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**Social interaction affects acquisition of lithium chloride-induced
conditioned taste aversion: identification of underlying changes in
neuronal activation in key forebrain areas**

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Abstract

A conditioned taste aversion (CTA) develops when exposure to a novel tastant is followed by sickness/malaise. Recently, it has been shown that a CTA can be reduced if the animal is placed in a social environment, i.e., in the presence of a conspecific, during the CTA acquisition phase. The current project was aimed to expand on our understanding of this phenomenon by (a) identifying the magnitude of an aversive response to a saccharin solution induced by lithium chloride (LiCl), a known emetic agent, in mice maintained in the social versus non-social setting; and (b) defining differences in LiCl-induced neuronal activation in the key CTA-related forebrain areas of animals in the social and non-social scenario. In mice lacking social stimulation, LiCl at a dose of 1 mEq induced a mild CTA to saccharin and the 6-mEq dose produced a profound aversive response. On the other hand, 6 mEq was the lowest effective dose in mice kept in the social setting. Immunohistochemical analysis of a neuronal activity marker, c-Fos, showed that alteration of activity in the paraventricular nucleus of the hypothalamus (PVN) and central nucleus of the amygdala (CEA) is associated with the observed changes. In LiCl-injected mice subjected to social stimulation, also the percentage of Fos-positive oxytocin (OT), but not vasopressin neurons in the PVN was higher than in LiCl-treated single-housed mice. These results are discussed in context of the effects that sociality has on the magnitude of responses to adverse associative stimuli and an involvement of specific elements of brain circuitry in mediating these effects.

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List of Abbreviations

- AP - Area postrema
- ARC - Arcuate nucleus
- BLA - Basolateral nucleus of the amygdala
- BBB - Blood brain barrier
- CNS - Central nervous system
- CEA - Central nucleus of the amygdala
- CCK - Cholecystokinin
- CTA - Conditioned taste aversion
- CRH - Corticotropin releasing hormone
- DMNV – Dorsal motor nucleus of the vagus
- GABA - Gamma-butyric acid
- GI - Gastrointestinal
- GLP-1 - Glucagon-like peptide-1
- HPA - Hypothalamic-pituitary-adrenal
- I.p. – intra-peritoneal
- LHA – Lateral hypothalamus
- LiCl - Lithium chloride
- mPFC - Medial prefrontal cortex
- mEq - milliequivalents
- MC - melanocortin
- NMDA - N-methyl D-aspartate
- NTS - Nucleus of the solitary tract
- OT - Oxytocin

- OT-A - Oxytocin antagonist
- OTR - Oxytocin receptor
- PBN – Parabrachial nucleus
- PFA – Perifornical hypothalamic area
- PL - Prelimbic
- PVN - Paraventricular nucleus of the hypothalamus
- PYY - Peptide YY
- SON - Supraoptic nucleus of the hypothalamus
- SCN - Suprachiasmatic nucleus
- VP - Vasopressin

Introduction

Food intake, while providing energy, nutrients and (to some extent) rewarding value – is a behavioural activity poised to expose an animal to the danger of toxicity. In the natural environment, the risk of edible plant/animal material harbouring toxins (oftentimes, not originating from the actual food source, but rather derived from external/secondary sources, such as bacterial/parasitic metabolites) is high [1]. Thus, the ability of the organism to avoid harmful substances is one of the key aspects of survival and is evolutionarily very well-conserved. Not surprisingly, behavioural avoidance of potentially toxic tastants has been shown in invertebrate and vertebrate species. Even unicellular protozoans reject bitter flavours that are typically associated with plant poisons [2].

There are several behavioural and physiological mechanisms that allow an organism to minimise the likelihood of encountering food-derived toxins. One of them is food neophobia, a phenomenon where novel food is tentatively consumed in minute amounts, and these amounts increase with subsequent exposure to this ingestant, but only if the initial intake of this food did not result in gastrointestinal (GI) sickness [3]. Moreover, innate flavour preferences tend to reflect food toxicity risks associated with them and so bitter tastes, being characteristic for tainted foods, are generally avoided [4, 5]. Furthermore, many animal species are capable of expelling from the gut foods that cause an unpleasant gastrointestinal sensation, for example via the emetic reflex. An alternative to emesis is pica, a process in which the animal exposed to food toxicity and GI discomfort, ingests large amounts of non-nutritive substances, such as clay or soil [6], in an

attempt to decrease the concentration of a presumed toxin in the GI tract and to provide additional stimulation to gut mechanoreceptors, possibly being conducive to emesis [7]. Finally, there is a complex learning-based mechanism through which a risk of consuming toxic foods can be reduced, termed a conditioned taste aversion (CTA). This association-based learning process, described in detail below, allows the animal to avoid tastants whose intake has previously been followed by sickness/malaise.

1.1. Conditioned Taste Aversion

In the natural environment, a CTA ensues when ingestion of a novel tastant leads to an unpleasant GI sensation within a relatively short timeframe. This facilitates a Pavlovian learning process that underlies avoidance of this particular food upon future presentation [8]. If the feeling of sickness occurs during a meal, it also promotes an immediate termination of ingestive behaviour. Subsequent presentations of an aversive food lead to complex responses underlying persistent hypophagia, including suppression of hunger, increase in anxiety and fear as well as memory-based avoidance. The CTA phenomenon is particularly crucial for those species (including rodents) that do not have an emetic reflex [9], and thus have to rely on a CTA as a key mechanism in a smaller repertoire of processes that minimise dangers of food-borne toxicity.

In the laboratory setting, a CTA is typically induced by pairing presentation of a novel food (the unconditioned stimulus) with an injection of a noxious substance; usually lithium chloride (LiCl) or copper sulphate (CuSO₄) are used to cause short-lived malaise (conditioned stimulus). The animal then associates the sickness with the novel food and will avoid it (conditioned

response) upon subsequent exposures [8]. A CTA test can be performed with any tastant of a characteristic flavour, however, the vast majority of the studies available in the literature use novel liquid tastants, especially the palatable and non-caloric 0.1% saccharin solution. Acquisition of a CTA is assessed by giving the animal a two-bottle choice test [10], in which saccharin versus water intakes are measured. Non-CTA animals drink more of the palatable saccharin, whereas aversive individuals consume mainly water in this choice scenario [11]. While CTAs form readily towards novel foods, they can also develop to foods that have been encountered before, however, it usually requires a much more robust sickness response and/or repetitions of associations between malaise and a previously consumed tastant [12].

A CTA was first described by Garcia, who found that fluid and food intakes in rats were suppressed after exposure to sickness inducing low levels of gamma radiation [13]. Subsequent pairing of the radiation with food and water would further depress consumption [13]. Further studies showed that the only the GI discomfort-related type of conditioned stimulus could lead to the acquisition of a CTA – for example, electric shocks paired with a meal did not cause a CTA, however, associations with toxin injections or x-ray exposure produced a CTA [14]. Interestingly, aversion can also be induced by forced exercise that leads to GI motility changes [15]. A 2006 study by Masaki and Nahajima compared the relative magnitudes of CTA developed by rats that were either forced to run, swim, run voluntarily, run optionally, or were treated with LiCl. While the injection generated the strongest aversion, forced exercise also invoked a CTA, with forced

running giving the greatest magnitude of an aversive response [16]. It was initially hypothesised that activity-based CTA was induced through physiological stress [17] or the energy expenditure [18]. However, a study in male rats that examined whether fighting between conspecifics would promote a CTA found that no aversion developed [19]. An alternative hypothesis to activity-based CTA is that there is gustatory discomfort: exercise inhibits emptying of the stomach, therefore, it is not the stress of exercise that causes the CTA, but rather the GI discomfort [20].

As mentioned in the introductory paragraphs to this thesis, a CTA is a complex phenomenon whose development relies on a vast array of intertwined processes, including stress, memory and reduced appetite.

Intuitively, as any sickness is taxing (thus, stressful) for an organism, GI discomfort and malaise induce the activity of the hypothalamic-pituitary-adrenal (HPA) axis [21, 22]. In fact, even transient GI tract discomfort may lead to long-lasting altered behaviours and digestive outcomes that persist despite complete resolution of the initial symptoms [23, 24]. Therefore, the CTA includes a stress component. For example, it has been shown that malaise-inducing doses of LiCl produce c-Fos immunoreactivity in the brain regions implicated in CTA, as well as activating the HPA axis and elevating plasma corticosterone levels in rats [25]. Administration of synthetic glucocorticoids at the time of LiCl CTA acquisition attenuates the development of aversion [26]. On the other hand, adrenalectomy does not affect CTA acquisition, but it does delay extinction unless corticosterone levels are restored via exogenous administration [25]. Aside from the HPA axis, the amygdala complex modulates CTA-driven hypophagia by

providing additional stress- and fear-related processing that aids in the proper manifestation of a CTA. The basolateral nucleus of the amygdala (BLA) is the receiver of sensory data that allows for the acquisition and expression of the fear response, whilst the central nucleus of the amygdala (CEA) is thought to be the generator of fear responses [27]. The CEA and BLA receive inputs from the hippocampus, hypothalamus, brainstem, and gustatory areas, and the CEA sends information back to the hypothalamus and brainstem, using information from other nuclei of the amygdala [28]. Activation of the BLA during aversion has been shown through Fos activation [29], and some lesion studies suggested that BLA is necessary for CTA acquisition [30-34]. While some studies show the link between a CTA and these two nuclei of the amygdala, the results are somewhat conflicting [35]. For example, Zimmerman et al found that when the BLA was lesioned, the CEA could still facilitate conditional fear responses after overtraining [36].

Glucocorticoids are not just critical in stress-related processing, but also affect another component of a CTA, memory [37] (the multi-faceted role of glucocorticoids serves as an example of a functional overlap of the neuroendocrine systems that contribute to the complexity of aversive responses). The key anatomical site for CTA memory – especially the memory for the initial excitatory conditioning - is the insular cortex through which brain-derived neurotrophic factor increases an aversive memory-trace [38, 39]. On the other hand, N-methyl D-aspartate (NMDA) receptors in the dorsal medial prefrontal cortex are needed for CTA memory consolidation, highlighting the complexity of medial pre-frontal cortex

functions [40]. Surprisingly, the role of the hippocampus in CTA memory is relatively limited. Hippocampal lesions do not block a CTA, but promote increased sensitivity to latent inhibition [41, 42]. There is no evidence linking the hippocampus with CTA memory retrieval [43], but this memory-related brain region does facilitate the renewal of CTA following extinction [44].

Finally, a successful avoidance of tainted food requires an abrupt termination of food intake: this hypophagic response should occur despite the energy needs of the organism as well as despite the palatability (and consequently motivating) value of the potentially tainted food. Thus, intricate neural and neuroendocrine mechanisms that ensure an immediate cessation of ingestive behaviour are the particular interest of research focused on unravelling the role of critical factors modifying aversive responses that go well beyond the classically envisioned basic learning paradigm. The following sections delineate the foundations of mechanisms that affect food intake and describe how these mechanisms tie with aversive responses.

1.1.1. Appetite regulation: basic concepts

Food intake is driven to a large extent by energy needs as well by the rewarding (palatability) aspects of consumption. Therefore, neural circuits responsible for the regulation of food intake encompass pathways that control the hedonic (pleasure) and the homeostatic (caloric) facets of eating behaviour [45]. These pathways are summarised in Figures Figure 1 and Figure 2, where Figure 1 depicts the hedonic circuit, and Figure 2 gives an overall view of the chief brain pathways regulating food intake.

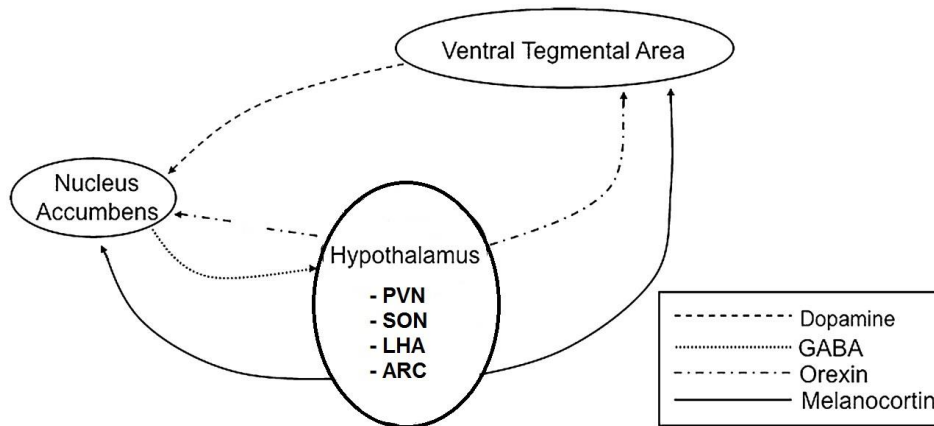


Figure 1: Schematic representation of neural circuits that regulate feeding. Dopaminergic neurons originating in the VTA project to neurons within the nucleus accumbens of the ventral striatum. The lateral hypothalamus receives input from GABAergic projections from the nucleus accumbens as well as melanocortinergic neurons from the Arc of the hypothalamus. In addition, melanocortin receptors are also found on neurons in the VTA and the nucleus accumbens. Figure modified from *Homeostatic and hedonic signals interact in the regulation of food intake* [45]

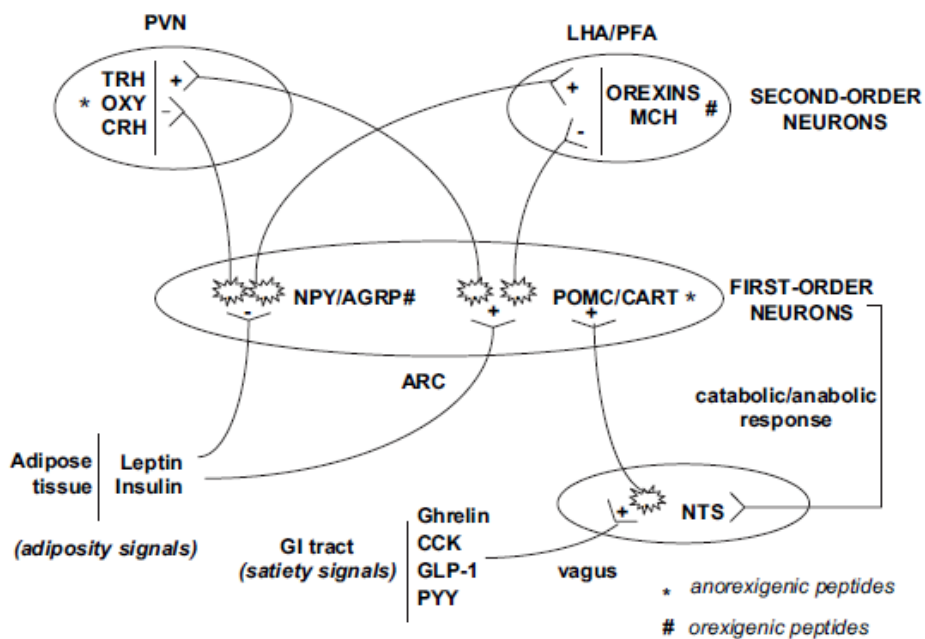


Figure 2: A schematic representation of the chief brain pathways involved in the regulation of eating behaviour. ARC, arcuate nucleus; NTS, nucleus of the solitary tract; CCK, cholecystokinin; GLP-1, glucagon-like peptide 1; PYY, peptide YY. PVN, paraventricular nucleus; LHA, lateral hypothalamic area; PFA, perifornical area; NPY, neuropeptide Y; AGRP, Agouti-related peptide; POMC, pro-opiomelanocortin; CART, cocaine- and amphetamine-related transcript; CRH, corticotrophin-releasing hormone; TRH, thyrotropin-releasing hormone; OX, oxytocin; MCH, melanin-concentrating hormone. From *Neuronendocrine control of food intake* [46]

The key components of the homeostatic network include the host of brainstem and hypothalamic sites, with the hindbrain acting as a relay station for peripheral signals [47] and the hypothalamus providing neuroendocrine feedback into the periphery as well as the input into other Central Nervous System (CNS) areas. There are many specific pathways within the brainstem-hypothalamic circuit involved in this process. One of them is activated by signalling from the gastrointestinal (GI) tract (Figure 2), which stimulates (via vagal afferents) neurons in the nucleus of the solitary tract (NTS), which then provide information onto primary-order neurons in the arcuate nucleus (ARC). The ARC also contains cells that express leptin receptors and glucose sensing molecules, thereby being capable of receiving direct information from the periphery regarding energy stores and energy availability [48]. Thus, second order neurons from the paraventricular nucleus of the hypothalamus (PVN) or the lateral or perifornical hypothalamic area (LHA/PFA) can be activated via the ARC-derived input in response to signals from adipose sources. The PVN and LHA/PFA neurons can also be activated directly by projections from the NTS and from other brainstem sites, such as the area postrema and dorsal motor nucleus of the vagus [46]. This hypothalamic-brainstem pathway provides dispersed input into a vast network of other brain sites that are not directly involved in the regulation of energy balance, and these areas (via reciprocal innervation with the brainstem-hypothalamic pathway and with each other) modulate feeding responses by incorporating other processes that shape ingestive behaviour including stress, reward, and sociality [46].

Rewarding aspects of consumption are mediated via the hedonic pathway whose key component is the mesolimbic system [49]. Activity of the reward system is increased upon exposure to stimulation associated with pleasure (aside from food intake, it also occurs in conjunction with any rewarding behavioural activity, including reproductive behaviours, social behaviours, etc). This activity is further increased upon repeated experiences with rewarding stimuli so that eventually the activity of the reward system itself motivates the animal to seek them [50]. In line with that, the reward system is responsible for increasing behaviours that enhance an animal's search for and intake of palatable foods [51]. The brainstem-hypothalamic pathway is – to some degree – a gateway into the reward system. Food intake stimulates the ARC: the ARC activates the mesolimbic pathway via projections sent directly to the VTA that initiate the cascade of neural events which leads to the release of VTA-derived dopamine in the nucleus accumbens [45]. This is the basis for reinforcement of eating behaviour by food via dopamine release. The VTA also receives oxytocin (OT) innervation from the PVN [52], which is thought to promote termination of intake of palatable tastants [53].

1.1.2. Appetite-related neural and neuroendocrine systems in CTA development

As mentioned earlier, an immediate termination and/or subsequent hunger reduction in the presence of an aversive tastant are the fundamental aspects of a CTA. Therefore, many of the key sites known to be involved in this process include those that regulate appetite. When a sickness/malaise is induced, the brain networks responsible for

termination of food intake are stimulated. C-Fos data show that, following the injection of LiCl, brainstem and hypothalamic areas exhibit enhanced activity [54, 55]. This reflects the fact that GI discomfort is mediated vagally whereas chemoreception of blood-borne toxins occurs via microcirculation around the hindbrain areas where the blood brain barrier (BBB) is weak. Consequently, the peripheral signalling related to a CTA affects the dorsal vagal complex (DMNV and NTS – for vagally mediated changes; and AP for plasma toxicity) [56]. The area postrema (AP) plays a critical role in inducing emesis [57]. This effect was discovered in 1953 by Borison and Wang, who discovered that AP ablation causes a decrease in vomiting rates and this stems from the fact that the AP contains chemoreceptors [58]. Berger et al. investigated whether thermal lesions of the AP alleviate a CTA (what they termed ‘bait shyness’). They saw that lesioned rats were less likely to develop “bait shyness” to those that were sham lesioned, confirming that the AP is critical for the development of CTA [59]. This attenuation of CTA through AP ablation has been further corroborated by multiple studies [60-65]. A recent report showed that lesions of the AP lead to a significantly lower level of brain activity (defined through immunohistochemical detection of an immediate-early gene product, c-Fos) in the amygdala, PVN, supraoptic nucleus of the hypothalamus (SON), and parabrachial nucleus (PBN), regions that therefore may depend on the AP input to properly integrate the full strength of a CTA signal. The magnitude of activation of these regions was correlated with the magnitude of a CTA [55].

Aversive treatments have been shown to induce c-Fos also the NTS [54, 66]. Bilateral lesions in the NTS impair taste aversion learning, however these lesions do not affect a CTA if the aversion is exclusively due to blood-borne substances, which evoke the response via the AP without affecting the vagal component of the CTA pathways [67].

Fibres from the NTS project to the PBN [54]. A 2014 study showed that interactions between the PBN and the amygdala were essential for developing a normal CTA, whereas lateral hypothalamic-PBN pathways were crucial for the speed of acquisition and rate of extinction of a CTA as shown in asymmetric LHA-PBN lesion studies [68]. The hypothalamus hosts two other sites that shape aversive responsiveness, the PVN and SON. Both of these sites encompass OT neurons and they show heightened c-Fos immunoreactivity after treatments that cause a CTA [69-71].

While the brainstem-hypothalamic circuit plays a key role in an immediate cessation of consummatory behaviour, it also reciprocally communicates with – among others - the amygdala and prefrontal cortex, thereby ensuring the consolidation of various facets of CTA-related activity (i.e., stress-, memory- and appetite-related) within the dispersed neuronal network [72-74].

Interestingly, many anorexigenic peptides at the peripheral and CNS level serve as mediators of hypophagia in CTA. In the periphery, cholecystokinin (CCK), glucagon-like peptide-1 (GLP-1), and peptide YY (PYY) seem to be crucial in evoking aversion-driven termination of

ingestive behaviour. CCK, released from the gut, decreases food intake via afferent vagal pathways, primarily by inhibiting gastric acid secretions and gastric emptying [75, 76]. Intravenous (IV) CCK infusions induce the release of OT, a hypothalamic peptide that reduces consumption for energy, consumption for reward as well as promoting discontinuation of feeding when internal milieu is jeopardised (see section 2.2 for more detail). Interestingly, taste stimuli previously paired with IV CCK induce neurohypophyseal release of OT even in anaesthetised rats, which underscores the likely involvement of the CCK-OT functional (multisynaptic) pathway in associative learning of food avoidance [77]. It should be noted that CCK itself is capable of inducing a CTA [78], and this effect can be prolonged through coupling CCK to a 10 kDa polyethylene glycol, which increases the time CCK is present in the general circulation and thus enhances exposure time of CCK1 receptor to its ligand [79]. Overall, the CCK findings exemplify the fact that endocrine processing of aversive responses originates already at the gut level, and the GI tract acts as the gateway to processing behavioural responses to potentially dangerous foods. This notion is supported by data pertaining to other gut molecules. PYY is released from the GI tract into circulation following a meal, and it reduces food intake and gastric motility when administered peripherally [80-82]. Similar to CCK, peripheral PYY can induce a dose-dependent CTA, and thus its appetite reducing effects (under some circumstances) may be due to its aversive properties [83]. Similarly, gut-derived GLP-1, a GI motility inhibitor released at the end of a meal [84],

induces a CTA [85], though its aversive properties seem to be mediated by a very select subset of brain sites [86].

At the CNS level, a number of anorexigenic peptides have been shown to be linked to aversive responsiveness as well. Oxytocin (described in detail in section 1.2.1) is the final component of the circuitry that ensures the development of a CTA. Melanocortin (MC) receptor agonists (especially those that bind to MC3 receptors) have been found to cause a CTA [87]. Furthermore, corticotropin releasing hormone (CRH), one of the most potent anorexigens defined to date, has also been found to cause aversive consequences [88].

Overall, the fact that such a vast array of anorexigenic peptides (both peripheral and central) support aversion and induce cessation of consummatory behaviour that endangers internal milieu strongly indicates that there is a functional overlap in aversion and hypophagia promoting mechanisms. This reflects the necessity for the aversive and anorexigenic processes to be at least somewhat coordinated via common pathways.

Interestingly, while anorexigenic peptides aid in shaping the full spectrum of CTA responsiveness, orexigenic molecules oftentimes appear to prevent the development of aversion. It has been shown that an endogenous MC3/MC4 receptor antagonist, Agouti-related protein (AgRP) alleviates aversive consequences of pairing LiCl administration with an intake of a novel ingestant [89]. The reward system seems to be particularly effective in suppressing anorexigenic responses to toxic foods. This is most likely facilitated by inhibitory input of opioid peptides that

mediate reward by silencing the activity of satiety pathways, including those of the brainstem and the hypothalamus. For example, Olszewski et al (2000) found that various subclasses of opioid peptides suppress acquisition of LiCl-induced CTA in rats acquainted with opioid ligand treatment [90]. In line with those findings, Flanagan et al [91] reported that opioid receptor blockade with naloxone potentiates anorexigenic and aversive consequences of LiCl and CCK treatments.

1.1.2.1. Oxytocin and Vasopressin as Key

Neuroendocrine Systems involved in CTA formation

Oxytocin (OT) and vasopressin (VP) are structurally similar neurohypophysial hormones, whose molecules differ only by two amino acids (Figure 3).

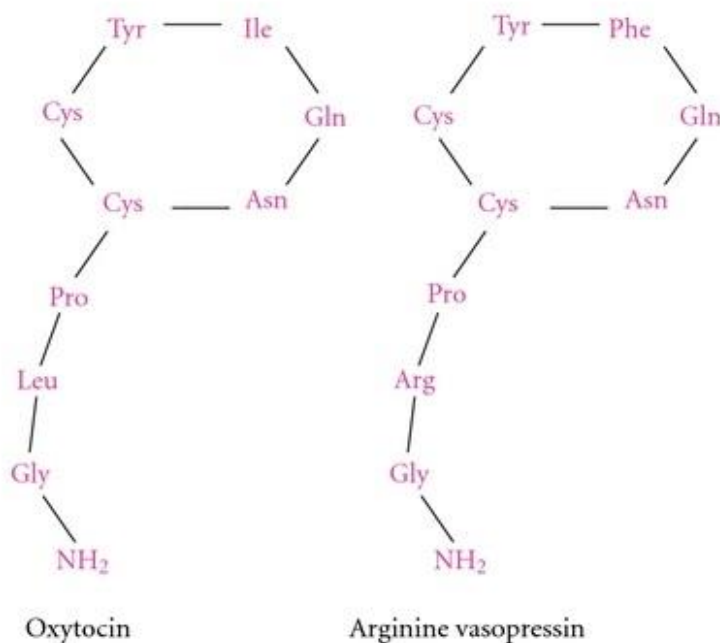


Figure 3: Amino acid sequences of oxytocin (OT) and vasopressin (VP). Figure modified from *The Oxytocin-Oxytocin Receptor System and Its Antagonists as Tocolytic Agents* [92]

These highly conserved peptides are present in all vertebrates except cyclostomes [93, 94]. Gene duplication that occurred early in the evolution gave rise to the mammalian OT-VP system [95]. Similar to the peptide molecules, their respective receptors are thought to have arisen through gene duplication and subsequent divergence to produce the unique receptors. These receptors belong to the G protein-coupled family, four of which are found in humans and rodents: three for VP (V1a-R, V1b-R, and V2-R), and one for OT (OTR). OTR and V1a-R are most abundantly expressed within the brain [96], though V1b-R receptors are also found in the anterior pituitary [97]. While OTR has equal affinity for both OT and VP, the V1a-R receptor has greater selectivity, with a 30-fold greater affinity for VP over OT [98].

OT and VP are synthesised mainly in the hypothalamus: in the PVN and SON, in the accessory nuclei between these two regions, as well as – in the case of VP – in the suprachiasmatic nucleus (SCN) [99, 100]. The magnocellular neurons of the PVN and SON deliver OT and VP to the neurohypophysis and throughout the CNS, whereas parvocellular neurons of the PVN provide only innervation within the CNS [96, 101, 102].

Classically, OT is described as a peptide that promotes parturition and milk ejection, whereas VP is known to have anti-diuretic properties through its action on water reabsorption in the kidney [103-105]. Importantly, OT and VP support termination of ingestive behaviour and participate in the formation of a CTA.

As anorexigens, OT and VP appear to terminate feeding in order to protect homeostasis. For example, OT release occurs upon excessive stomach distension caused by voracious consummatory activity [106]. The OT system is also stimulated by an increase in plasma osmolality – a typical occurrence associated with consumption of salty tastants. Lesions of the PVN, where OT neurons are amassed, cause a loss-of-control overeating [107, 108], and this excessive consumption continues despite a gradual decline in parameters signifying internal milieu. OT also reduces feeding for reward – especially intake of carbohydrates and sweet non-carbohydrate tastants is affected [109-112]. The role of VP in the regulation of consumption has been mainly linked with the control of osmotic balance, and this homeostatic role fits in the general function of VP in kidney processes and blood pressure regulation [113-115]. In line with those findings, intra-peritoneal VP injections induce inhibition of food intake, but they also affect thirst [116].

Both OT and VP support CTA development. Neurohypophysial secretion of OT and VP has been observed following administration of aversion inducing toxins [117]. Aversive treatments stimulate activation of OT and VP neurons in the PVN and SON (defined by c-Fos expression analysis), and pharmacological strategies that reduce CTA decrease activation of OT and VP cells [90]. Furthermore, blocking OT receptors blunts the magnitude of LiCl-induced aversion, and a reduction in activation levels of the CEA is a likely culprit [71].

1.1.2.2. A cross-link between the regulation of food intake and social behaviour: do oxytocin and vasopressin play a role?

The vast majority of data on how OT and VP affect CTA development and food intake have been collected in studies on single-housed animals. However, it should be noted that consumption in social species usually has a social component. In fact, how much animals eat, when they eat, whether they consider food as safe, and when they stop eating, is to at least some extent driven by social interactions. Because of that phenomenon, there has been a recent surge in studies defining the link between appetite and the social context of consumption. Simultaneously, neuroendocrine systems that affect both food intake and sociality have attracted a lot of attention. Interestingly, OT and VP belong to this category.

Oxytocin promotes social behaviour by supporting the development of mother-child interactions, pair bonding as well as in-group social behaviours [118-120]. OT induces behaviours that have been described initially as “altruistic”, although the current consensus is that these are in fact behaviours that benefit a given group of individuals [121-123]. Interestingly, disruption of the OT signalling by administration of the OT receptor blocker leads to abnormal feeding behaviour in mice given access to a meal in a social setting [124].

Similarly to OT, VP plays roles in maternal stress, social behaviour, and sexual motivation [125]. VP receptor blockade in the olfactory bulb impairs social recognition in rodents [126]. VP-null Brattleboro rats given VP via

microdialysis show improved social recognition [127]. Increasing the VP tone in rats improves social memory [128]. Mice lacking V1a-R receptors have poor social recognition [129].

When VP is injected centrally via the lateral ventricle, extinction rate of LiCl-induced CTA rapidly increased [130]. This was also confirmed via peripheral administration of VP [131], but these were in low doses that did not induce a CTA on their own. High doses of VP given after LiCl injections delay extinction of the CTA, and also strengthen its acquisition, and can also induce a CTA when injected alone. Hayes and Chambers believe that this was due to avoidance-inducing properties of VP [132].

Despite this link with sociality, it is not known whether a social context of a CTA might affect OT or VP signalling, thereby modifying aversive responsiveness of socially housed animals that learn to associate an unpleasant GI sensation with a novel food. This is a striking gap in our knowledge especially considering the fact that a 2015 short communication by Hishimura revealed that the ability to interact with conspecifics during a CTA acquisition phase reduces the magnitude of an aversive response [133]. Thus, a certain social buffering system – possibly associated with OT and/or VP – might be underlying a blunted sensitivity to unpleasant GI sensation that would otherwise lead to a reduction in the intake of a tainted ingestant.

1.2. Overarching Goal and Specific Aims

The development of a CTA is a complex process that relies on a combination of appetite, memory, and stress processes. Recently, a short communication has been published in which it was shown that interaction with a conspecific reduces the magnitude of a CTA response. In the current set of studies, I wished to provide a detailed characterisation of the phenomenon of social environment as a factor modifying responsiveness to aversive stimuli. I have therefore sought to:

1. Determine the minimum dose of an aversive agent (LiCl) that is required to induce a CTA in a social vs non-social setting;
2. Determine LiCl-induced patterns of brain activity (defined by marking c-Fos immunoreactivity) in CTA-related forebrain sites in a social vs non-social setting;
3. Determine activation of OT and VP neurons induced by the same dose of an aversive agent (LiCl) in a social vs non-social setting.

Materials and Methods

2.1. Experiment 1: Effect of LiCl on the acquisition of a CTA in mice housed in a non-social environment

Animals:

40 male C57 mice aged 12 weeks old were single-housed in standard mouse cages with wire tops in a temperature-controlled (22°C) animal facility with a 12:12 hour light:dark cycle (lights on at 0700). Food (Sharpe's Feed) and water were available *ad libitum* unless stated otherwise.

Experimental Procedure:

Animals were divided into five treatment groups (n=8): control (saline), 0.6, 1, 3, and 6 mEq of LiCl. Each group was exposed for one hour to a 0.1% saccharin solution after overnight water deprivation solution, and then immediately injected intraperitoneally (i.p.) with vehicle (saline) or LiCl. Three days later, both saccharin and water were presented to the animals in a 2-bottle choice test after overnight water deprivation and the amount of saccharin consumed by each animal was recorded.

2.2. Experiment 2: Effect of LiCl on the acquisition of a CTA in social mice

Animals:

40 male C57 mice aged 12 weeks old were pair housed in standard mouse cages with wire tops in a temperature-controlled (22°C) animal facility with a 12:12 hour light:dark cycle (lights on at 0700). Cages were

divided by a wire mesh such that mice had partial social contact in order to avoid agonistic interactions. Food (Sharpes Feed) and water were available *ad libitum* unless stated otherwise.

Experimental Procedure:

Animals were divided into five treatment groups (n=8): control (saline), 0.6, 1, 3, and 6 mEq of LiCl. Each group was exposed to a 0.1% saccharin solution after overnight water deprivation solution, and then immediately injected i.p. with vehicle (saline) or LiCl. Three days later, both saccharin and water were presented to the animals in a 2-bottle choice test after overnight water deprivation and the amount of saccharin consumed by each animal was recorded.

2.3. Experiment 3: Effect of Social Housing versus Non-Social Housing on neuronal activity in mice following injection of Lithium Chloride

Animals:

24 male C57 mice aged 12 weeks old were pair housed in standard plastic cages with wire tops in a temperature-controlled (22°C) animal facility with a 12:12 hour light:dark cycle (lights on at 0700). Food (Sharpes Feed) and water were available *ad libitum* unless stated otherwise.

Experimental Procedure:

Mice were pair housed for a minimum of seven days and 12 mice were divided into two groups (n=6): 1) pair-housed LiCl and 2) pair-housed Saline (Sal). Group 1 received a 1 mL 6 mEq LiCl solution injection, and Group 2 received a 1mL saline injection. Food was removed immediately

after the injection and euthanasia of groups 1 and 2 was performed 60 minutes after injections (see euthanasia below).

The remaining 12 mice were single-housed for seven days, and were divided into two groups (n=6): 3) Single-housed LiCl and 4) Single-housed Sal. Group 3 received a 1mL 6 mEq LiCl solution injection, and Group 4 received a 1 mL saline injection. Food was removed immediately after the injection and euthanasia of groups 3 and 4 was performed 60 minutes after injections (see euthanasia below).

Euthanasia and Perfusion:

Sixty minutes post injection, mice were deeply anaesthetised with 1 mL 35% urethane, and perfused intracardially with 50 mL of 4% paraformaldehyde (PFA) in a 0.1 mol L⁻¹ phosphate buffer (pH 7.4), and their brains were dissected out.

Immunohistochemistry:

Coronal sections of 60 µM were cut on a Vibratome (Leica), and processed as free-floating sections. All intermediate tissue rinsing was done four times with Tris-buffered saline (TBS) on a rocking table, and all incubation procedures were done in a Supermix solution of 0.25% gelatin (Sigma) and 0.5% Triton X-100 (Sigma) in TBS.

Single staining for c-Fos: Tissue was first treated for 10 minutes in 3% H₂O₂ in 10% methanol (MeOH) (in TBS), then rinsed and incubated overnight at 4°C in polyclonal goat anti-c-Fos antibody (1: 1000, Santa Cruz). After rinsing, sections were incubated for 1 hour at room temperature in a secondary biotinylated rabbit-anti-goat antibody (Vector

Laboratories) (1:400 in Supermix), then rinsed and subsequently incubated for 1 hr in the avidin-biotin complex (Vector Laboratories). After additional rinsing, sections were placed in a solution of 0.05% diaminobenzidine (DAB) (Sigma), 0.01% H₂O₂ and 0.2% nickel sulphate in TBS, in order to develop staining.

Mounting:

Sections were mounted on gelatinised microscope slides, dried, and dehydrated in ascending concentrations of ethanol, then soaked in xylene, and embedded in Entellan.

Analysis:

Sections were examined under a light microscope, acquiring images with a camera (Olympus DP70), and analysed using Scion image software. Four areas of interest were outlined using the neuroanatomical atlas by Paxinos and Watson - these areas were the paraventricular nucleus of the hypothalamus (PVN), nucleus accumbens (NAcc), basolateral nucleus of the amygdala (BLA), and central nucleus of the amygdala (CEA). Each region was counted using ImageJ software cell counter plugin and the area was recorded using scale. Percentages of active neurons per square mm were calculated. The data was averaged per animal, and then per experimental group.

2.4. Experiment 4: Effect of Social Housing versus Non-Social Housing on OT and VP neuronal activation in mice following injection of Lithium Chloride

The perfused and sectioned brain tissue from experiment three was used to perform double-immunohistochemistry with c-Fos and OT as well as c-Fos and VP.

Double staining for c-Fos and Oxytocin: The method outlined above for single-staining of c-Fos was performed. The procedure for the oxytocin double-staining involved repeating the same procedure after the completion of c-Fos staining, with overnight incubation in rabbit anti-oxytocin antibody (Vector Laboratories) followed by incubation in secondary biotinylated goat anti-rabbit (Vector Laboratories) antibody. The oxytocin staining was developed in a 0.05% diaminobenzidine (DAB), 0.01% H₂O₂ in TBS solution without nickel sulphate.

Double staining for c-Fos and Vasopressin: The method outlined above for single-staining of c-Fos was performed. The procedure for the vasopressin double-staining involved repeating the same procedure after the completion of c-Fos staining, with overnight incubation in polyclonal sheep anti-vasopressin antibody (Millipore) followed by incubation in secondary biotinylated rabbit anti-sheep (Vector Laboratories) antibody. The vasopressin staining was developed in a 0.05% diaminobenzidine (DAB), 0.01% H₂O₂ in TBS solution without nickel sulphate.

Mounting:

Sections were mounted on gelatinised microscope slides, dried, and dehydrated in ascending concentrations of ethanol, then soaked in xylene, and embedded in Entellan.

Analysis:

Slides were examined under a light microscope, and a cell count was performed by the experimenter. Areas of interest (PVN) were identified and outlined using the neuroanatomical atlas were identified, and the total numbers of oxytocin/vasopressin neurons were counted. Subsequently, using the c-Fos staining, total numbers of active oxytocin/vasopressin neurons were counted. A neuron was considered active if the c-Fos stain fell within the oxytocin/vasopressin stain, signalling an active nucleus of that neuron. The total numbers of active oxytocin/vasopressin neurons were calculated as a percentage of the total number of oxytocin/vasopressin neurons.

Statistics:

Results are presented as means \pm SEM. All data was analysed using two-way ANOVA. Post hoc comparisons were performed using a Fisher's test for Experiment 1 and 2, and a Tukey test for experiment 3, with a significance level of $p < 0.05$. Values were considered significantly different when $p < 0.05$.

All experiments had prior approval of the Animal Ethics Committee at the University of Waikato.

Results

3.1. Experiment 1: LiCl reduces consumption of 0.1% saccharin in mice housed in a non-social environment

LiCl injections paired with exposure to a novel diet (saccharin) in non-socially housed mice induced a CTA at concentrations of 1 mEq LiCl and above, which was confirmed three days later in a two-bottle choice test. The reduction of saccharin consumption as a result of the LiCl injection follows a dose-response curve (Figure 4).

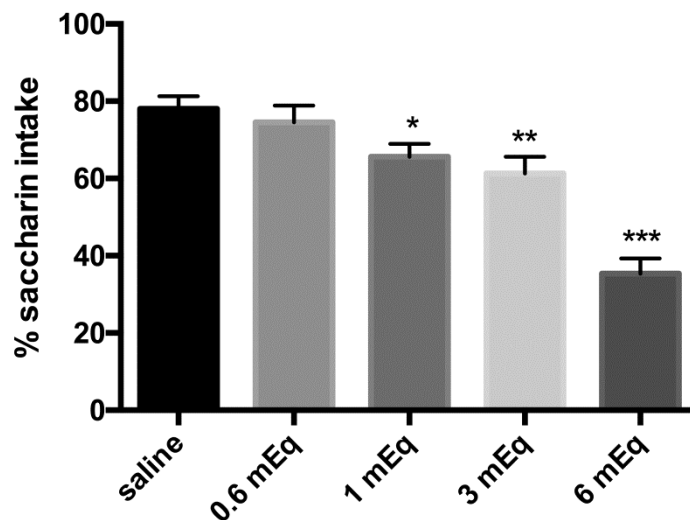


Figure 4: The effect of 0 (saline), 0.6, 1, 3 and 6 mEq of LiCl on acquisition of a CTA to a 0.1% saccharin solution in mice housed in a non-social setting. LiCl was administered IP just after the initial exposure to the saccharin solution. A CTA was established in a two-bottle test in which a choice between water and saccharin was given. The data are shown as % of saccharin solution intake in the cumulative fluid intake. *, **, *** = significantly from saline ($p = <0.05$, <0.01 , and <0.001 respectively).

3.2. Experiment 2: A higher dose of LiCl is necessary to induce a CTA in mice housed in a social environment

LiCl injections paired with exposure to a novel diet (saccharin) in socially housed mice failed to induce a CTA at the same concentrations as in mice housed in a non-social environment. Only at the highest concentration of LiCl, 6 mEq, was a reduction in saccharin intake 3 days after the injection measured (Figure 5).

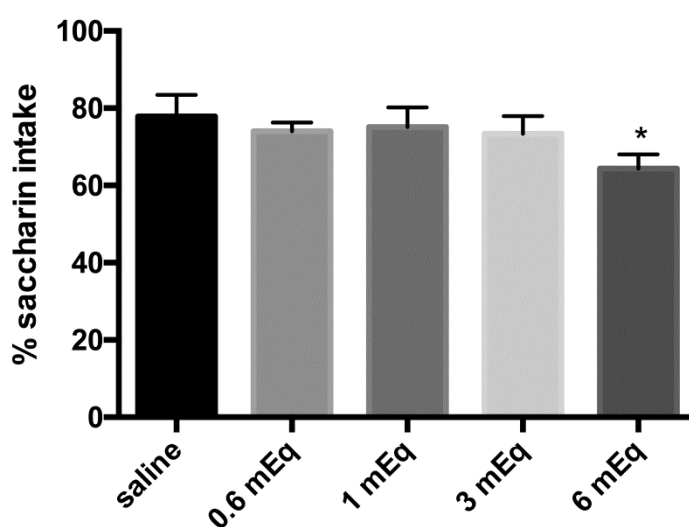


Figure 5: The effect of 0 (saline), 0.6, 1, 3, and 6 mEq of LiCl on acquisition of a CTA to a 0.1% saccharin solution in mice housed in a social setting. LiCl was administered IP just after the initial exposure to the saccharin solution. A CTA was established in a two-bottle test in which a choice between water and saccharin was given. The data are shown as % of saccharin solution intake in the cumulative fluid intake. *, **, *** = significantly from saline ($p < 0.05$, < 0.01 , and < 0.001 respectively).

3.3. Experiment 3: Social versus non-social housing differentially affects c-Fos IR in LiCl-treated mice

A significant increase in c-Fos immunoreactivity (IR) was observed in the PVN and CEA ($p = 0.05$ and < 0.01 respectively), but none in the NAcc or BLA in non-socially housed mice injected with LiCl compared to socially housed mice injected with saline. The same increase in c-Fos IR in those areas was found in LiCl-treated mice housed in a social environment ($p =$

<0.01 for PVN, and 0.02 for CEA). For animals injected with LiCl, c-Fos IR was greater within the PVN and a significantly reduced in the CEA in mice that were socially housed compared to those that were isolated ($p = 0.01$ and <0.01 respectively). This decrease of activation in the CEA was comparable to the isolated saline levels (Figure X). The socially housed saline treatment group had a decrease in activation of the NAcc compared to non-socially housed saline treatment group ($p = <0.05$). These results are summarised in Figure 6.

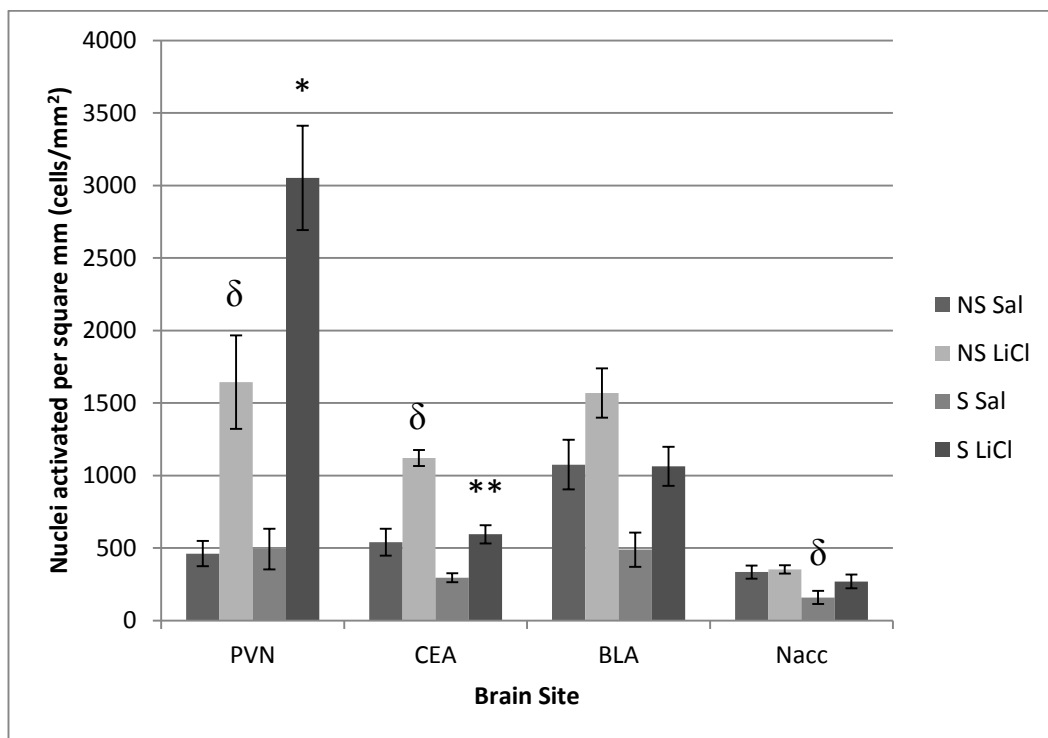


Figure 6: Density of c-Fos nuclei in areas implicated in the development of a CTA. PVN – paraventricular nucleus of the hypothalamus; CEA – central nucleus of the amygdala; BLA – basolateral amygdala of the nucleus; NAcc – nucleus accumbens. δ = significantly different from Single Sal ($p = <0.05$). NS = Non-Socially housed, S = Socially Housed, Sal = saline, LiCl = lithium chloride. *, ** = significantly from saline ($p = <0.05$ and <0.01 respectively)

3.4. Experiment 4: A social environment enhances LiCl-induced activity increase of OT neurons compared to a non-social environment

Looking at the OT activation in PVN, there was no significant difference between the saline groups. Non-socially housed LiCl mice had greater activation of OT neurons in the PVN than non-socially housed saline ($p < 0.01$), and socially housed LiCl had a greater activation of OT neurons compared to non-socially housed LiCl ($p = < 0.05$). On the other hand, activation of VP neurons was only significantly different between non-socially housed saline and non-socially housed LiCl/paired LiCl ($p = < 0.01$ and < 0.05 respectively). Figure 7 Figure 8 illustrate these results.

Examples of photomicrographs used to collect data for experiments 3 and 4 are illustrated in Figure 9 Figure 10.

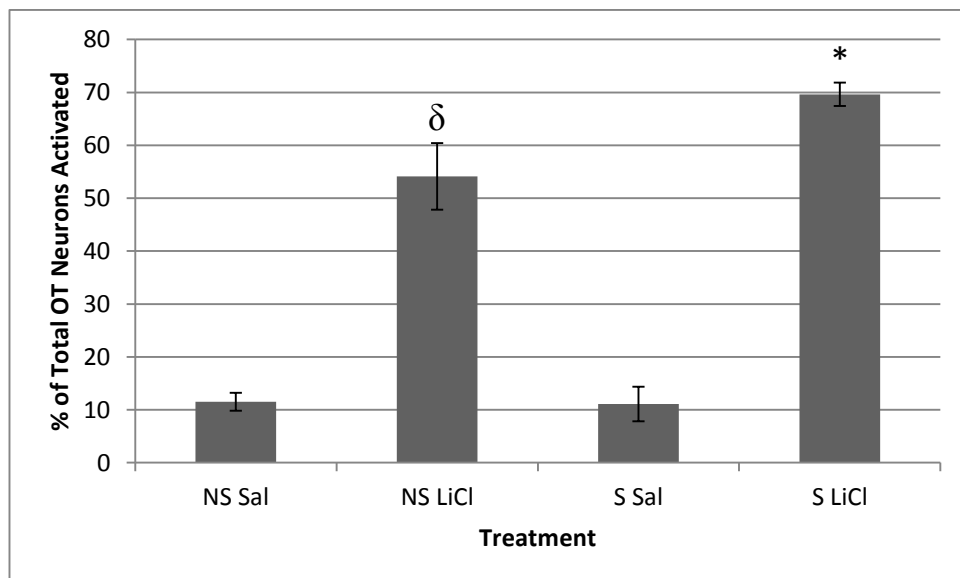


Figure 7: Effect of LiCl versus Saline on activity of OT neurons within the PVN in animals housed in a social versus non-social setting. NS = Non-Socially housed, S = Socially Housed, Sal = saline, LiCl = lithium chloride. δ = significantly different from Single Sal ($p = < 0.05$), * = significantly from Single LiCl ($p = 0.05$).

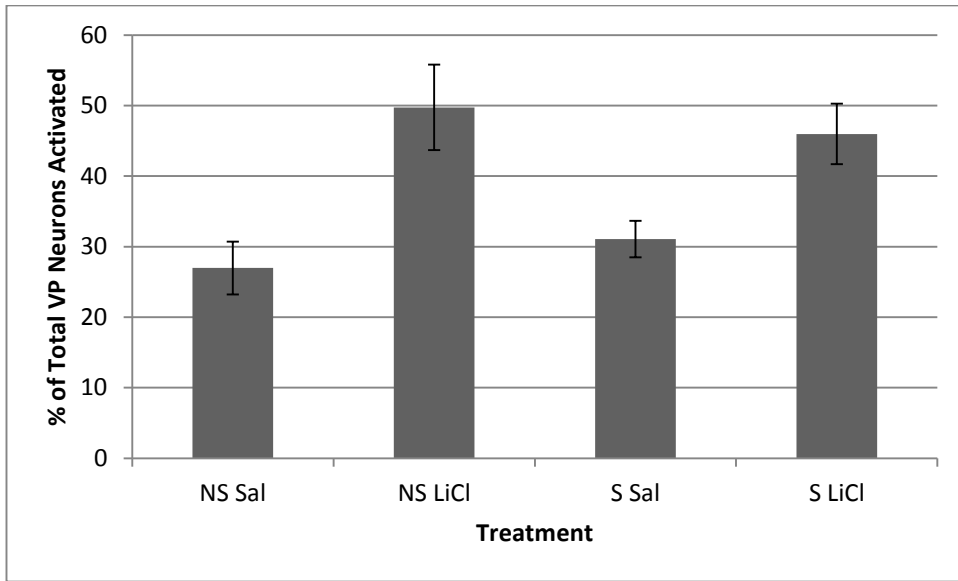


Figure 8: Effect of LiCl versus Saline on activity of VP neurons within the PVN in animals housed in a social versus non-social setting. No significant effects found within social comparison or non-social comparisons

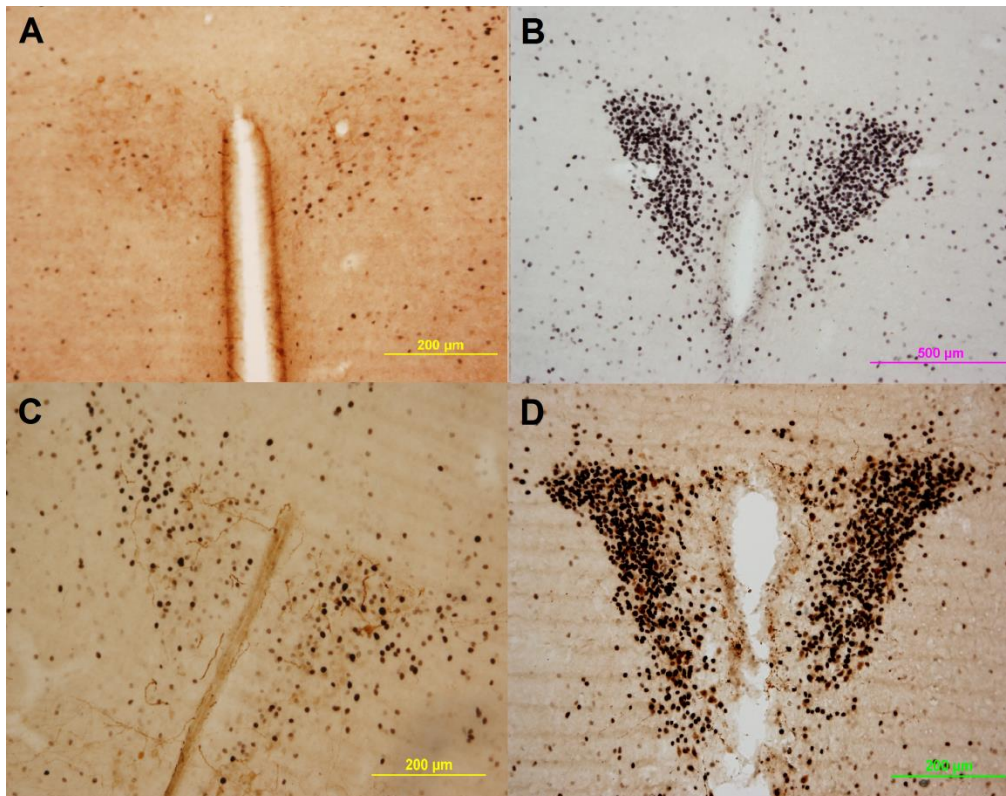


Figure 9: Photomicrographs depicting c-Fos immunoreactivity within the paraventricular nucleus of the hypothalamus (PVN) of mice injected with saline and non-socially housed (A), LiCl and non-socially housed (B), saline and socially housed (C), and LiCl and socially housed (D).

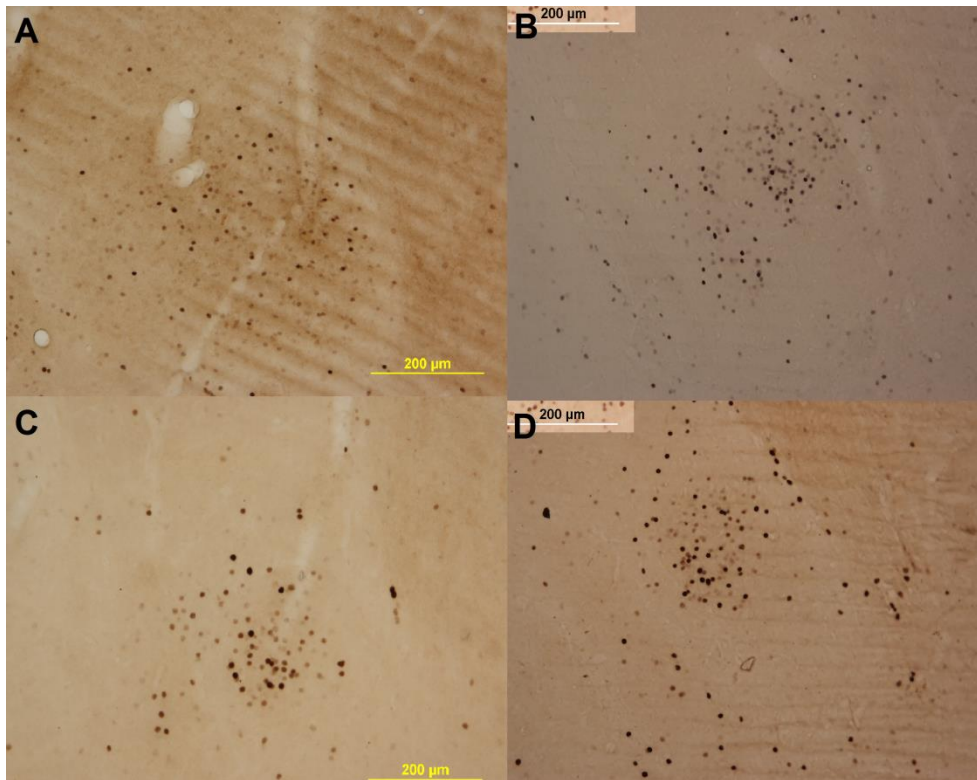


Figure 10: Photomicrographs depicting c-Fos immunoreactivity within the central nucleus of the amygdala (CEA) of mice injected with saline and non-socially housed (A), LiCl and non-socially housed (B), saline and socially housed (C), and LiCl and socially housed (D).

Discussion

An adverse event of either physiological or environmental nature (the latter, being subsequently “translated” to a certain set of physiological processes) typically triggers protective mechanisms whose goals include ensuring that the consequences of this event do not jeopardise homeostasis and that – if possible – the risks that this adverse event might reoccur are minimised. In appetite regulation, behavioural and neuroendocrine changes are in the forefront of processes preventing the animal from consuming foods that pose threat to internal milieu. What seems to be almost counterintuitive is that food-borne risk avoidance is extremely dynamic and can be modified by a variety of factors. The most commonly encountered aspect of this phenomenon is voracious overeating, which occurs (in humans and laboratory animals alike) upon presentation of highly palatable foods, especially in the intermittent fashion [134, 135]. Such foods are consumed in extremely large quantities upon multiple exposures, even though each of the previous overeating episodes would have resulted in excessive stomach distension and changes in plasma osmolality that activated a plethora of homeostatic mechanisms and produced sickness/malaise [45]. One can easily understand the need for such behavioural plasticity: finding the intricate balance between potential gains versus negative consequences of key behaviours, including food intake, is the key aspect of survival, especially in the evolutionary context of adaptation to the environment in which food is a scarce resource [135, 136]. Interestingly, we know very little about factors that are capable of shifting the risk-versus-gain scale by diminishing the

avoidance response. The current project has therefore offered a unique opportunity to examine social behavioural processes that influence acquisition of a conditioned taste aversion and define neural mechanisms that underlie the changed aversive response in LiCl-treated animals subjected to different social environment scenarios.

At the first stage of the experimental trials, I established dose response curves for LiCl-induced CTA in socially housed versus non-socially housed mice. The results showed that animals that did not have social exposure were more sensitive to aversive properties of LiCl as they needed a much lower (6 times) dose of the drug to reduce consumption of saccharin in a two-bottle test, compared to the mice in the social environment.

In 1955, Garcia made the initial observation that water and food intakes are suppressed following low doses of gamma radiation, but only if the ingestants are offered in containers that were used in the radiation chamber. Then, in order to study whether it was an associative learning phenomenon that took place, he developed a model of CTA in which rats were exposed to a 0.1% saccharin solution during a radiation treatment and he found that when the animals were later given simultaneous access to the saccharin solution and water (this time, in containers different from the ones used in the radiation chamber), they showed a significantly decreased preference for palatable saccharin [13]. When saccharin and water were presented to the rats daily for the next 60 days, the CTA animals gradually extinguished their aversive response, though the entire extinction process would require approximately 30 days. A CTA to saccharin was dose-dependent – the higher the dose, the greater

magnitude of an aversive response [13]. This paradigm in which a non-caloric and palatable saccharin solution is used in a choice scenario against gustatorily neutral water was quickly adopted by other investigators interested in aversion and it has been successfully used since (included in the experiments presented here) to define aversive properties of treatments [10]. Pharmacological agents replaced radiation as standard means of inducing a CTA. Among them is LiCl that – upon intraperitoneal administration – consistently generates gastrointestinal discomfort, which is sufficient to produce a conditioned response in choice paradigms, including saccharin-water two-bottle tests. In fact, LiCl CTA has become a standard CTA model, most frequently used in biomedical and biopsychological studies involving laboratory animals [137]. Despite the consensus on the reliability of LiCl as a CTA inducing agent, some variability in responsiveness to LiCl has been noted. For example, a magnitude of an aversive response is not only species-, but also strain-dependent: Long-Evans Hooded rats showed display a greater avoidance of a saccharin solution after an associative learning treatment (thus, a stronger CTA) than Sprague-Dawley rats [138]. The type of test used to determine the acquisition of a CTA to LiCl also affects a magnitude of the CTA – the use of a two-bottle choice test rather than just giving access just to a single tastant produces more pronounced outcomes [139]. For instance, the same dose of the aversive agent that produces a CTA in a two-bottle test is ineffective in reducing consumption of that tastant if the animal has no other choice of fluid [140]. Some sex differences occur when assessing CTA acquisition in rodents: Chambers and Sengstake

found that in females extinction of an LiCl-induced CTA to sucrose was faster than in males [141]. This discrepancy between sex and CTA development seems to be dependent partly on testosterone – gonadectomised males were less likely to develop a CTA compared to intact males, and administering testosterone to these males counteracted this effect [142]. Furthermore, younger animals are more resistant to CTA development, which is possibly due to the fact that taste information is more readily forgotten as it is not as salient in younger animals as in adults [143]. Another factor that changes the strength of a CTA is the number of conditioning trials – three trials versus one in mice induces a greater level of saccharin preference suppression, which is also reflected by further elevated c-Fos immunoreactivity in the NTS, PBN and CEA [144].

Nachman and Ashe showed that the threshold dose for acquisition of a CTA to a saccharin solution using intraperitoneal injections of LiCl in rats was 0.15 mEq/kg, and that the strongest aversion to saccharin occurred at around 3 mEq/kg [145]. Most authors report that 1-3 mEq/kg range is effective in inducing aversion in rodent species. This is consistent with the results obtained herein for mice in the non-social environment: the 1 mEq dose of LiCl was sufficient to generate a CTA. The maximum response (i.e., the highest levels of statistical significance when compared to saline controls as well as the greatest percentage change) was achieved with 6 mEq of LiCl. Similarly to other studies, once more pronounced aversive responses are achieved (such as with 6 mEq in the non-social scenario here), a preference for the palatable saccharin solution decreases by 50% or more.

One of the most common features of the vast majority of CTA experiments available in the current literature is that they were performed in animals individually housed, and without any clear and predictable social contact. In such studies, single-housed animals are typically given access to a novel food, and following presentation of this stimulus, they are injected with LiCl. What seems to be a standard procedure may in fact constitute a confound per se, because it has been shown that social isolation has a profound impact on a vast array of responses to negative stimuli, as – for example - it affects depressive- and anxiety-like profile of an individual. Thus, a 2015 paper by Hishimura has produced a groundbreaking discovery demonstrating that a social environment affects the acquisition of a CTA. In that paper, a single LiCl dose (0.2 M LiCl at 20 ml/kg b. wt.) was given right after exposure to a novel 0.2% saccharin solution in male mice, and the animals were either kept in isolation or they were placed in a social setting (with a male conspecific). Development of a CTA to saccharin was tested through a two-bottle test over the course of 3 days. LiCl-treated mice paired with their conspecifics drank as much saccharin as non-CTA saline controls paired with conspecifics [133]. Importantly, animals maintained in isolation after the aversive treatment, developed a typical aversive response [133]. In the current set of experiments, we expanded on the Hishimura study and generated a full dose-response curve for LiCl-CTA in animals that were in the non-social environment. We then followed with the experiment in which the same dose range was tested in mice that had been housed socially. We found that, in non-social cohort, a 1-mEq dose was sufficient to develop a mild

CTA, and the 6-mEq dose decreased saccharin preference by 50%. On the other hand, in the social scenario, 6 mEq LiCl was the lowest effective dose. Not only was the dose higher, but also the magnitude of the aversive response was hampered: saccharin consumption dropped only by about 15%.

Thus, the dose needed to induce a CTA when there is social contact is about 6 times higher than when the animal is isolated. It suggests that sociality is an important modifying factor in CTA acquisition and that social exposure appears to promote anti-aversive effects. It is still uncertain though what the exact mechanism underlying this interaction between aversion and sociality might be. Hishimura hypothesised that this effect could have been caused social stress producing analgesia [133]. He proposed that – since male mice show a number of agonistic behaviours upon social reintroduction [146] – anxiogenic aspects of the social environment might overshadow the process of associating the malaise with the novel saccharin solution. Therefore, according to this hypothesis, social stimulation would not have reduced aversion through mechanisms positive for the animal from the behavioural and physiological contexts, but rather would be processed as a more deleterious event than the GI discomfort associated with the novel flavour. Hishimura's notion was based on the fact that in his study, there was full physical contact between the animals, allowing the mice to exhibit a complete spectrum of behaviours, including the agonistic ones [133]. In order to address this issue, the current set of experiments utilised a modified approach, in which social contact was only partially retained (through the wire mesh partition

of the cage), but the occurrence of agonistic behaviours and consequences of those was eliminated. Despite that, the anti-aversive effect of social stimulation persisted, which strongly indicates that agonistic facets of social interactions play (if at all) a much lesser role than initially proposed by Hishimura.

The c-Fos data obtained here shed more light on neural and neuroendocrine processes underlying the hampered CTA responsiveness in the social setting.

Our c-Fos data in the non-socially housed animals confirm earlier findings: activation of the PVN and CEA is elevated upon administration of LiCl at aversion promoting doses [32, 55, 69, 144]. Recently, an increase in the c-Fos expression of another neuroanatomical subdivision of the amygdala, the BLA, along with the CEA, PVN, and SON, has been reported in response to LiCl treatment [71]. In this set of experiments, only a trend suggesting an increase was detected in the BLA, but even though it has only neared significance, it reflected overall changes in the hypothalamic and amygdala circuitry during CTA acquisition. Assuming this slight difference is not caused by an individual variability component of cohorts of animals used in those two distinct studies, another possibility could be that the BLA is more critical for the retrieval of CTA [147] – this is particularly likely since the earlier report looked at neuronal activity following CTA trials. However, in this experiment only one injection of LiCl was given and this was prior to perfusion, no actual CTA conditioning trials were performed, hence the BLA may not have been activated purely because of the lack of the associative learning context normally present in

the CTA. In fact, the injection of LiCl in absence of the associative scenario might have been more reflective of the anorexigenic aspect of an aversive response (rather than associative one), and therefore as a stimulus it was not sufficient to engage the BLA.

This is in line with the unpublished findings from our laboratory showing that injections of an anorexigen, OT, in the BLA do not reduce energy- or palatability-driven food intake. On the other hand, the BLA participates in generating emotional value to events via associative learning [148].

One of the candidate molecules I wished to investigate was OT, because of its pleiotropic role in food intake, social behaviour, and anxiety. While in the introduction it was stated that OT is an anorexigenic peptide, this is not entirely true. Instead, OT appears to modulate consumption of specific macronutrients, namely reduction of consumption of carbohydrates. Mice with OT gene deletions experience an enhanced intake of sucrose solutions both initially and sustained compared to mice without the gene deletion [112], and this increased preference is not seen for fats (intralipid) in these knockouts [149]. Administration of an OT-A increases sucrose intake in wild type mice, whether the sucrose solution contains intralipid or not. It had no effect on consumption of intralipid solutions alone, or on total calorie intake [149]. It is unclear whether it is specifically sweet tastes that OT inhibits consumption of, or if it is carbohydrate related. One study showed that OT knockout mice consumed greater amounts of saccharin, a non-nutritive sweetener, compared to wild type mice at all times of the dark:light cycle [150]. A more recent study showed that the use of an OT-A increased consumption of multiple sweet solutions (both nutritive and non-

nutritive), and that gene expression of OT was increased after sucrose exposure, but not saccharin exposure. Sucrose was seen to be particularly sensitive to inhibition of OTR [151].

In the present set of studies we observed a significant increase in activity of not only PVN neurons, but particularly of OT neurons within the PVN in both LiCl-treated groups: non-socially housed mice and mice exposed to social stimulation. This increase in OT neuronal activity is not surprising, as already Verbalis and colleagues in 1986 noted an increase in OT and - to a much lesser degree - VP release in response to LiCl in CTA animals [117]. However, our results indicate a much larger increase in OT neuronal activity in the socially housed LiCl-injected group compared to non-socially housed animals. OT neuronal activity is higher in animals housed in a social setting [152, 153], which means that the social component has a different influence on OT neurons and that in this case, we likely observed an additive effect of two distinct subpopulations of OT neurons: the first one mediating hyponeophagia, and the second one mediating sociality. Further studies are needed to decipher this effect, however, the increase in OT neuronal activity in socially stimulated animals may cause anxiolysis, resulting in a milder aversion following LiCl exposure. Administration of exogenous OT in rats causes anxiolysis [154]. It is possible that this effect is caused by the action of OT on the OTR in serotonergic neurons – Yoshida et al. demonstrated that the OTR was present in serotonergic neurons in mice, and when OT was infused centrally, serotonin was released from the median raphe nucleus, reducing anxiety-like behaviour. The anxiolytic effect of OT was blocked when a

serotonin receptor antagonist was infused, suggesting that OT can directly activate serotonergic neurons [155]. Another area of interest when looking at the anxiolytic effect of OT is the medial pre-frontal cortex (mPFC) – bilateral administration of OT into the prelimbic (PL) region of the mPFC in rats (both male and female) reduced anxiety-like behaviours, an effect that was not seen when VP or OT-A were administered [156]. The PL mPFC contains OTR-expressing neurons and receives long range OT projections from the hypothalamus – a 2015 thesis by Shirley Dong showed that exogenous OT in the PL mPFC reduced anxiety behaviours in rats, and this effect was blocked by GABA-receptor antagonists. The conclusion from this thesis was that attenuation of anxiety related behaviour by OT was performed through a GABAergic system [157]. In addition, another recent study demonstrated that intra-nasal administration of OT in humans enhanced connectivity between the mPFC and amygdala [158]. We did observe a decrease in c-Fos activity in the CEA in socially housed animals in the present set of studies. The CEA is the major output from the amygdala to the autonomic nervous system for expression of conditioned fear responses [159]. The CEA innervates both the hypothalamus and brainstem via efferent neurons [160]. It is necessary to establish fear learning – if inactivated, fear learning is disrupted, and protein synthesis inhibition within the CEA disrupts consolidation of memories relating to the fear learning [159]. Both the CEA and BLA are well established in the literature as being necessary for fear conditioning – electrolytic and ibotenic lesions of the CEA and NMDA-induced lesions of the BLA block fear-potentiated startles to conditioned stimuli [161, 162]. When rats were

made aversive to a strawberry flavoured solution, glutamate (a common excitatory neurotransmitter within the CNS) was released in the amygdala when re-presentation of the solution occurred, indicating activation of the amygdala in processing the response to this stimulus [163].

The CEA is innervated by both OT and VP neurons [164] – however, in the social LiCl group, only OT neuron activation was significantly different in the PVN, not VP. This indicates that the underlying connection of social buffering is modulated by OT and not VP.

László and colleagues recently published their findings demonstrating that direct administration of 10 ng OT bilaterally into the CEA attenuates anxiety in rats during elevated plus maze tests, and develops positive reinforcement during conditioned place preference testing [165]. In addition, a publication by Knobloch et al. revealed that when OT expressing axons were stimulated *in vitro*, a GABAergic circuit was activated that decreased output in the CEA. These findings were then confirmed *in vivo* through freezing responses in fear-conditioned rats [166]. GABA is an inhibitory neurotransmitter in the CNS, and is known for decreasing excitation neural circuitry.

Within the CEA, the medial part is the region from which projections to the hypothalamus and brainstem stem [167]. The inhibitory effect of OT on the CEA acts through these medial neurons via activating GABA circuits.

These GABA circuits are found in the capsular lateral parts of the CEA [164]. The behavioural and physiological effects of fear conditioning are regulated through the CEA by distinct neuron populations – OT affects the

behavioural response, without affecting the physiological response [72]. It has been shown that there are OT-positive axon terminals in the CEA, and that application of OT on the CEA can attenuate fear-related behaviours [168], indicating that our observations of decreased CEA activity in socially housed animals did not stem from hyponeophagia, but from mediating anxiolysis.

In further studies, it would be beneficial to look at injecting an OT antagonist alongside LiCl and look at relative OT activation in the PVN and SON, and cFos activation in the CEA, to see if there is a drop despite there being social contact. This would help narrow down the correlation we see being caused by OT and not some unrelated mechanism. Alongside this, anxiety tests could also be measured in order to assess behaviourally whether anxiolysis is the main effect

While in this thesis the focus of brain sites was the forebrain, it may be worth looking into hindbrain structures implicit in acquisition of a CTA, such as the AP and NTS. While it is unlikely that social stimulation has a profound effect in these regions, it may be necessary to look at, especially considering the reciprocity of connections between the PVN and brainstem structures. Future studies would look into whether the brainstem displays different activity after social treatment.

Other regions within the forebrain could also be investigated. The medial pre-optic area (MPOA) is a region associated with sociality and bonding [169, 170], and OT injections into this regions facilitate social recognition in rats [171]. The PL mPFC, which is involved in anxiety and expresses

OTR's [157] may also play a role in the attenuation of CTA acquisition through innervation of the amygdala and may need to be investigated.

Another direction to take this research could be to look at the role of sociality in extinction of a CTA. An experiment could be designed such that animals are separated after bonding, then made aversive to a novel food, and then look at whether returning social stimulation in the presence of this food will increase the rate of extinction, versus the social stimulation being absent. In this scenario, the BLA may also have varied activation, as it is involved in the retrieval of CTA memories, and this function might change when social stimulation is present.

Conclusions

Overall, our data expand on our understanding in how sociality plays a role in attenuating the development of a CTA. Our results show that a 6–times higher LiCl dose is required to induce a CTA in socially housed animals compared to single-housed mice. Immunohistochemical analysis of neuronal activity marker, c-Fos, showed that alteration of activity in the paraventricular nucleus of the hypothalamus (PVN) and central nucleus of the amygdala (CEA) is associated with the observed changes in responsiveness to the aversive treatment. Furthermore, in LiCl-injected mice subjected to social stimulation, also the percentage of Fos-positive OT, but not vasopressin neurons in the PVN is higher than in LiCl-treated single-housed mice. It underscores the importance of OT pathways in both sociality and aversive processes, however, it also suggests that distinct subpopulations of OT neurons might be contributing to each of these mechanisms in an independent manner.

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