminals which are situated at the pore entrance of connexons[41]. Amino acid residues on the N-terminal domain have been shown to be either positively or negatively charged, and when these amino acids are altered experimentally, the polarity of the gating is affected[38]. It is hypothesized that the N terminals are flexible and stabilized by hydrogen bonds

forming a funnel to open the channel, and then migrate across the channel and form a plug to close the channel[42].

1.5.2 Calcium

It is thought that the closure and uncoupling of gap junction channels in response to intracellular Ca2+ functions as a way to protect healthy cells. Increased Ca2+ occurs as a result of the malfunction or breakdown of cells, therefore isolating these cells prevents healthy cells from becoming affected[3]. Experiments illustrating the effect of Ca2+ on gap junctions were performed by injections of large concentrations of Ca2+ into the cytoplasm of gap junction joined cells, which induced uncoupling[43]. Ca2+ itself may not directly act upon connexins, rather the protein calmodulin (CaM) could be the intermediary molecule responsible for the inhibitory effects[3]. CaM has been shown to bind to various parts of the cytoplasmic structure of connexins depending on the isoform. In Cx36, CaM binds to the c-terminal in response to increased Ca2+[44], whereas it binds to the cytoplasmic loop in Cx43[45]. The involvement of CaM has been further confirmed by studies using blockers such as calmidazolium which prevented the Ca2+ induced uncoupling of cells, and even increased coupling[46]. CaM is thought to function by physically blocking the pore and thereby closing the channel.

1.5.3 pH

Decreasing the intracellular level of pH causes a closure of gap junctions. pH sensitivity to acidification is highly sensitive but variable, depending on the connexin isoform involved. Confounding this is conflicting data in neuronal Cx36 channels which may be unique to Cx36 alone. Studies showed gap junction closure with decreased pH[47], but others later found Cx36 uncoupling as a result of alkalinisation which normally increases coupling in other connexin channels. It has been proposed that Cx36 channels are different in that they may have both

acidification and alkalinisation gating mechanisms[48]. Because some connexins can be highly specific in their expression, certain tissues will be more susceptible to intracellular acidification. In contrast, extracellular acidification appears to have no effect on gap junction communication[49]. Despite this, open uncoupled connexin hemichannels respond to low extracellular pH by closing, however this is likely to occur when H+ ions enter through the hemichannel and close the channel from within the cytoplasm by binding to the same internal site as in formed gap junctions[50]. Causes of acidification can be due to glycolysis and phosphorylation, and as a result of anaerobic energy production. Unlike gap junction regulation by Ca2+, it is likely that H+ ions react directly with connexins. This is expected as the uncoupling of gap junctions and closure of uncoupled connexon hemichannels has been shown to be instantaneous when acidification was induced by CO2[51]. Protonation of Cx43 was seen by Beahm and Hall, which highlights the ability of connexins to react directly with H+ ions without the use of an intermediary compound, unlike Ca2+ gap junction regulation[50]. The location of the connexin responsible for pH regulation is the C-terminal and the intracellular loop. Further to this, elimination of the C-terminal of Cx43 resulted in a complete lack of sensitivity to acidification, leaving the channel open[52]. Finally, other compounds with the ability to bind to the same location have been shown to partially inhibit pH induced uncoupling[53]. This direct binding may alter binding of the C terminal to the cytoplasmic loop to act as a 'ball and chain' where the C terminal; the ball, obstructs the pore channel. Interestingly, coexpression of CX43 and Cx40 in heterotypic gap junctions of Xenopus oocytes shows an elevated sensitivity to lowered pH than either Cx43 or Cx40 alone[54].

1.5.4 Phosphorylation

Phosphorylation is crucial to a number of regulatory mechanisms: connexin synthesis, connexon assembly, trafficking, membrane insertion and gating

activity[55]. During connexon oligomerization and trafficking to the cell membrane, phosphorylation ensures the hemichannels remain in a closed state until they are activated as hemichannels or coupled as gap junctions[56]. Gap junction communication is affected when phosphorylation alters both the opening and closing of gap junctions[55]. A number of protein kinases are implicated in post-translational modification of connexins, including serine/threonine-specific protein kinases such as; protein kinase C (PKC), cAMP-dependent protein kinase (PKA), Ca2+/calmodulin-dependent protein kinase II (CaMKII) and mitogen-activated protein kinase (MAPK)[57]. The site of connexin phosphorylation is the C-terminal as this domain contains a number of serine and tyrosine residues available for phosphorylation, of which multiple serine residues are primarily targeted. The C terminal location has been determined by sequence scanning, although the cytoplasmic loop is also a target for connexins Cx26, Cx56 and Cx36[58,59,60]. Cx26 is the only known connexin without C terminal phosphorylation sites as unlike all other connexins; it is not a phosphoprotein[61]. No phosphorylation of the N terminal has been found thus far in any connexin. Due to the variability in the C terminal of specific connexin isoforms, phosphorylation differentially regulates gap junction functionality depending on their constitution.

1.6 Cx36

Of all connexins discovered, Cx36 is the predominant connexin found in the mammalian central nervous system. It was initially discovered in 1998 as an ortholog to Cx35, which was previously found expressed in the retinal gap junctions of skate[62]. Cx36 is ubiquitously expressed throughout the CNS; in the hippocampus, cerebellum, thalamus, amygdala, ventral tegmental area, and nucleus accumbens along with others[63,]. Being a part of numerous regions responsible for many functions shows the importance of Cx36 to a large range of

neuronal mechanisms. The hippocampus is important in memory and learning processes, and the presence of Cx36 suggest a vital role in these as experiments on Cx36 knockout mice have demonstrated impairment in both memory and learning[64]. CX36 is expressed in neuroendocrine cells of the hypothalamus that produce hormones such as oxytocin (OT), vasopressin (VP), gonadotropin releasing hormone (GnRH), corticotrophin releasing hormone (CRH) and growth hormone releasing hormone (GHRH)[65]. Due to the wide-ranging effects and systems these hormones control and interact with, it is reasonable to presume that Cx36 is likely to be implicated in many of these, however research into the role of Cx36 in the hypothalamus is currently limited.

The temporal expression of Cx36 illustrates a role in the development of the CNS. In mice, Cx36 is first expressed in the mouse embryo forebrain at 9.5 days which is the beginning of neurogenesis. The peak occurs at the second postnatal week which is thought to correspond with early environmental stimuli. From this point expression decreases until it reaches the lower levels seen in the adult brain[66].

1.7 Cx36 Structure

Cx36 has a 321 amino acid sequence, and contains the intracellular loop; identified as the site for the majority of regulatory interaction, which is a 99 amino acid chain containing a glycine rich sequence of 18 amino acids.

The Cx36 or *gap junction delta-2 protein* (GJD2) gene is highly conserved across identified species, where the human Cx36 gene is 98% identical to both the mouse and rat Cx36 gene. Interestingly this is also 80% identical to the skate and 83% identical to perch Cx35[67]. In the Cx35/Cx36 connexin group, the gene is constructed of two exons separated by a 1.05 kb intron that begins in the coding region 71 bp after the initial translation site. Most other connexin genes in

comparison consist of an intron in an untranslated region, with the entire uninterrupted coding sequence in a single exon[68]. In mice the gene has been mapped to chromosome 2F3 which shows homology to 15q14, which is where the location has been mapped in human Cx36.[69]

Cx36 is exclusively assembled into homomeric connexons, and dock with identical connexons on adjacent cells to form homotypic gap junctions[22]. This exclusivity means that Cx36 gap junctions retain the same specific properties wherever they are expressed. Cx36 is only found in GABAergic interneurons, and no other connexin isotype has been found in GABAergic interneurons[70]. Voltage sensitivity and single channel conductance are both extremely low in comparison to other connexins, which may have the benefit of the channel remaining in the open state over a larger range of neuronal activity.

1.8 Cx36 and eating behaviour

The role of the reward system in the consumption of palatable foods has been most shown in studies linking obesity and food addiction to the same neural pathways as other more established substance based addictions[71,72,73]. It is these same brain regions and pathways in which Cx36 may play a crucial role.

The reward system of the brain evolved as a mechanism to enhance survival and reproduction. Behaviours that are needed for this such as eating, drinking and sex, stimulate the reward regions resulting in subjective feelings of wellbeing. The behaviour becomes associated with these feelings, acting as a reinforcer, thereby ensuring its future repetition[74].

Anatomical structures of the mesolimbic pathway of the reward system are the prefrontal cortex, amygdala, and most importantly the ventral tegmental area (VTA) and nucleus accumbens (NAc) which are the main reward centres[75]. The

primary mediator of this pathway is dopamine (DA). The VTA sends dopaminergic projections to the NAc, which release DA into the NAc and is responsible for feelings of wellbeing[76,77]. GABAergic neurons of the VTA however, act as inhibitors of dopaminergic neurons, controlling the amount of DA released in the NAc[78].

Exogenous drugs responsible for addiction hijack the reward system, often stimulating it to a degree far beyond that of the natural stimuli it evolved to respond to. Addictive drugs do not all act in the same way, but ultimately they all increase the amount of DA in the NAc[79]. An example of this is cocaine, which prevents reuptake of dopamine in the synaptic cleft of dopaminergic neurons, leaving more DA circulating in the synaptic cleft[80]. Another example is opiate based drugs. These bind to natural opiate receptors and are believed to act on GABAergic neurons in the VTA, preventing their inhibitory effects on DA release into the NAc by dopaminergic neurons, again resulting in increased DA in the NAc[81].

The intake of palatable food is mediated by the mesolimbic dopamine pathway, and even natural palatable stimuli can cause abnormal regulation as in the case of food addiction and its physical manifestation, obesity[82]. Pleasure derived from palatable food is normal and does not indicate dysregulation, as food intake is controlled by a number of other systems. Hunger and satiety signals controlling energy balance interact with the reward system by respectively inducing neuronal response to food during hunger, and reducing the response as a result of satiety[83]. There is a complex interplay between neural and neuroendocrine regulators of metabolism initiated in the periphery to maintain energy and bodyweight homeostasis[84]. These signals are integrated within various regions of the hypothalamus such as the arcuate nucleus. Examples of these regulatory compounds are Ghrelin; a major orexigenic hormone released by the anterior

pituitary which increases the motivation to eat, and leptin; a peptide released by adipose tissue which initiates feeding termination. Leptin acts on the hypothalamus and directly on VTA dopaminergic neurons reducing the reward value of palatable foods[85]. Rats administered ghrelin had an increased activation of the reward system, enhancing the hedonic value of platable food[86]. Conversely, leptin directly injected into the VTA caused inhibition of DA, consequently reducing food intake[85].

As with increased duration of reward-stimulating drug administration, long-term consumption of highly palatable foods results in a decreased responsiveness by the reward system. This tolerance is a compensation of the brain to attempt to regain homeostasis; however, the decreased reward can further motivate overconsumption in an effort to avoid the negative effects of reward withdrawal[87]. The consumption of alcohol increases dopamine levels via both directly exciting VTA dopaminergic neurons projecting to the NAc[88], and indirectly; by inhibiting GABAergic neurons that inhibit dopamine release[89]. Some of the strongest evidence implicating Cx36 gap junctions in the mediation of reward has been studies comparing the impact of alcohol administration to Cx36 knockout (KO) and wild-type (WT) mice. KO mice drank significantly less alcohol than WT, and additionally, WT mice treated with the CX36 antagonist mefloquine also drank less than untreated WT mice[90]. It was concluded that CX36 gap junction uncoupling between VTA GABAergic neurons allowed increased DA neuron firing, and a permanent increase in NAc dopamine. Considering alcohol was no longer as rewarding when Cx36 was either absent or blocked, it is hypothesized that it is via Cx36 gap junction electrical synapses that VTA GABAergic neurons communicate to regulate DA synthesis in the NAc.

Aversive response is at the opposing end of the same continuum as reward, therefore many of the same brain regions, pathways and peripheral mechanisms

are involved in each. A conditioned taste aversion (CTA) is a phenomenon that occurs when a novel tastant is temporally associated with malaise, resulting in the novel food being avoided upon future presentation to protect against repeated illness. These can be created experimentally by pairing a rewarding novel food with a toxic stimulus. The aversion is thereafter elicited in order to avoid the toxic tastant.

Considering the effect of Cx36 on rewarding substances, its anatomical location in the mesolimbic system, the neural and neuroendocrine effect of food and other rewarding stimuli which ultimately guide reward seeking behaviour; it is presumed that Cx36 will be linked to the intake of food driven by the reward system. However, Cx36 is not only implicated in reward mechanisms, but it is also located in some of the pivotal regions governing metabolic neural and endocrine mechanisms, potentially linking Cx36 to non-reward related regulation of food intake.

1.9 Aims

In contrast to studies investigating the metabolic mechanisms driving feeding intake dysregulation, the role of the reward system will also be researched. Normal energy balance homeostasis can be overridden in eating disorders such as obesity, where food intake is determined by the reward received by palatable foods rather than feelings of hunger and satiety. The purpose of these studies is to find pathways controlling the ingestive response to palatable foods to further elucidate pathways by which we can control this response with the goal of reducing obesity.

The first specific aim we will look at is whether Cx36 has a role in the intake of palatable foods. We will test this by determining whether the KO of Cx36 has an

effect on sweet palatable food consumption in both short-term and long-term exposures.

Next we will investigate whether Cx36 plays a role in aversion to palatable foods. This will be examined by testing whether the knockout of Cx36 affects acquisition of a conditioned taste aversion to a novel sweet palatable tastant.

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2

2. Effect of Cx36 knockout on the consumption of rewarding tastants

Abstract

Food intake is controlled by both hedonic and postingestive mechanisms. The reward system is activated by consumption of sweet palatable foods, which are associated with pleasure, and thus drive consumption. Knockout (KO) of Connexin 36 (Cx36) gap junctions has recently been linked to disruption of sensitivity to rewarding stimuli; therefore it is proposed that connexin 36 may be crucial to reward response to sweet foods. Short-term and long-term feeding paradigms examining a number of sweet palatable tastants illustrated that deletion of Cx36 decreases sweet tastant consumption. Additionally, concurrent consumption of sweet tastants and bland chow increased chow intake. Current data suggest Cx36 is a component of feeding for reward.

2.1 Introduction

2.1.1 Regulation of food intake

Food intake controls are broadly separated into two mechanisms: postingestive regulation, and hedonic regulation which is responsible for the derivation of pleasure from the taste of palatable foods. The integration of these systems firstly in the hypothalamus culminates to enable energy balance and body weight homeostasis despite fluctuating changes in caloric ingestion and energy intake[1].

2.1.2 Postingestive regulation of food intake

Postingestive mechanisms respond to the caloric, macronutrient content, and volume of food, and act to mediate food intake by inducing hunger or satiety signals. Peripheral satiety is determined primarily by mechanical and endocrine responses to food intake[2,3]. Distension of the gastrointestinal wall in response to ingestive load engages mechanoreceptors which sense stretch and volume, relaying this information via vagus afferent signals to the brain, triggering satiety pathways and terminating feeding behaviour[4].

Energy balance and adiposity is signalled by leptin produced in adipocytes and gastric epithelial cells. It is thought that adipocyte synthesized leptin influences long-term food intake, and gastric secretion influences short-term intake in response to immediate local response to ingested food[5]. Leptin induces satiety centrally by releasing a number of peptides known to be anorexigenic such as alpha-melanocyte stimulating hormone (a-MSH), suppressing the orexigenic agouti-related protein (AGRP) and working synergistically with cholescystokinin (CCK) to inhibit hunger[6,7].

Ghrelin is the major hormone known to induce hunger, and is synthesized in the stomach and small intestine. Increases in ghrelin are anticipatory and surge before

meals, before decreasing again after consumption of caloric macronutrients[8], most strongly in response to carbohydrate consumption followed protein and fat. Central targets are neuropeptide Y (NPY) and AGRP neurons in the arcuate nucleus of the hypothalamus[9]. Long-term control of food intake is also influenced by ghrelin, and is due to the inverse relationship between ghrelin and bodyweight. An increase in hunger coincides with the loss of bodyweight in an attempt to maintain homeostasis[10].

2.1.3 Sweet taste and reward

Intake of sweet palatable foods can be induced independent of hunger and satiety mechanisms; this is by association of sweet taste to pleasure controlled by the reward system of the brain[11]. The mesolimbic dopamine system is the main focus in the study of the reward system, and it is this pathway which is also vital for the acquisition and maintenance of addictions, both behavioural and substance based[12,13]. In the presence of a food addiction, the normal homeostatic nature of food intake is suppressed, causing continued feeding behaviour even in the presence of satiety signalling[14]. Brain areas making up the mesolimbic DA pathway are the VTA and the NAc[12]. DA projections extend from the VTA to the NAc where an increase in DA induces pleasure, reinforcing the associated behaviour[15,16]. A preference for sweet taste is innate[17], and has most likely been evolutionarily selected for as increased consumption of sweet foods due to reward-seeking behaviour would have enhanced the chances of survival and reproductive success. A preference for sweet tastants has been shown for sugars and synthetic sweeteners[18]; but in studies of sweet taste indifferent mice, a preference over water was only shown by caloric sweet tastants[19], indicating the reward system may be activated by caloric sweet tastants in the mouth and also in the gastrointestinal tract. It is therefore apparent that non caloric sweet tastants only activate the reward system by oral sweet taste receptors.

2.1.4 Cx36 mediated reward

Gap junction channel protein Cx36 knockout (KO) mice have a decreased self-administered intake of alcohol in comparison to WT mice[20]. The expression of Cx36 in the VTA and NAc, and the alcohol intake reduction when Cx36 is genetically deleted in KO mice suggests Cx36 as a vital component of reward pathways.

A recent as yet unpublished study led by Dr Pawel Olszewski of the University of Waikato, New Zealand[21], has illustrated a decrease in consumption of sweet palatable tastants in Cx36 KO mice compared to their wild-type counterparts. All sweet tastants in this research were affected, with caloric sweeteners most profoundly so. Additionally, consumption of water was unaffected by the deletion of Cx36.

The aim of these experiments is to confirm recent investigations showing that Cx36 is implicated in the reward mediated consumption of sweet palatable tastants, and whether this effect extends to female mice.

2.2 Materials and Methods

2.2.1 Animals

Adult female WT and Cx36 KO mice were housed in individual cages in a temperature controlled room at 22°C on a 12:12 light dark cycle (light begins at 0600h). The mice weighed approximately 27g at the start of the experiment. Prior to the experiment mice had access to ad-libitum tap-water and bland chow (Teklad). Cx36 KO mice were originally created by Dr David Paul (Harvard Medical School, USA)[22], and our colony was generated using standard breeding

methodology as described therein. The procedures described herein were granted approval by the University of Waikato animal ethics committee.

2.2.2 Experiment 1. Food, palatable tastant and caloric consumption

Mice were fed chow pellets and water ad-libitum prior to the start of the experiment unless otherwise stated. Over a period of 4 days, mice (n=26 KO, 14 WT) were given chow and a sweet tastant solution ad-libitum. The liquid tastant solutions were tested sequentially (water, saccharin 0.1%, sucrose 10%, glucose 10%, fructose 10%), with each beginning at the start of a new week. Both chow and water were measured after each 24hr period, and spillage was accounted for. The mice were weighed at the same time as chow and sweet tastant each day (1100h), and daily consumption was calculated by gram of bodyweight. The effect of Cx36 on chow and sweet tastant intake was determined using t-tests. P values were considered significant when $P < 0.05$.

2.2.3 Experiment 2. Short-term palatable tastant consumption

A new set of adult female mice weighing approximately 24g were used to determine the effects of the same tastants used in Experiment 1 over a short-term exposure. Mice (n = 11 KO, 11-13 WT) were fed chow and tapwater ad-libitum unless otherwise stated. For 5 days mice were given 2 hour access to a sweet liquid tastant per day (1100h – 1300h), with chow and water removed; the remainder of each 24hr period mice had ad-libitum access to chow and water. The tastants (saccharin 0.1%, sucrose 10%, glucose 10%, fructose 10%) were tested sequentially, each beginning at the start of a new week. Mice began drinking immediately upon presentation of liquid tastants. Bottles were weighed and corrected for spillage at the start and end of each 2h test. The volume of liquid consumed was calculated

and recorded as ml/g of bodyweight. T-tests were performed to determine whether knockout of Cx36 affected the short-term consumption of sweet tastants. P values were considered significant when $P \leq 0.05$.

2.3 Results

2.3.1 Experiment 1. Food, palatable tastant and caloric consumption

Concurrent ad-libitum exposure to bland chow, palatable caloric and non-caloric fluids in Cx36 KO female mice is associated with overconsumption of bland chow and decreased intake of palatable fluids (Fig. 2.1). Consumption of all sweet tastants was decreased in Cx36 KO mice, with intake of saccharin, sucrose and most notably glucose significantly decreased. Fructose consumption showed a trend towards decreased intake, however did not reach the level considered significant. Total caloric intake increased in Cx36 KO mice when chow was presented with non-caloric liquids, and decreased when chow was presented in conjunction with sucrose and glucose. Concurrent chow and fructose consumption resulted in no change in caloric intake.

2.3.2 Experiment 2. Short-term palatable tastant consumption

Palatable tastants-only short-term exposure is associated with the reduction of glucose and sucrose solution intake in Cx36 KO mice, thus, the tastants that were most profoundly affected in Experiment 1 (Fig. 2.2). While fructose consumption showed a decreased yet insignificant intake during 24h concurrent exposure with chow in Experiment 1, it was unaffected during short-term palatable tastant exposure.

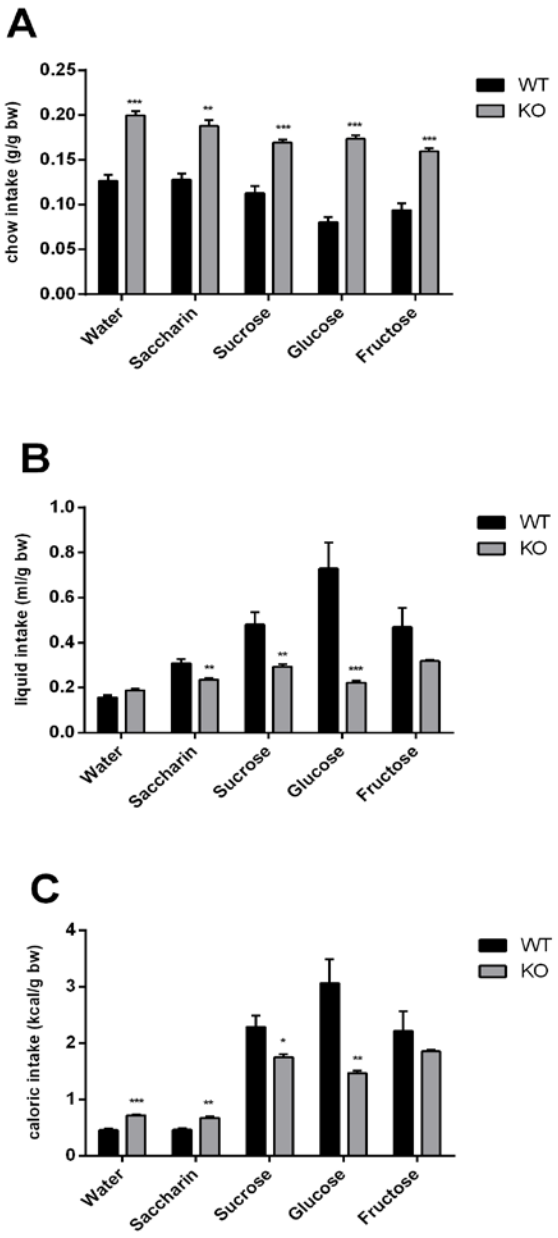

Figure 2.1 Chow and palatable tastant intake was determined during 24 hour concurrent administration comparing Cx36 KO and WT mice. **A** Cx36 KO increases consumption of chow administered with all liquids. **B** Consumption of sucrose, glucose and fructose is decreased by Cx36 KO. **C** Total caloric intake of concurrent liquid and chow administration is decreased with sucrose and glucose, and increased with non-caloric liquids. (*- $P \leq 0.05$, ** -$P \leq 0.01$ *** - $P \leq 0.001$).

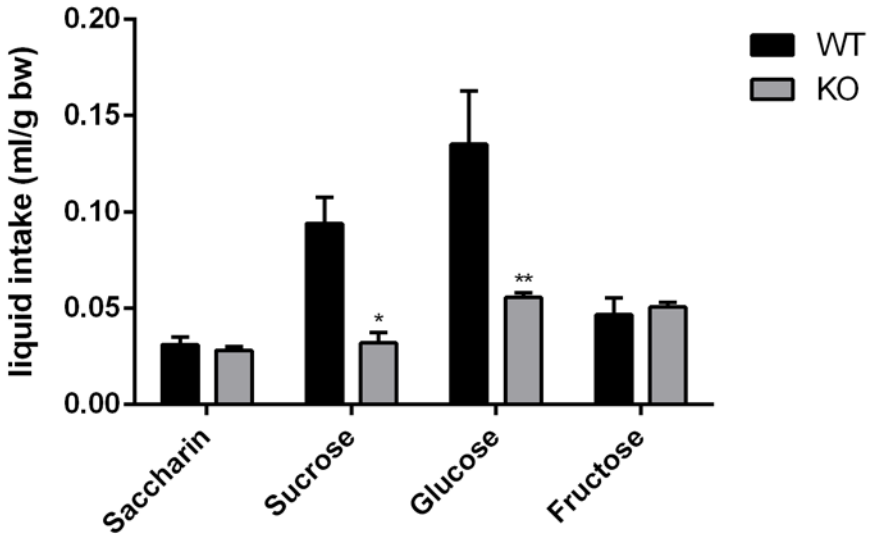

Figure 2.2 Knockout of Cx36 in female mice decreases consumption of glucose and sucrose during short-term exposure. Palatable tastant intake was measured after 2 hours of administration. (*- $P \leq 0.05$, ** -$P \leq 0.01$ *** - $P \leq 0.001$).

2.4 Discussion

When looking at the mechanisms regulating food intake, we can determine that the reward system plays a major role[1]. Dysregulation of the reward system can result in a loss of homeostatic control which regulates bodyweight by integrating hunger and satiety signals with pleasure and motivation[14]. A preference for sweet tasting food is evident in a range of human and other mammalian studies. Its intake activates the reward system, becoming associated with pleasure, and thus increases sweet food seeking behaviour[11]. Cx36 is found throughout brain regions implicated in appetite and reward, and has recently been demonstrated to alter intake of rewarding substances[20,21], therefore it is speculated Cx36 is also involved in sweet taste induced feeding reward.

The results of Cx36 deletion on long-term food intake show a decrease in the consumption of palatable sweet tastants. This suggests that Cx36 is a crucial component of reward mediated sweet taste signalling. Major reward regions of the brain show expression of Cx36, implicating this connexin in reward pathways[23]. Previous research studying the effect of Cx36 deletion on reward mediated alcohol consumption suggests CX36 gap junction channels enable the communication of adjacent VTA neurons, the abolishment of which interrupt DA release in the NAc[20]. The mechanism inducing alcohol reward is highly correlated with that of sweet taste reward. Thus, it is likely the decrease in sweet tastant consumption is the result of diminished cell-to-cell communication in the VTA and NAc.

Supporting the hypothesis that sweet taste is specifically affected and the response is not merely the feeding termination of all foods is the change in bland chow consumption when administered together with sweet tastants. In contrast to sweet tastants, Cx36 deletion increases chow consumption. This also suggests that various peripheral postingestive regulatory processes respond to a decrease in reward induced consumption, possibly by increasing consumption of chow to maintain homeostasis. Cx36 has been found in pancreatic islet β-cells, whose coupling is important for insulin secretion in response to glucose. While loss of β-cell Cx36 channels prevents glucose induced insulin release, basal insulin secretion is increased, as illustrated by Cx36 deletion[24]. This is equivalent to the function of β-cells in type 2 diabetes. Similarly, a high fat diet was fed to C57BL/6 mice that later became pre-diabetic and obese as measured by glucose sensitivity and insulin resistance. Pre-diabetic mice were shown to have a significantly decreased level of Cx36 gap junctions than control mice[25]. A close relationship is therefore defined between Cx36 and diabetes, as loss of Cx36 induces insulin response typical of type 2 diabetes, and a pre-diabetic condition reduces Cx36 gap junctions. For this reason, an impaired insulin response is possibly a causative factor in sugar consumption of Cx36 vs. WT mice.

When accounting for both the decrease in sweet tastants and increase of chow, there remains a net decrease in total caloric intake, excluding non-caloric water and saccharin tests. Results for long-term exposure mirrored that of the previous study where male Cx36 KO mice consumed less sweet tastants[21], confirming that the effects are not gender specific. Short-term exposure to sweet palatable tastants consequently produced similar results to long-term exposure. The only noticeable change was a shift from a non-significant trend in Cx36 KO fructose consumption, to no difference during the short-term exposure. In both long and short-term paradigms, Cx36 KO mice ingested similar amounts of all sweet tastants; therefore it is in WT mice that differences in sweet tastant consumption are evident. Rodents display differing preferences to various sugars which may account for the consumption differences in WT mice[26,27].

The reward system is comprised of a number of brain regions which also interact with appetite regulatory pathways[1]. These studies have illustrated that Cx36 participates in the regulation of feeding for reward. The increased consumption of chow is also suggestive of Cx36 involvement in metabolic processes in addition to reward processes. Further research should investigate which specific brain regions are affected by Cx36 during food intake. Immunohistochemical studies could now be undertaken where target antigens, such as the transcription factor protein c-Fos which is a marker of recent neuronal activity, are targeted by specific antibodies and stained to visualize regions of activity that may be involved in a particular mechanism[28].

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3

3. The effect of Cx36 knockout on a conditioned taste aversion

Abstract

Associating a malaise inducing stimuli to a novel food by classical conditioning creates a conditioned taste aversion (CTA) to the novel food. Aversion and reward processes are on opposite ends of the same continuum, though it is uncertain whether Cx36 gap junctions implicated in reward are also essential to a CTA. It was therefore proposed that knockout (KO) of Cx36 would increase aversive sensitivity to a sweet novel tastant in mice during a CTA reward paradigm. During a two bottle test of a sweet novel tastant vs. water, preference for a sweet tastant was decreased from 70% in wild-type (WT) mice to 27% in Cx36 KO mice when a LiCl was administered following sweet tastant consumption. Total liquid intake was unaffected by KO of Cx36 gap junctions. These results illustrate that Cx36 KO exaggerates the response of a LiCl induced CTA to a sweet novel tastant.

3.1 Introduction

A conditioned taste aversion (CTA) is acquired when the effects of a toxin are associated with novel tasting food. It is a form of classical conditioning where an unconditioned stimulus: a malaise inducing toxin, is paired with a conditioned stimulus: novel tasting food[1]. A novel stimulus is used because pre exposure may result in latent inhibition, preventing or diminishing the acquisition of a CTA[2,3].

CTAs are necessary to protect animals from poisoning. If the ingestion of a substance is followed by illness, an animal will avoid the suspected harmful substance to prevent further illness[4]. The association between the toxin and the novel food is long-lasting and results in future avoidance of the novel food when it is later presented[5,6]. One of the most used chemicals to cause gastrointestinal discomfort in experimental settings is lithium chloride (LiCl), which is highly effective at decreasing food consumption[7].

A range of processes are integrated to establish a CTA. Reward, fear learning, memory and peripheral postingestive mechanisms are all required for the acquisition and retrieval of CTAs[8,9,10]. Creating a CTA to sweet palatable tastants shifts the response from reward to aversiveness despite the tastant being intrinsically unchanged. The hedonic shift from positive to negative illustrates the proposition that reward and aversion are mechanisms at opposing ends of the same continuum. Reinforcing this view is evidence showing that brain regions in the reward system are also activated during acquisition and retrieval of a CTA, specifically the amygdala, the ventral palladium (VP), ventral tegmental area (VTA) and nucleus accumbens (NAc)[11,12].

Neuroendocrine studies in taste aversion also implicate oxytocin (OT), opioids, and dopamine (DA) in the mesolimbic reward pathway[13]. Pairing a sweet palatable solution with an aversive stimulus has the reverse effect on the VTA to NAc

pathway that the same sweet palatable has sans aversive stimuli. NAc DA is suppressed to a similar level as during bitter taste consumption[14].

OT is a satiety regulator, initiating termination of feeding, particularly associated with carbohydrate consumption[15,16]. Numerous studies have shown that deletion of the OT gene enhances consumption of sweet solutions, but leaves lipid based solution consumption unchanged[16,17]. In contrast, opioids increase consumption of palatable foods irrespective of caloric content, even in the presence of satiation, implicating the reward pathway[18]. Opioid agonists attenuate aversive response to LiCl, whereas blocking opioid receptors with the opioid receptor antagonists naltrexone and naloxone heighten the aversive response[19,20]. A relationship between opioids and OT cells in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus is apparent in the regulation of taste aversion. OT is released in these areas after injection of LiCl, and when various opioids are administered prior to LiCl, the LiCl induced CTA is attenuated as evidenced by decreased c-Fos expression in the PVN[13]. These same hypothalamic regions also express Cx36 which potentially links a CTA to Cx36 via the hypothalamus.

Taking into account the effect of Cx36 gap junctions on reward mediated sweet tastant intake as illustrated in chapter 1, and the evidence linking CTA to the same reward pathways, it is hypothesized that Cx36 mediated communication is crucial to reward related pathways therefore its deletion will alter aversive response. Hypothalamus regions mediating aversiveness also express Cx36 which may also affect aversive response by non-reward related mechanisms.

3.2 Materials and Methods

3.2.1 Animals

Adult male WT and Cx36 KO mice were housed in individual cages in a temperature controlled room at 22°C on a 12:12 light dark cycle (light on at 0600h). Mice weighed ~30g prior to the experiment and had access to ad-libitum tap-water and bland rodent chow (Teklad). Cx36 KO mice were originally created by Dr David Paul (Harvard Medical School, USA)[21], and our colony was generated using standard breeding methodology as described therein. The procedures described herein were granted approval by the University of Waikato animal ethics committee

3.2.2 Conditioned taste aversion

Mice were fed bland chow pellets and water ad-libitum for 5 days prior to the start of the experiment. For 2 days mice were then acclimatised to deprivation of chow and water for 5 hours (1300 – 1800h) to ensure hunger and thirst, with chow and water replaced immediately prior to the beginning of the 12h dark cycle. On day 3, a sweet novel tastant (pineapple juice, Golden Circle) was then administered for 1hour post deprivation for 2 hours (1800h – 2000h). WT and KO mice were each randomly divided into two groups (n=8 each): one group given intraperitoneal (IP) injections with 6 mEq/kg bw LiCl, and one group given IP saline solution injections. All injections were administered after 2hr of novel tastant consumption. Chow and water were replaced 30min after injections. All mice had 24 hour ad-libitum access to water and chow on day 4 to allow recovery from the effects of LiCl. On day 5 mice were deprived of food and water for 5 hours (1300h – 1800h), before being administered a 2-bottle test to assess acquisition of a CTA to the novel tastant. The bottles were weighed and corrected for spillage to

ascertain consumption. The amount of novel tastant consumed in ml/g of bodyweight and the percentage of total fluid intake were analysed using t-tests and were considered significant when $P \leq 0.05$.

3.3 Results

In a two bottle test of water and a sweet novel tastant, Cx36 KO mice display increased sensitivity to aversive treatments: a threshold dose of LiCl (i.e., sufficient to induce a very mild CTA in WT mice) causes pronounced aversion in KO mice (Fig. 3.1). There was a trend towards decreased total liquid intake in Cx36 KO mice; however this did not reach significance. The CTA was shown in both WT and KO mice by the volume of novel tastant consumed, with the increase in aversiveness by KOs most evident in the percentage of novel tastant intake. WT consumed 70% of total liquid intake as sweet tastant while Cx36 KO mice consumed 27% as sweet tastant.

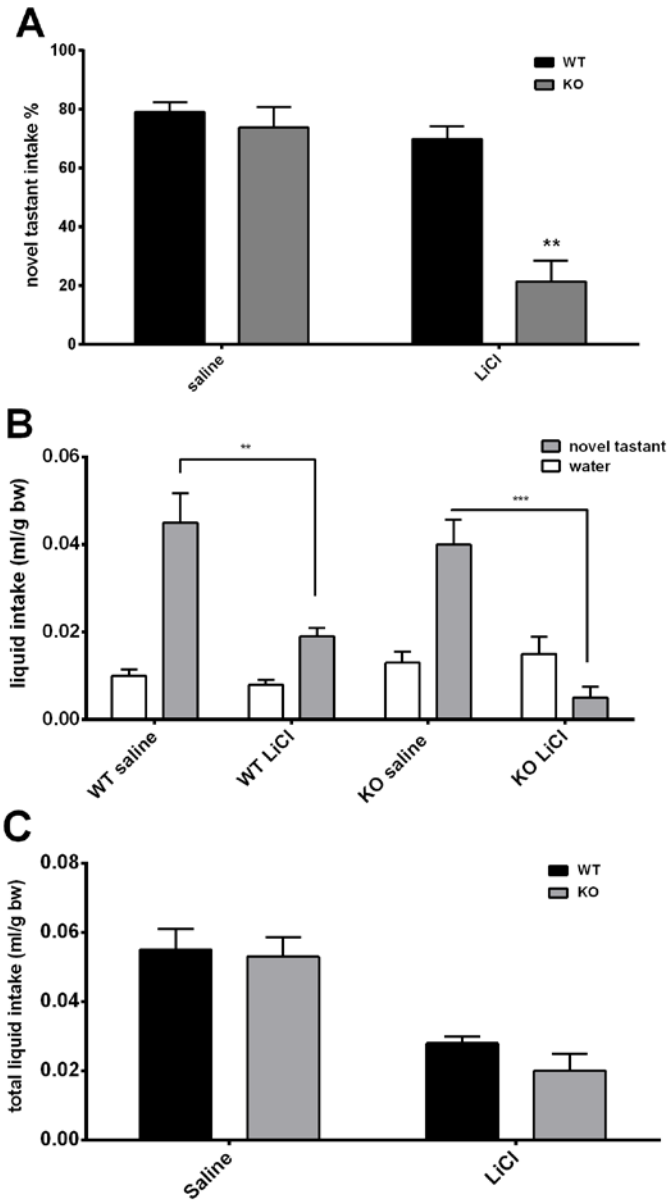

Figure 3.1 Knockout of Cx36 in male mice increases the magnitude of aversive response during a LiCl induced CTA to a sweet novel tastant, but does not affect total liquid intake. **A** Novel tastant consumption as a percentage of total liquid intake. **B** Intake of individual liquids (ml/g bw) **C** Total liquid intake (ml/g bw). Novel tastant and water intakes were established during a standard two-bottle test (novel tastant vs. water) in female mice treated with either 6 mEq/kg bw. LiCl or saline. (*- $P \leq 0.05$, ** -$P \leq 0.01$ *** - $P \leq 0.001$).

3.4 Discussion

Gap junction communication is ubiquitous throughout mammalian tissue and is as diverse functionally as it is distributionally[22]. Cx36 gap junctions have been illustrated to participate in reward related behaviour in response to a number of associated stimuli. Most recently the studies from chapter one demonstrate that lack of Cx36 decreases reward, possibly by diminishing the hedonic value of sweet palatable foods.

A CTA, like reward, is a complex interaction of multiple brain regions and pathways where toxicity is associated to a previously neutral stimulus, reducing or eliminating its consumption[1]. Some of these pathways are shared between reward and aversion[11,12]; when one mechanism is stimulated it causes an increase in reward response, conversely when that same mechanism is blocked it causes an increase in aversive response. If the subsequent proposition is true that both reward and aversion are opposites of the same spectrum, it is hypothesized that since Cx36 deletion decreases the reward related consumption of sweet foods, it will also increase aversive sensitivity to malaise associated foods. Despite some new research into Cx36 in the reward system, the relative recency of its discovery and broad range of functions and tissues in which it is expressed means that there is as yet limited knowledge as to its possible function in aversion.

In a two bottle test of water vs. a sweet novel tastant, both Cx36 KO and WT mice had a decreased total liquid intake after LiCl induction of a CTA to the sweet tastant. There were no significant differences in total intake between KO and WT in either the LiCl or saline treated groups, suggesting Cx36 had no effect on thirst as a consequence of a CTA.

The percentage of sweet novel tastant consumption between saline injected KO and WT was statistically insignificant, however, Cx36 KO mice consumed 27%

sweet tastant and WT consumed 70%. Differences in consumption of the sweet malaise-associated tastant only, and not in total liquid intake suggests Cx36 does in fact generate remarkably increased sensitivity to aversive stimuli as hypothesized. There was a diminished preference for the aversive conditioned sweet tastant while leaving total liquid intake unchanged suggesting there is an aspect of the reward system affected by knockout of Cx36.

There are a number of mechanisms that Cx36 could act upon. Based on the results from feeding studies in chapter one, it is likely that the mesolimbic dopamine pathway within which Cx36 is found plays a role, but this may not be the only mechanism Cx36 acts upon. Previous research has found OT cells in the PVN and SON to mediate acquisition of a LiCl induced CTA. An alternative explanation for the enhanced aversive response to sweet tastants is that Cx36 found within these neuronal populations alters the endocrine mediated aspects of a CTA.

A CTA is not the result of activation of a single pathway, nor is Cx36 expressed in a single pathway. Memory, fear learning, reward and postingestive endocrine regulation are all involved in acquisition of a CTA, and Cx36 is expressed throughout neurons of multiple CTA correlated brain regions. Elucidation of which parts of these processes Cx36 influences therefore requires investigation of these individually. I have found Cx36 is important in sweet tastant consumption and LiCl induced aversion to sweet tastants, but it is uncertain by which neuronal populations and neuroendocrine factors this occurs. That Cx36 is implicated is herein confirmed, how it is implicated needs to now be explored.

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4. Conclusions and Perspectives

The results within this thesis have expanded the knowledge of connexin 36 (Cx36) as an important component in the regulation of consummatory behaviour. There is currently limited research into Cx36 and consummatory behaviour; however, recent research has implicated Cx36 in consumption of alcohol[1], and preliminary studies have linked CX36 knockout (KO) to diminished sweet solution consumption in male mice[2]. In my current experiments, Cx36 knockout (KO) was shown to decrease the consumption of sweet palatable tastants and increase consumption of bland chow compared to wild-type (WT) counterparts. Also shown is that during a conditioned taste aversion (CTA), Cx36 KO increases the aversive response to a sweet novel tastant.

Cx36 KO and WT mice were exposed to sweet tastants, resulting in a decrease in consumption of sucrose and glucose during short term intake in KO mice, and decrease in consumption of saccharin, sucrose and glucose during long term intake. In addition to the decrease in sweet tastant intake was an increase in bland chow consumption when consumed concurrently. Knockout of Cx36 decreases intake of sweet foods, possibly by diminishing their hedonic value which suggests the necessity of Cx36 for the proper communication between neuronal cell populations within the reward system. However, an increase in bland chow

consumption by Cx36 KO mice is suggestive of homeostatic postingestive regulation responding to caloric content, macronutrient content and volume of gastric load.

To test whether Cx36 affects acquisition of a CTA, a novel sweet tastant was administered immediately prior to injection of LiCl to create a CTA to the sweet tastant. Malaise caused by the LiCl was consequently associated with the sweet tastant, decreasing its consumption. A mild aversion was acquired by WT mice, but Cx36 KO mice showed significantly increased aversive responsiveness to the sweet tastant.

That reward and aversion are opposing on the same continuum is thereby supported by the results of the CTA when combined with the rewarding sweet tastant consumption results. It was predicted that Cx36 would be involved in the consumption of rewarding foods, altering their intake, but the results may now also implicate Cx36 in postingestive mechanisms of consummatory behaviour. It is unclear whether Cx36 is a component of these mechanisms inducing bland food intake, or if this increased intake is merely a homeostatic response to a decreased gastric volume and caloric intake.

The implication of Cx36 in feeding reward opens the possibility of manipulation to treat eating disorders such as obesity, or related diseases including diabetes; as Cx36 is a component of insulin secretory mechanisms[2,3]. Investigations could begin with genome wide association studies to indentify whether any single nucleotide polymorphisms in the Cx36 GJD2 gene are associated with obesity or other eating disorders[4,5,]. If there is associated genetic variation in GJD2 it may be possible to develop pharmacological interventions. Selective blockade of Cx36 channels by the creation of synthetic ligands may consequently modify appetite which is disrupted in eating disorders. Further to this, the results from the current studies of food intake and those associated with alcohol consumption suggest

CX36 may be associated with the wider phenomenon of addiction. Modulation of Cx36 could treat a variety of addictions both behavioural and substance based. In the case of addictive-like overeating, which has neurobiological similarities to substance addiction[6,7], the health implications of obesity could be mitigated by decreasing the underlying hedonic value of palatable foods that drives excessive consumption. Current pharmaceuticals used to treat addictive-like conditions show some efficacy[8,9], however, drugs with higher specificity and efficacy could be produced.

In these studies I have investigated the role of Cx36 in consummatory behaviour, and have found that Cx36 KO diminishes intake of sweet tastants and increases intake of bland chow during concurrent consumption. Furthermore, a LiCl induced CTA is enhanced by KO of Cx36.

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