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Conditioned Taste Aversion Dependent Regulation of Amygdala Gene Expression

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1. Introduction

Feeding is an important activity for all animals providing nutrients essential for survival and reproduction. Not surprisingly, learning plays a critical role in feeding behavior through the establishment and strengthening of food preferences and aversions. That is, when an animal eats, food-related signals (e.g. taste) are associated with post-ingestive visceral signals related to the consequences of its ingestion. If consumption is followed by negative gastrointestinal consequences (e.g. nausea, sickness, or vomiting), the taste cue becomes an aversive signal. This form of taste memory is called conditioned taste aversion (CTA) and discourages consumption upon subsequent exposure to that or any similar taste. Experimentally, this form of learning is often induced by pairing intake of a novel taste stimulus with peripheral LiCl administration [1–3].

Neural processing in the parabrachial nucleus (PBN) is obligate for the integration of orosensory and visceral signals responsible for CTA. From the PBN, one rostral projecting pathway carries axons to the gustatory and visceral cortical areas via the thalamus and another directly to ventral forebrain areas such as the lateral hypothalamus, amygdala, and bed nucleus of the stria terminalis [4–11]. Because lesions in the thalamus have no obvious effect on CTA [12;13], the direct PBN pathways to ventral forebrain structures contribute to formation of aversion gustatory memories.

Of these forebrain areas, the role of the amygdala in CTA has been investigated the most. Lesion studies demonstrate a prominent role for the basolateral nucleus of the amygdala (BLA), but are not in full agreement regarding the effects of damage to the central nucleus (CeA). Some studies unveiled no effect of CeA lesions on CTA [14–16], while others have shown that CeA lesions placed prior to conditioning disrupts learning [17;18]. Based on neural recordings, however, the response characteristics of CeA and BLA neurons to a conditioned taste stimulus are altered [19;20]. Indeed, LiCl administration itself induces expression of AP-1 transcription factors in both amygdala nuclei [21;22], and disruption of cAMP response element-binding protein (CREB) or c-fos activity in this region during the

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time of taste/LiCl pairing, but not after, impairs learning [23–25]. Similar results have been observed following transient blockade of CREB and c-fos associated intracellular signaling cascades, protein kinase A and C [26;27]. Together these studies provide compelling evidence that neural processing and subsequent activation of cAMP/Ca²⁺/CREB pathways in the CeA and BLA plays a critical role in CTA. The activation of cAMP/Ca²⁺/CREB pathways during the early taste/visceral associative phase of CTA implies that formation of long-term memory requires protein synthesis. Consistent with this notion, administration of anisomycin, a protein synthesis inhibitor, into the amygdala impairs CTA [23]. Despite these recent advances, a major gap in knowledge remains regarding later changes in downstream gene expression.

Expression profiling with DNA microarrays can be used to develop gene/gene product association networks that may underlie complex behavioral attributes. For example, microarrays have been widely used to study altered gene activity associated with ethanol exposure leading to a testable set of hypotheses regarding underlying molecular events [28–30]. To the best of our knowledge only a single study has used this approach to examine genes correlated with CTA behavior. In pond snails, Azami and colleagues [31] identified 2 known genes and 40 unknown genes that changed their expression levels following CTA memory formation. One of the known genes, molluscan insulin-related peptide, plays a role in neurite formation [32], and its up-regulation following CTA is hypothesized to contribute to altered synaptic morphology.

The aim of the present study was to characterize late CTA responsive genes in the CeA and BLA of rats using gene expression arrays. We chose a training procedure consisting of two CS-US pairings to ensure maximal suppression of CS intake. We hypothesized that changes in gene expression underlying the long-lasting behavioral plasticity associated with CTA would be persistent, as has been shown for some mRNA associated with long-term memory for sensitization of the gill- and siphon-withdrawal reflexes in *Aplysia californica* [33] and psychostimulant-elicited plasticity in genetic mouse models [34]. Subsequent qRT-PCR, Western blot, and ELISA analyses were used to independently assess the microarray data for a subset of genes. Our results revealed that largely distinct sets of genes were associated with long-term CTA memory compared to non-contingent LiCl treatment that does not support development of CTA. To further support the idea that we identified genes and their gene products associated with CTA, we combined behavioral experiments with intra-cerebroventricular injections of an insulin receptor antagonist to investigate the role of insulin signaling.

2. Materials and Methods

2.1. Subjects

All the experiments in the present investigation used adult male Sprague-Dawley rats that weighed 300–375 g at the time experiments began (n=55). All animals were individually housed in plastic cages placed in a temperature- and humidity-controlled room with food *ad libitum*. Behavioral experiments were performed in the home cage. All procedures complied with National Institutes of Health guidelines and were approved by the University of Louisville Institutional Animal Care and Use Committee.

2.2 Tissue collection

In experiments 1–3 below, animals were administered a lethal dose of Nembutal (150 mg/kg) immediately following the sucrose intake test on day 12 and decapitated after termination of respiration using a laboratory guillotine. Brains were extracted under chilled (4 °C) oxygenated artificial cerebrospinal fluid (in mM: 123 NaCl, 5 KCl, 1.2 KH₂PO₄, 15 glucose, 30 NaHCO₃, 1.3 MgSO₄, 2.4 CaCl₂) adjusted to pH 7.4, blocked for tissue

containing the amygdala, rapidly frozen on dry ice, and stored at -80°C until further use. Tissue samples for RNA (Experiments 1 & 2) and protein (Experiment 3) analyses were obtained from thick cryostat cut sections ($250\text{ }\mu\text{m}$). Both the central and basolateral nuclei were dissected bilaterally from six frozen sections using established anatomical landmarks (i.e. bounded dorsal by the striatum, medial by the optic tract, and lateral by the external capsule). In experiment 2 below, an additional brain area was included for isolation of total RNA, visual cortex. In rodents, neural processing in visual cortex does not play a role in CTA and, thus, allowed assessment of non specific changes in gene expression. The instruments and working area were sprayed with RNase Zap (Ambion/Applied Biosystems, CA, USA) to ensure a RNA-free environment.

2.3. Experiment 1: Whole genome microarray

2.3.1. Treatment groups—Animals were divided into three different groups (sucrose/NaCl, sucrose/LiCl and LiCl/sucrose). After 5 days acclimation to fluid restriction where drinking water from a graduated cylinder fitted with a sipper tube was available for 15 min in the morning and 1 hr in the afternoon, all animals had access to 0.3 M sucrose instead of water in the morning on Day 6. Thirty minutes later, animals in the sucrose/NaCl group ($n=5$) received an intraperitoneal (ip) injection of saline (control group), while those in the sucrose/LiCl group ($n=5$) received ip LiCl (0.15 M, 13.3 ml/kg, contingent LiCl group). Animals in the LiCl/sucrose group ($n=5$) received ip LiCl the day prior to sucrose intake (i.e. following morning water intake on day 5; non-contingent LiCl group). For the next two days animals had access to water for 15 min in the morning and 1 hr in the afternoon. This sequence was repeated a second time followed by an intake test on Day 12 where all groups had access to sucrose for 15 min in the morning in the absence of ip injections. Data were presented as mean sucrose intake ($\pm$ SE) and analyzed with repeated measures ANOVA. Post hoc contrast analyses (LSD) were used to determine the source of statistically significant differences. P values < 0.05 were considered statistically significant.

2.3.2. RNA extraction and gene chip hybridization—The excised brain tissue samples were placed in QIAzol lysis reagent (Qiagen, CA, USA) on ice then total RNA isolated using RNeasy lipid tissue mini kit (Qiagen). Briefly, $200\text{ }\mu\text{L}$ of chloroform was added to the homogenate, gently agitated at room temperature for 5 min, and then centrifuged at 15,800 rpm for 15–20 min at 4°C . An equal volume of 70% ethanol in DEPC water ($600\text{ }\mu\text{L}$ each) was added to the aqueous phase and the mixture was immediately transferred to mini columns and centrifuged at 10,000 rpm for 15 sec at room temperature. The RNA quantity and quality were measured with the aid of a NanoDrop 1000 (Thermo Scientific, MA, USA). RNA from two animals in each group was not used because the 260/230 emission ratio indicated poor quality (e.g. < 1.5). Thus, microarray analyses were conducted using amygdala RNA isolated from three rats per group. A separate microarray chip was used for each animal.

A single-color Low RNA Input Linear Amplification kit (Agilent Technologies, CA, USA) generated fluorescent labeled cRNA that was purified using the RNeasy Mini Elute kit (Qiagen). A minimum of $1.65\text{ }\mu\text{g}$ of each labeled cRNA sample was hybridized to $4 \times 44\text{K}$ 60-mer oligonucleotide whole Rat Genome arrays (Agilent Technologies). The slides were scanned with a G2565BA microarray scanner (Agilent Technologies) using a one-color setting (green channel) and $5\text{ }\mu\text{m}$ resolution. The raw data files were imported into GeneSpring software (GX 7.3) and normalization performed using a per-chip 75th percentile method, which allows for comparison among chips. Then, a per-gene to median normalization was performed that normalizes the expression of each gene to its median among samples. The gene sets that showed up- or down-regulation of at least 2 fold were identified and entered into separate data sheets (i.e. contingent LiCl/non-contingent LiCl or

non-contingent LiCl/saline). The genes with a $P < 0.05$ were identified and gene annotation performed for these probe sets. Gene networks were generated by uploading the filtered data to Ingenuity Pathway Analysis (IPA 5.0) software, a web-delivered bioinformatics tool (Naga Prasad et al., 2009).

2.4. Experiment 2: Analyses of mRNA and protein levels

2.4.1. Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR)

—Animals were divided into sucrose/LiCl (contingent LiCl; $n=3$) and LiCl/sucrose (non-contingent LiCl; $n=3$) groups as described above in section 2.3.1. As a control for differences in sucrose intake between contingent and non-contingent groups, additional animals were divided into sucrose/LiCl (contingent LiCl; $n=3$) and LiCl/sucrose (non-contingent LiCl; $n=3$) with sucrose intake of the non-contingent LiCl group clamped to that of the contingent LiCl group (i.e. sucrose intake on Trial 2 and Test was no more than 1 ml). Total RNA was isolated from individual animals and cDNA synthesis by reverse transcription was performed using 1 μg of DNaseI (Ambion Inc., CA, USA) treated RNA and iScript cDNA synthesis kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's protocol. qRT-PCR was performed using a 20 μL reaction volume containing 1 μL of cDNA, 1 μL of each forward and reverse sequence specific primers (Table 1), 10 μL of supermix (Bio-Rad Laboratories), and 7 μL of nuclease free water. For quantification, standard curves were obtained for rat 18S rRNA using a ten-fold serial dilution of pooled cDNA from all samples. The quantity of target mRNA expression in individual samples was normalized to the level of rat 18S rRNA. Based on availability, two different qRT-PCR detection systems were used: 1) MyiQ single color real-time PCR machine that calculates relative quantity (Bio-Rad Laboratories) and 2) ABI Prism 7900 (Applied Biosystems, CA, USA) that calculates expression ratio which represents fold change. Thus, data for treatment groups were presented as mean expression levels or expression ratio relative to 18S rRNA ($\pm$ SE) and analyzed with independent-samples t-tests. P values < 0.05 were considered statistically significant.

2.4.2. Western blot and ELISA

—Animals were divided into sucrose/LiCl (contingent LiCl; $n=3$) and LiCl/sucrose (non-contingent LiCl; $n=3$) groups as described above in section 2.3.1. The excised amygdala tissue was homogenized in 300 μL of lysis buffer (150 mM Sodium Chloride, 1% Triton X-100 and 50 mM Tris, pH 8.0). The samples from animals within a given treatment group were pooled and $\sim 100 \mu\text{g}$ of total protein loaded per well (one blot/antibody). Samples were separated by gel electrophoresis according to standard protocols [35]. The proteins were transferred onto a nitrocellulose membrane (Millipore, MA, USA) in a cold room for 2–3 hr at 90 V. The membrane was processed using standard Western blot immunohistochemical techniques. Primary antibodies were purchased from Abcam, MA, USA (major histocompatibility complex class I-C (1:10,000, ab52922), glucagon (1:1,000, ab53704), and insulin 1 (1:200, ab7842)), while HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, PA, USA and used at 1:10,000 dilution (donkey, anti-rabbit (711-035-152) and anti-guinea pig (706-035-148)). The blocking and antibody dilution buffer consisted of 5% dried milk in 1x TBST (0.15 M NaCl, 10 mM Tris-HCl, 0.1% Tween-20, pH 8.0). The processed membrane was exposed to substrate solution (ECL kit, GE Health Care, NJ, USA) for 2 min in the dark for chemiluminescence then exposed to x-ray film (Kodak, USA). Tubulin (Sigma-Aldrich, MO, USA) was used to normalize band densities. For oxytocin, enzyme-linked immunoabsorbent assay (ELISA; Phoenix Pharmaceuticals Inc., CA, USA) was used for quantification in separate groups of contingent ($n=3$) and non-contingent ($n=3$) LiCl treated animals. Sufficient protein was available for duplicate ELISA measurements and the mean concentration of unknown samples ($\pm$ SE) was derived from the graph depicting the standard peptide concentration curve using GraphPad Prism 5 software.

2.5. Experiment 3: Central blockade of insulin receptors

Prior to behavioral testing, animals were anesthetized using Nembutal (50 mg/kg, intraperitoneal, ip), secured in a stereotaxic apparatus, and a midline incision made to reveal bregma and lambda cranial sutures. A hole was drilled through the bone overlying the right lateral ventricle (−0.85 mm posterior and −1.4 mm lateral to bregma), a cannula lowered −3.8 mm below the skull surface (21-gauge stainless steel tubing, Plastics One, Inc.), and secured using stainless steel screws and dental acrylic. We chose ventricular administration to globally block insulin receptors because we do not know as of yet the site or sites of action within the brain. The analgesic buprenex (0.1 ml) was administered for at least two days post surgery. Rats were allowed to recover for one week with water and food available *ad libitum*.

Animals were divided into four different groups (saline/S961_33.3, LiCl/vehicle, LiCl/S961_6.6 and LiCl_33.3). The behavioral procedures were the same as described above in section 2.3.1 with the exception that rats received an intracerebroventricular injection (icv, 2.5 μ l volumes at 0.5 μ l/min) of vehicle (0.9% saline) or the insulin receptor antagonist S961 (6.6 or 33.3 nmol dissolved in vehicle) immediately after sucrose intake on days 6 and 9. Animals in the saline/S961_33.3 group (n=4) had sucrose paired with ip saline and treated icv with 33.3 nmol S961, while those in the LiCl/vehicle (n=4), LiCl/S961_6.6 (n=4), and LiCl_33.3 (n=4) groups had sucrose paired with ip LiCl and treated icv with vehicle, 6.6 nmol S961, or 33.3 nmol S961, respectively. The insulin receptor antagonist was a generous gift from Dr. Lauge Schaffer at Novo Nordisk. S961 has been shown to exhibit high affinity for rat insulin receptors and at the dose range used here inhibits peripheral insulin stimulated lipogenesis and blood glucose uptake [36;37]. The choice of time for S961 treatment was based on the findings that the peripheral effects persist for at least 6 hrs, which would be sufficient time to influence the transition from short-term to long-term memory [26].

To assess the rate of extinction, rats were given simultaneous access to 2 bottles in the morning on day 15, one contained sucrose and the other water (i.e. two-bottle preference test). The preference test was followed by morning single-bottle tests with sucrose alone on days 16 and 17. This sequence of a two-bottle preference test followed by two single-bottle tests was repeated three times followed by the last two-bottle test on day 27. Sucrose preference was calculated using the following formula: sucrose intake/sucrose + water intake. Each day fluid intake and body weight was measured to the nearest milliliter or gram, as appropriate. Data were presented as mean sucrose intake or percent preference ($\pm$ SE) and analyzed with repeated measures ANOVA. Post hoc contrast analyses (LSD) were used to determine the source of statistically significant differences. P values < 0.05 were considered statistically significant.

Upon completion of behavioral testing, all rats received an icv injection of black India ink (1 μ l) followed by a lethal dose of Nembutal (150 mg/kg). Immediately after termination of respiration the animals were perfused through the ascending aorta, with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, blocked, and post fixed overnight at 4 °C in 30% sucrose. Coronal sections (50 μ m) were cut using a freezing microtome, collected in phosphate buffered saline, mounted on slides, counter stained with Cresyl Violet, and cover slipped using Permount.

3. Results

3.1. Histology

Representative photomicrographs of brain sections following excision of tissue containing the central and basolateral nuclei of the amygdala are shown in Figures 1A-D. The amygdala tissue removed was consistent across treatment groups. Representative photomicrographs of

ventricular cannula placement are shown in Figures 1E and 1F. Histological examination showed proper cannula placement in each animal.

3.2. Experiment 1. Whole genome microarray

3.2.1. Sucrose Intakes—Sucrose intake across trials for each treatment group used in microarray analyses is shown in Figure 2. Repeated measures ANOVA revealed that intake varied as a function of group and trial ($F_{4,12} = 9.1$, $P < 0.01$). Control and non-contingent groups consumed similar amounts of sucrose across all trials (P values > 0.7), while the contingent LiCl group consumed significantly less sucrose on Trial 2 and Test compared to control and non-contingent groups (P values < 0.01). Thus, contingent sucrose/LiCl pairing supported development of a CTA evidenced by avoidance of sucrose, while non-contingent LiCl/sucrose and saline/sucrose pairing did not.

3.2.2. Genes regulated by CTA learning—With the aid of microarray chips, we were able to demonstrate that CTA significantly ($p < 0.05$) affected the expression of 4,897 genes (2,417 up-regulated and 2,480 down-regulated) compared to non-contingent LiCl treatment. Filtering the data set resulted in 72 genes up-regulated and 96 down-regulated. IPA software analysis revealed that 97 of the genes had known annotations with 76 being eligible for network analyses. Table 2 lists the 20 up-regulated and the 56 down-regulated network-eligible genes. Some 15 of these genes (underlined) were represented in IPA's functional category of 'behavior'. The majority of these 'behavior' related genes encode neuropeptides and G protein-coupled receptors such as insulin 1 (INS1), oxytocin (OXT), glucagon (GCG), corticotrophin releasing hormone (CRH), prodynorphin (PDYN), dopamine receptor 2 (DRD2), glycine receptor, alpha 2 subunit (GLRA2), adrenergic, alpha-1D-, receptor (ADRA1D), and adenosine A2a receptor (ADORA2A).

3.2.3. Genes regulated by non-contingent LiCl—We were able to demonstrate that non-contingent LiCl treatment significantly ($p < 0.05$) affected the expression of 7,264 genes (3,921 up-regulated and 3,343 down-regulated) compared to saline treatment. Filtering the data set resulted in 228 genes up-regulated and 229 down-regulated. IPA software analysis revealed that 241 of the genes had known annotations with 191 being eligible for network analyses. Supplemental Table 1 (website) lists the 146 up-regulated and the 45 down-regulated network-eligible genes. Some 80 of these genes (underlined) were represented in IPA's functional category of 'neurological disease'.

Compared to the set of genes associated with CTA, non-contingent LiCl treatment influenced the expression of nearly 3 times more genes. Moreover, there was a considerable difference in the direction of gene expression with 76% of genes responsive to LiCl alone up-regulated (146 of 191) versus 26% of those responsive to CTA (20 of 76). Examination of Table 2 and Supplemental Table 1 revealed regulation of 30 common genes (indicated by asterisks in Table 2). The majority (20 of 30 genes) can influence ERK 1/2, p38MAPK, and/or PI3K signaling pathways (Figure 3, IPA software generated), but in opposite direction dependent upon the contingency of sucrose intake and LiCl treatment. A list of all genes from this microarray experiment can be found in the ArrayExpress database with accession number E-MEXP-3029 (<http://www.ebi.ac.uk/microarray-as/ae/>).

3.3. Experiment 2. Analyses of mRNA and protein levels

Seven genes from Table 2 were selected for qRT-PCR. Four of these genes were represented in IPA's functional category of 'behavior'; INS1, OXT, GCG, and DRD2. In addition, we selected the most highly up-regulated gene, major histocompatibility complex, class I, C (HLA-C), one of the weakest down-regulated genes, protein phosphatase 3, regulatory

subunit B, alpha isoform (PPP3R1), and one gene that was regulated both by contingent and non-contingent LiCl treatment, GLRA2.

In amygdala tissue, but not visual cortex, independent-samples t-tests revealed that the mRNA levels of INS1 ($t(4)=-7.1$, $p < 0.01$), OXT ($t(4)=-3.6$, $p < 0.01$), HLA-C ($t(4)=-4.7$, $p < 0.01$), DRD2 ($t(4)=-19.8$, $p < 0.01$), GLRA2 ($t(4)=-2.6$, $p = 0.03$), and GCG ($t(4)=2.3$, $p = 0.04$) were significantly regulated by CTA (Figure 4). However, altered expression of DRD2 was in the opposite direction (up-regulated) compared to microarray analysis (down-regulated). A significant change in expression of PPP3R1 mRNA was not observed ($t(4)=-0.7$, $p = 0.25$). Moreover, the protein levels of HLA-C, INS1, and OXT were increased, whereas the level of GCG was unchanged (Figure 5). Changes in protein level of GLRA2 was not examined, but subsequent qRT-PCR analyses showed that expression of this mRNA was unaltered when sucrose intake was clamped ($t(4)=-0.5$, $p = 0.61$), whereas the expression of HLA-C ($t(4)=-3.7$, $p = 0.02$), INS1 ($t(4)=-3.5$, $p = 0.02$), and OXT ($t(4)=-7.0$, $p < 0.01$) mRNA remained significantly up-regulated (Table 3).

3.4. Experiment 3. Central blockade of insulin receptors

Sucrose intake during acquisition and extinction trials (Figure 6) varied as a function of group and trial (acquisition, $F_{6,24}=10.5$, $P < 0.01$; single-bottle extinction, $F_{21,84}=6.6$, $P < 0.01$; two-bottle extinction, $F_{12,48}=16.8$, $P < 0.01$). Briefly, sucrose intake decreased on Trial 2 and Test compared to Trial 1 ($P = 0.03$), albeit modestly for the saline/S961 group. Nevertheless, the LiCl/S961 animals consumed more sucrose compared to LiCl/vehicle ($P = 0.03$). A weaker CTA was supported further by the more rapid rate of extinction in the LiCl/S961 groups. Sucrose preference of LiCl/S961 and saline/S961 animals was nearly identical at ~70% during the last two-bottle test, while the preference of LiCl/vehicle animals remained low at ~20%.

4. Discussion

We investigated late response gene expression in the amygdala, a brain region involved in various forms of learning including conditioned taste aversion (CTA). Previous investigations provide compelling evidence that early activation of cAMP/Ca²⁺/CREB pathways in the amygdala mediate gene expression involved in CTA [21;23–27;38]. Our microarray work extends these findings by demonstrating enduring regulation of downstream genes encoding several neuropeptides, cytokines, phosphatases, and kinases following consolidation of long-term CTA memory. For a subset of genes -- INS1, OXT, and HLA-C -- further evidence for a role in CTA comes from confirmation that altered mRNA expression was translated into correspondingly appropriate changes in protein level and independent of actual sucrose consumption (i.e. intake clamped experiments).

To test the hypothesis that we identified relevant genes, we assessed the effects of interfering with the activity of one of these gene products on CTA learning. Insulin was chosen because its receptors are widely distributed in the brain and it influences diverse behaviors such as food intake and other forms of learning [39–42]. We showed that blockade of central INS1 receptors during the time of taste/visceral pairing produced a weaker CTA that was less resistant to extinction. The finding that INS1 receptor blockade reduced, but did not eliminate, CTA memory formation might be due to the mode of injection. That is, intraventricular administration might require higher doses than used here to maintain sufficient concentrations to block endogenous INS released in the brain. Alternatively, blockade of INS1 receptors alone is not sufficient to completely block CTA. This notion is supported by a number of previous studies showing that blocking activity in a single pathway whether it be CREB, c-fos, ERK1/2 or PKC impairs CTA memory

formation, but does not eliminate it [23–25;27;43]. Thus, CTA goes beyond the idea of a single gene for acquisition and expression of the behavior.

To the best of our knowledge, the present study is the first to show a role for insulin signaling in CTA, and local synthesis in the amygdala. Insulin mRNA has been identified in the rat and rabbit hippocampus, as well as rabbit medial prefrontal cortex, entorhinal cortex, perirhinal cortex, thalamus, and granule cell layer of the olfactory bulbs [44;45]. Alternatively, fibers of insulin expressing neurons that innervate the amygdala may be the source of INS1 mRNA and protein in our study. These possibilities are not mutually exclusive, but await future investigations.

HLA-C was the most highly up-regulated gene following CTA and is a member of the class I major histocompatibility complex (MHCI) transmembrane molecules. These molecules have diverse actions including activity-dependent changes in synaptic strength and connectivity [46–49]. In addition, we found up-regulation of CaMKII- α , a Ca^{2+} -calmodulin-dependent protein kinase highly expressed in dendrites, regulated by synaptic activity, and important for neuronal plasticity, learning, and memory [50–54]. Recent research has shown that CTA learning increased postsynaptic density length in insular cortex neurons [55] and, thus, it is likely that CTA associated changes in synaptic structure and efficacy occur in the amygdala as well. This notion is consistent with altered responsiveness of CeA and BLA neurons to a conditioned taste stimulus [19;20].

Our results implicating OXT in CTA are complimentary to previous research in so far as they support a central action. For instance, circulating OXT is increased by treatments that serve as an US in CTA such as LiCl; however, peripheral treatment with OXT does not produce a CTA nor does peripheral blockade of endogenous OXT interfere with LiCl-induced CTA [56]. Moreover, central administration of OXT has been shown to influence memory retention in a different, passive-avoidance learning paradigm [57]. Alternatively, increased OXT expression in the present study might simply reflect the stressful experience of the CTA procedure because OXT can dampen stress responses that involve the amygdala [58]. The fact that the contingent and non-contingent animals were matched for LiCl exposure and sucrose intake, presumably differing little in paradigm-induced stress, argues against such an interpretation. The source of OXT mRNA and protein in our study is likely from fibers of OXT expressing neurons that innervate the amygdala [59;60].

The present study further demonstrated that the expression of 30 genes was correlated with non-contingent (unconditioned response) and contingent (CTA learning) LiCl treatment. In each case, however, altered expressions were in opposite directions. This is likely because non-contingent animals served as the control group to which fold change in contingent animals was calculated (i.e. contingent/non-contingent), while non-contingent animals were compared to saline treated animals (i.e. non-contingent/saline). No change in expression was observed when contingent animals were compared to those treated with saline (i.e. contingent/saline). One possibility is that up-regulation of this set of genes that can influence ERK 1/2, p38MAPK, and/or PI3K activity is involved in signaling the unconditioned response to LiCl exposure. Indeed, previous research shows that LiCl treatment increases the phosphorylation of ERK in the central nucleus of the amygdala and insular cortex [61]. It is also well known that CTA are more readily acquired when consuming novel taste stimuli than familiar. In this context, a previous study has shown that exposure to an unfamiliar, but not familiar, saccharin solution increased the activated form of ERK 1/2, and inhibiting kinase activity attenuated long-term taste aversion memory [43]. Thus, increased activation of ERK 1/2 signaling pathway likely functions in early CS-US integration, but is down regulated to basal levels once long-term memory has been formed

Given the compelling evidence that cAMP/Ca²⁺/CREB-mediated gene expression in the amygdala plays a role in the early taste/visceral associative phase of CTA [21;23–25;38], it is not surprising that each of the gene products discussed above, with the exception of OXT, has been shown to influence or be influenced by CREB activity. For instance, transcription of INS and HLA-C genes involve protein binding to the CRE element of their promoter [62–64]. Conversely, INS and CaMKII can influence transcriptional activity via phosphorylation of CREB, and, at least for INS, this involves activation of ERK 1/2 signaling pathway [65;66]. The OXT promoter apparently does not contain a CRE element; however, there is evidence suggesting that c-fos participates in transcriptional regulation of OXT gene in hypothalamic nuclei following dehydration [67]. In the amygdala, c-fos activation during the time of taste/LiCl pairing plays a role in CTA [23;25].

In conclusion, our studies point to a complex up- and down-regulation of distinct sets of late response genes following consolidation of CTA memory. Consistent with the emerging role of insulin in learning and memory, the results revealed a novel function for brain insulin in CTA and an additional source of local synthesis, the central and/or basolateral nuclei of the amygdala. Similar approaches will be relevant for understanding regulation of gene expression in situations where learning promotes consumption, e.g., sodium appetite and learned flavor preference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference List

1. Nachman M, Ashe JH. Learned taste aversions in rats as a function of dosage, concentration, and route of administration of LiCl. *Physiol Behav.* 1973; 10:73–78. [PubMed: 4697023]
2. Grill HJ, Norgren R. Chronically decerebrate rats demonstrate satiation but not bait shyness. *Science.* 1978; 201:267–269. [PubMed: 663655]
3. Lubow, RE. Conditioned Taste Aversion and Latent Inhibition: A Review. In: Reilly, S.; Schachtman, TR., editors. *Conditioned Taste Aversion: Behavioral and Neural Processes*. New York: Oxford University Press Inc; 2009. p. 37-57.
4. Nishijo H, Uwano T, Tamura R, Ono T. Gustatory and multimodal neuronal responses in the amygdala during licking and discrimination of sensory stimuli in awake rats. *J Neurophysiol.* 1998; 79:21–36. [PubMed: 9425173]
5. Norgren R. Taste pathways to hypothalamus and amygdala. *J Comp Neurol.* 1976; 166:17–30. [PubMed: 1262547]
6. Li CS, Cho YK. Efferent projection from the bed nucleus of the stria terminalis suppresses activity of taste-responsive neurons in the hamster parabrachial nuclei. *Am J Physiol Regul Integr Comp Physiol.* 2006; 291:R914–R926. [PubMed: 16966389]
7. Bernard JF, Alden M, Besson JM. The organization of the efferent projections from the pontine parabrachial area to the amygdaloid complex: a Phaseolus vulgaris leucoagglutinin (PHA-L) study in the rat. *J Comp Neurol.* 1993; 329:201–229. [PubMed: 8454730]
8. Alden M, Besson JM, Bernard JF. Organization of the efferent projections from the pontine parabrachial area to the bed nucleus of the stria terminalis and neighboring regions: a PHA-L study in the rat. *J Comp Neurol.* 1994; 341:289–314. [PubMed: 7515078]

9. Bester H, Matsumoto N, Besson JM, Bernard JF. Further evidence for the involvement of the spinoparabrachial pathway in nociceptive processes: a c-Fos study in the rat. *J Comp Neurol.* 1997; 383:439–458. [PubMed: 9208992]
10. Bester H, Besson JM, Bernard JF. Organization of efferent projections from the parabrachial area to the hypothalamus: a Phaseolus vulgaris-leucoagglutinin study in the rat. *J Comp Neurol.* 1997; 383:245–281. [PubMed: 9205041]
11. Bester H, Bourgeais L, Villanueva L, Besson JM, Bernard JF. Differential projections to the intralaminar and gustatory thalamus from the parabrachial area: a PHA-L study in the rat. *J Comp Neurol.* 1999; 405:421–449. [PubMed: 10098938]
12. Scalera G, Grigson PS, Norgren R. Gustatory functions, sodium appetite, and conditioned taste aversion survive excitotoxic lesions of the thalamic taste area. *Behav Neurosci.* 1997; 111:633–645. [PubMed: 9189278]
13. Reilly S, Pritchard TC. Gustatory thalamus lesions in the rat: II. Aversive and appetitive taste conditioning. *Behav Neurosci.* 1996; 110:746–759. [PubMed: 8864266]
14. Galaverna OG, Seeley RJ, Berridge KC, Grill HJ, Epstein AN, Schulkin J. Lesions of the central nucleus of the amygdala I: Effects on taste reactivity, taste aversion learning and sodium appetite. *Behav Brain Res.* 1993; 59:11–17. [PubMed: 8155277]
15. Yamamoto T, Fujimoto Y, Shimura T, Sakai N. Conditioned taste aversion in rats with excitotoxic brain lesions. *Neurosci Res.* 1995; 22:31–49. [PubMed: 7792081]
16. St Andre J, Reilly S. Effects of central and basolateral amygdala lesions on conditioned taste aversion and latent inhibition. *Behav Neurosci.* 2007; 121:90–99. [PubMed: 17324053]
17. Gallo M, Roldan G, Bures J. Differential involvement of gustatory insular cortex and amygdala in the acquisition and retrieval of conditioned taste aversion in rats. *Behav Brain Res.* 1992; 52:91–97. [PubMed: 1335264]
18. Roldan G, Bures J. Tetrodotoxin blockade of amygdala overlapping with poisoning impairs acquisition of conditioned taste aversion in rats. *Behav Brain Res.* 1994; 65:213–219. [PubMed: 7718154]
19. Grossman SE, Fontanini A, Wieskopf JS, Katz DB. Learning-related plasticity of temporal coding in simultaneously recorded amygdala-cortical ensembles. *J Neurosci.* 2008; 28:2864–2873. [PubMed: 18337417]
20. Yasoshima Y, Shimura T, Yamamoto T. Single unit responses of the amygdala after conditioned taste aversion in conscious rats. *Neuroreport.* 1995; 6:2424–2428. [PubMed: 8747167]
21. Kwon B, Goltz M, Houpt TA. Expression of AP-1 family transcription factors in the amygdala during conditioned taste aversion learning: role for Fra-2. *Brain Res.* 2008; 1207:128–141. [PubMed: 18374904]
22. Spencer CM, Houpt TA. Dynamics of c-fos and ICER mRNA expression in rat forebrain following lithium chloride injection. *Brain Res Mol Brain Res.* 2001; 93:113–126. [PubMed: 11589989]
23. Lamprecht R, Dudai Y. Transient expression of c-Fos in rat amygdala during training is required for encoding conditioned taste aversion memory. *Learn Mem.* 1996; 3:31–41. [PubMed: 10456074]
24. Lamprecht R, Hazvi S, Dudai Y. cAMP response element-binding protein in the amygdala is required for long- but not short-term conditioned taste aversion memory. *J Neurosci.* 1997; 17:8443–8450. [PubMed: 9334416]
25. Yasoshima Y, Sako N, Senba E, Yamamoto T. Acute suppression, but not chronic genetic deficiency, of c-fos gene expression impairs long-term memory in aversive taste learning. *Proc Natl Acad Sci U S A.* 2006; 103:7106–7111. [PubMed: 16636292]
26. Koh MT, Thiele TE, Bernstein IL. Inhibition of protein kinase A activity interferes with long-term, but not short-term, memory of conditioned taste aversions. *Behav Neurosci.* 2002; 116:1070–1074. [PubMed: 12492305]
27. Yasoshima Y, Yamamoto T. Rat gustatory memory requires protein kinase C activity in the amygdala and cortical gustatory area. *Neuroreport.* 1997; 8:1363–1367. [PubMed: 9172136]
28. Kerns RT, Ravindranathan A, Hassan S, Cage MP, York T, Sikela JM, Williams RW, Miles MF. Ethanol-responsive brain region expression networks: implications for behavioral responses to

- acute ethanol in DBA/2J versus C57BL/6J mice. *J Neurosci*. 2005; 25:2255–2266. [PubMed: 15745951]
29. Daniels GM, Buck KJ. Expression profiling identifies strain-specific changes associated with ethanol withdrawal in mice. *Genes Brain Behav*. 2002; 1:35–45. [PubMed: 12886948]
 30. Rimondini R, Arlinde C, Sommer W, Heilig M. Long-lasting increase in voluntary ethanol consumption and transcriptional regulation in the rat brain after intermittent exposure to alcohol. *FASEB J*. 2002; 16:27–35. [PubMed: 11772933]
 31. Azami S, Wagatsuma A, Sadamoto H, Hatakeyama D, Usami T, Fujie M, Koyanagi R, Azumi K, Fujito Y, Lukowiak K, Ito E. Altered gene activity correlated with long-term memory formation of conditioned taste aversion in *Lymnaea*. *J Neurosci Res*. 2006; 84:1610–1620. [PubMed: 16941636]
 32. Smit AB, Vreugdenhil E, Ebberink RH, Geraerts WP, Klootwijk J, Joosse J. Growth-controlling molluscan neurons produce the precursor of an insulin-related peptide. *Nature*. 1988; 331:535–538. [PubMed: 3340203]
 33. Kuhl D, Kennedy TE, Barzilai A, Kandel ER. Long-term sensitization training in *Aplysia* leads to an increase in the expression of BiP, the major protein chaperon of the ER. *J Cell Biol*. 1992; 119:1069–1076. [PubMed: 1360013]
 34. Yao WD, Gainetdinov RR, Arbuckle MI, Sotnikova TD, Cyr M, Beaulieu JM, Torres GE, Grant SG, Caron MG. Identification of PSD-95 as a regulator of dopamine-mediated synaptic and behavioral plasticity. *Neuron*. 2004; 41:625–638. [PubMed: 14980210]
 35. Panguluri SK, Kakar SS. Effect of PTTG on endogenous gene expression in HEK 293 cells. *BMC Genomics*. 2009; 10:577. [PubMed: 19958546]
 36. Schaffer L, Brand CL, Hansen BF, Ribel U, Shaw AC, Slaaby R, Sturis J. A novel high-affinity peptide antagonist to the insulin receptor. *Biochem Biophys Res Commun*. 2008; 376:380–383. [PubMed: 18782558]
 37. Vikram A, Jena G. S961, an insulin receptor antagonist causes hyperinsulinemia, insulin-resistance and depletion of energy stores in rats. *Biochem Biophys Res Commun*. 2010; 398:260–265. [PubMed: 20599729]
 38. Oberbeck DL, McCormack S, Houpt TA. Intra-amygdalar okadaic acid enhances conditioned taste aversion learning and CREB phosphorylation in rats. *Brain Res*. 2010; 1348:84–94. [PubMed: 20599840]
 39. Baskin DG, Figlewicz LD, Seeley RJ, Woods SC, Porte D Jr, Schwartz MW. Insulin and leptin: dual adiposity signals to the brain for the regulation of food intake and body weight. *Brain Res*. 1999; 848:114–123. [PubMed: 10612703]
 40. Zhao WQ, Chen H, Quon MJ, Alkon DL. Insulin and the insulin receptor in experimental models of learning and memory. *Eur J Pharmacol*. 2004; 490:71–81. [PubMed: 15094074]
 41. Park CR, Seeley RJ, Craft S, Woods SC. Intracerebroventricular insulin enhances memory in a passive-avoidance task. *Physiol Behav*. 2000; 68:509–514. [PubMed: 10713291]
 42. Dou JT, Chen M, Dufour F, Alkon DL, Zhao WQ. Insulin receptor signaling in long-term memory consolidation following spatial learning. *Learn Mem*. 2005; 12:646–655. [PubMed: 16287721]
 43. Berman DE, Hazvi S, Rosenblum K, Seger R, Dudai Y. Specific and differential activation of mitogen-activated protein kinase cascades by unfamiliar taste in the insular cortex of the behaving rat. *J Neurosci*. 1998; 18:10037–10044. [PubMed: 9822758]
 44. Devaskar SU, Giddings SJ, Rajakumar PA, Carnaghi LR, Menon RK, Zahm DS. Insulin gene expression and insulin synthesis in mammalian neuronal cells. *J Biol Chem*. 1994; 269:8445–8454. [PubMed: 8132571]
 45. Zhao, WQ.; Dou, JT.; Liu, QY.; Alkon, DL. Neuroscience Meeting Planner. Orlando, FL: Society for Neuroscience; 2002. Evidence for locally produced insulin in the adult rat brain as a neuroactive peptide. Online Program No. 375.12 (2002)
 46. Zhong J, Zhang T, Bloch LM. Dendritic mRNAs encode diversified functionalities in hippocampal pyramidal neurons. *BMC Neurosci*. 2006; 7:17. [PubMed: 16503994]
 47. Huh GS, Boulanger LM, Du H, Riquelme PA, Brotz TM, Shatz CJ. Functional requirement for class I MHC in CNS development and plasticity. *Science*. 2000; 290:2155–2159. [PubMed: 11118151]

48. Goddard CA, Butts DA, Shatz CJ. Regulation of CNS synapses by neuronal MHC class I. *Proc Natl Acad Sci U S A*. 2007; 104:6828–6833. [PubMed: 17420446]
49. Shatz CJ. MHC class I: an unexpected role in neuronal plasticity. *Neuron*. 2009; 64:40–45. [PubMed: 19840547]
50. Mayford M, Wang J, Kandel ER, O'Dell TJ. CaMKII regulates the frequency-response function of hippocampal synapses for the production of both LTD and LTP. *Cell*. 1995; 81:891–904. [PubMed: 7781066]
51. Bach ME, Hawkins RD, Osman M, Kandel ER, Mayford M. Impairment of spatial but not contextual memory in CaMKII mutant mice with a selective loss of hippocampal LTP in the range of the theta frequency. *Cell*. 1995; 81:905–915. [PubMed: 7781067]
52. Glazewski S, Giese KP, Silva A, Fox K. The role of alpha-CaMKII autophosphorylation in neocortical experience-dependent plasticity. *Nat Neurosci*. 2000; 3:911–918. [PubMed: 10966622]
53. Elgersma Y, Sweatt JD, Giese KP. Mouse genetic approaches to investigating calcium/calmodulin-dependent protein kinase II function in plasticity and cognition. *J Neurosci*. 2004; 24:8410–8415. [PubMed: 15456813]
54. Ouyang Y, Rosenstein A, Kreiman G, Schuman EM, Kennedy MB. Tetanic stimulation leads to increased accumulation of Ca(2+)/calmodulin-dependent protein kinase II via dendritic protein synthesis in hippocampal neurons. *J Neurosci*. 1999; 19:7823–7833. [PubMed: 10479685]
55. Bi AL, Wang Y, Li BQ, Wang QQ, Ma L, Yu H, Zhao L, Chen ZY. Region-specific involvement of actin rearrangement-related synaptic structure alterations in conditioned taste aversion memory. *Learn Mem*. 2010; 17:420–427. [PubMed: 20729290]
56. Verbalis JG, McHale CM, Gardiner TW, Stricker EM. Oxytocin and vasopressin secretion in response to stimuli producing learned taste aversions in rats. *Behav Neurosci*. 1986; 100:466–475. [PubMed: 3017374]
57. Kovacs GL, Bohus B, Versteeg DH, de Kloet ER, de WD. Effect of oxytocin and vasopressin on memory consolidation: sites of action and catecholaminergic correlates after local microinjection into limbic-midbrain structures. *Brain Res*. 1979; 175:303–314. [PubMed: 487159]
58. Lee HJ, Macbeth AH, Pagani JH, Young WS III. Oxytocin: the great facilitator of life. *Prog Neurobiol*. 2009; 88:127–151. [PubMed: 19482229]
59. Paredes J, Winters RW, Schneiderman N, McCabe PM. Afferents to the central nucleus of the amygdala and functional subdivisions of the periaqueductal gray: neuroanatomical substrates for affective behavior. *Brain Res*. 2000; 887:157–173. [PubMed: 11134600]
60. Chung SK, McCabe JT, Pfaff DW. Estrogen influences on oxytocin mRNA expression in preoptic and anterior hypothalamic regions studied by in situ hybridization. *J Comp Neurol*. 1991; 307:281–295. [PubMed: 1856326]
61. Swank MW. Phosphorylation of MAP kinase and CREB in mouse cortex and amygdala during taste aversion learning. *Neuroreport*. 2000; 11:1625–1630. [PubMed: 10852213]
62. Melloul D, Marshak S, Cerasi E. Regulation of insulin gene transcription. *Diabetologia*. 2002; 45:309–326. [PubMed: 11914736]
63. Docherty K, Clark AR. Nutrient regulation of insulin gene expression. *FASEB J*. 1994; 8:20–27. [PubMed: 8299887]
64. Gobin SJ, Mvan Z, Westerheide SD, Boss JM, van den Elsen PJ. The MHC-specific enhanceosome and its role in MHC class I and beta(2)-microglobulin gene transactivation. *J Immunol*. 2001; 167:5175–5184. [PubMed: 11673530]
65. Klemm DJ, Roesler WJ, Boras T, Colton LA, Felder K, Reusch JE. Insulin stimulates cAMP-response element binding protein activity in HepG2 and 3T3-L1 cell lines. *J Biol Chem*. 1998; 273:917–923. [PubMed: 9422750]
66. Takeda H, Kitaoka Y, Hayashi Y, Kumai T, Munemasa Y, Fujino H, Kobayashi S, Ueno S. Calcium/calmodulin-dependent protein kinase II regulates the phosphorylation of CREB in NMDA-induced retinal neurotoxicity. *Brain Res*. 2007; 1184:306–315. [PubMed: 17961520]
67. da Silveira LT, Junta CM, Monesi N, de Oliveira-Pelegrin GR, Passos GA, Rocha MJ. Time course of c-fos, vasopressin and oxytocin mRNA expression in the hypothalamus following long-term dehydration. *Cell Mol Neurobiol*. 2007; 27:575–584. [PubMed: 17393298]

Highlights

- Late response genes associated with conditioned taste aversion were examined.
- mRNA was translated into correspondingly appropriate changes in protein level.
- Central insulin receptor inhibition disrupted CTA learning.

Figure 1.

(A-D) Photomontage of cresyl violet stained brain sections (gray scale) showing excision of amygdala tissue from one animal in the contingent LiCl treatment group that acquired a CTA (A, C) and one from the non-contingent LiCl group that does not support development of a CTA (B, D). The approximate coordinates relative to bregma cranial suture was -1.80 mm (A, B) and -3.14 mm (C, D) (Paxinos and Watson, 1998). The black dots outline the extent of tissue extraction. (E, F) Photomontage of cresyl violet stained brain sections (gray scale) showing cannula placement in the right lateral ventricle of a sucrose/LiCl paired animal treated icv with 33.3 nm S961 (E) and a sucrose/saline paired animal treated icv with the same dose of S961 (F). The asterisk in each image indicates the guide cannula track. Scale bars equal 1 mm. Abbreviations: ac, anterior commissure; cc, corpus callosum; CPu, caudate putamen (striatum); ec, external capsule; ic, internal capsule; LV, lateral ventricle; ot, optic tract.

Figure 2. Mean intake of sucrose during 15 min morning access on acquisition (Trial 1 and Trial 2) and test trials for saline control (circles), non-contingent LiCl treated (unfilled squares), and contingent LiCl treated animals (filled squares). *, significantly different from saline control and non-contingent groups.

Commonly Regulated Genes

Figure 3.

The top ranked network associated with genes commonly regulated by LiCl treatment alone (top panel) and CTA learning involved 2 mitogen-activated protein kinase signaling pathways (MAPK), ERK 1/2 and p38MAPK. The solid lines connecting molecules here represents a direct relationship and dotted lines an indirect relationship. The gene network presented here was generated with IPA software tool using the criteria $p\text{-value} < 0.05$ and fold change ≥ 2 . Red colored symbols indicate up-regulated genes and green symbols genes that were down-regulated.

Figure 4. Mean relative expression of glucagon (GCG), protein phosphatase 3, regulatory subunit B, alpha isoform (PPP3R1), dopamine receptor 2 (DRD2), major histocompatibility complex, class I, C (HLA-C), glycine receptor, alpha 2 subunit (GLRA2), insulin 1 (INS1), and oxytocin (OXT) in the amygdala and visual cortex from non-contingent (open bars) and contingent (filled bars) LiCl treated animals. *, significantly different from non-contingent group.

Figure 5.

Western blot (A-C) and ELISA (D) analyses of major histocompatibility complex, class I, C (HLA-C), insulin 1 (INS1), glucagon (GCG), and oxytocin (OXT) protein level in amygdala tissue from non-contingent (open bars) and contingent (filled bars) LiCl treated animals. (A-C) Bars represent band density relative to tubulin protein level. The numbers above the filled bars correspond to fold change in protein expression relative to non-contingent LiCl treated animals. (D) Bars represent the mean concentration of oxytocin in replicate samples.

Figure 6.

Mean intake of sucrose for saline/S961 (unfilled circles), LiCl/vehicle (unfilled bars), and LiCl/S961 (6.6nmol, filled squares; 33.3nmol, shaded triangles) animals during Acquisition and Extinction trials. For Extinction trials, the left part of the graph represents intake for 8 single-bottle trials. A two-bottle test with water and sucrose was given following two consecutive single bottle tests (arrowheads). The preference score for sucrose relative to water during each of the 5 two-bottle tests is shown on the right. *, 33.3nmol S961 group significantly different from LiCl/vehicle; **, both S961 dose groups significantly different from LiCl/vehicle.

Table 1

Primer sequences and their NCBI reference numbers of the selected genes used for the validation by quantitative real-time RT-PCR.

Gene	NCBI Ref. No.	Forward Primer	Reverse primer
Glucagon (GCG)	NM_012707	5'-AAC AAC ATT GCC AAA CGT CA-3'	5'-CAG CTA TGG CGA CTT CTT CC-3'
Dopamine receptor 2 (DRD2)	NM_012547	5'-ATC CAC TGA ACC TGT CCT GG-3'	5'-GTG GTC TGC AAA GCC TTC TC-3'
Insulin 1 (INS1)	NM_019129	5'-CAA GCA GGT CAT TGT TCC AA-3'	5'-ACC AGG TGA GGA CCA CAA AG-3'
Oxytocin (OXT)	NM_012996	5'-GGA GGA GAA CTA CCT GCC CT-3'	5'-AGG TAT CAT CAC AAA GCG GG-3'
Major histocompatibility complex, class I, C (HLA-C)	NM_001008837	5'-AGA TGA CCC GAA ACA AGT GG-3'	5'-ACA TCA CCT TCA GGT CTG GG-3'
Protein phosphatase 3, regulatory subunit B, alpha isoform (PPP3R1)	NM_017309	5'-TCT GTG CTG TTG TAG GTG GC-3'	5'-TGT TGG AAA ATG TGG CTT CA-3'
Glycine receptor, alpha 2 subunit (GLRA2)	NM_012568	5'-TGG CCT TCC TCA TTT TCA AC-3'	5'-GGT CCC AGG GTC AAT AGG AT-3'
18S RNA	V01270	5'-TGC GAA TGG CTC ATT AAA TC-3'	5'-GGC GAC TAC CAT CGA AAG TT-3'

Table 2

List of differentially expressed genes associated with CTA learning by cDNA microarray with $p < 0.05$ and fold ≥ 2 .

Gene Name	p-value	Fold	Gene Description
HLA-C	6.23E-05	97.208	major histocompatibility complex, class I, C
<u>OXT</u>	1.44E-02	6.457	oxytocin, prepropeptide
AMY2-3 *	1.86E-02	3.623	amylase 2-3, pancreatic
<u>INS1</u>	1.32E-02	3.496	insulin I
AMY2A *	1.28E-02	3.476	amylase, alpha 2A (pancreatic)
UBE2B *	4.79E-03	3.267	ubiquitin-conjugating enzyme E2B (RAD6 homolog)
<u>CAMK2A</u>	4.74E-03	2.708	calcium/calmodulin-dependent protein kinase II alpha
KIF15	8.68E-03	2.692	kinesin family member 15
SLC17A6	4.51E-03	2.648	solute carrier family 17 (sodium-dependent inorganic phosphate cotransporter), member 6
LHX9	2.42E-02	2.561	LIM homeobox 9
ADCY9 *	1.19E-03	2.424	adenylate cyclase 9
CFD *	3.01E-04	2.414	complement factor D (adipsin)
ZFP161 *	3.05E-02	2.369	zinc finger protein 161 homolog (mouse)
<u>APBA1</u>	1.84E-02	2.360	amyloid beta (A4) precursor protein-binding, family A, member 1
GLRA2 *	1.12E-02	2.225	glycine receptor, alpha 2
ERAS	1.10E-02	2.128	ES cell expressed Ras
SCN10A	3.47E-02	2.105	sodium channel, voltage-gated, type X, alpha subunit
PAH	2.58E-02	2.086	phenylalanine hydroxylase
PELI2	2.95E-02	2.059	pellino homolog 2 (Drosophila)
LHX3 *	1.48E-02	2.050	LIM homeobox 3
ALAS2	2.66E-02	-2.003	aminolevulinate, delta-, synthase 2
TYRO3 *	1.70E-03	-2.007	TYRO3 protein tyrosine kinase
PPP3R1	1.36E-03	-2.011	protein phosphatase 3, regulatory subunit B, alpha
CACNG1	4.07E-03	-2.022	calcium channel, voltage-dependent, gamma subunit 1
<u>DRD2</u>	1.45E-02	-2.028	dopamine receptor D2
SETD5	2.83E-03	-2.029	SET domain containing 5

Gene Name	p-value	Fold	Gene Description
GYPC	2.32E-02	-2.030	glycophorin C (Gerbich blood group)
KCNG3 *	1.54E-02	-2.039	potassium voltage-gated channel, subfamily G, member 3
BAT2	6.81E-03	-2.041	HLA-B associated transcript 2
RASL11B *	1.21E-02	-2.076	RAS-like, family 11, member B
CD52	1.97E-04	-2.077	CD52 molecule
NUDT3	1.38E-02	-2.079	nudix (nucleoside diphosphate linked moiety X)-type motif 3
<u>ADRA1D</u> *	4.81E-02	-2.089	adrenergic, alpha-1D-, receptor
ACADS	9.23E-03	-2.090	acyl-CoA dehydrogenase, C-2 to C-3 short chain
SLC38A4	1.41E-02	-2.092	solute carrier family 38, member 4
CHST11	1.73E-02	-2.106	carbohydrate (chondroitin 4) sulfotransferase 11
NPTX2 *	5.85E-03	-2.110	neuronal pentraxin II
HCLS1	3.97E-02	-2.125	hematopoietic cell-specific Lyn substrate 1
DUSP9 *	1.81E-02	-2.136	dual specificity phosphatase 9
CRABP1 *	4.63E-03	-2.151	cellular retinoic acid binding protein 1
STK32C	3.48E-03	-2.159	serine/threonine kinase 32C
<u>CRH</u>	3.44E-04	-2.180	corticotropin releasing hormone
CABP7 *	2.15E-03	-2.188	calcium binding protein 7
HLA-DRB1	1.72E-03	-2.208	major histocompatibility complex, class II, DR beta 1
SNW1	6.59E-03	-2.223	SNW domain containing 1
LASS5	1.16E-02	-2.240	LAG1 homolog, ceramide synthase 5
BST2	2.13E-02	-2.251	bone marrow stromal cell antigen 2
TRIM54 *	3.87E-02	-2.253	tripartite motif-containing 54
LRRC4 *	2.28E-02	-2.262	leucine rich repeat containing 4
DHX9	1.57E-02	-2.267	DEAH (Asp-Glu-Ala-His) box polypeptide 9
PRSS12 *	3.89E-03	-2.291	protease, serine, 12 (neurotrypsin, motopsin)
CYP11B1 *	1.86E-02	-2.327	cytochrome P450, family 11, subfamily B, polypeptide 1
RGL1	3.35E-03	-2.351	ral guanine nucleotide dissociation stimulator-like 1
HES6	2.20E-02	-2.353	hairy and enhancer of split 6 (Drosophila)

Gene Name	p-value	Fold	Gene Description
GPR6	9.24E-03	-2.354	G protein-coupled receptor 6
<u>MAPK8IP1</u>	4.26E-02	-2.355	mitogen-activated protein kinase 8 interacting protein 1
RHOA	2.10E-03	-2.416	ras homolog gene family, member A
FAM38A	1.41E-02	-2.418	family with sequence similarity 38, member A
<u>PDYN</u>	1.88E-03	-2.427	prodynorphin
<u>MAG</u>	9.45E-03	-2.471	myelin associated glycoprotein
MOCOS	1.20E-02	-2.503	molybdenum cofactor sulfurase
<u>GCG</u>	1.15E-02	-2.512	glucagon
DIO3 *	3.80E-04	-2.522	deiodinase, iodothyronine, type III
PLCH2 *	4.37E-04	-2.601	phospholipase C, eta 2
<u>IL1RN</u>	1.41E-02	-2.672	interleukin 1 receptor antagonist
<u>EGR2</u> *	2.14E-02	-2.691	early growth response 2
TBC1D10C *	8.68E-03	-2.728	TBC1 domain family, member 10C
HAL	1.63E-02	-2.733	histidine ammonia-lyase
RAB5C	1.11E-02	-2.736	RAB5C, member RAS oncogene family
KCNJ4 *	2.82E-03	-2.884	potassium inwardly-rectifying channel, subfamily J, member 4
CLCF1 *	5.82E-03	-3.020	cardiotrophin-like cytokine factor 1
GPR88 *	8.46E-04	-3.061	G protein-coupled receptor 88
CHMP4B	2.31E-03	-3.157	chromatin modifying protein 4B
<u>PPP1R1B</u> *	2.18E-04	-3.822	protein phosphatase 1, regulatory (inhibitor) subunit 1B
SCN4B *	8.64E-03	-3.823	sodium channel, voltage-gated, type IV, beta
ADORA2A *	1.25E-03	-4.446	adenosine A2a receptor

The underlined genes were represented in IPA's functional category of 'behavior', while those marked with an asterisk indicate genes associated both with CTA and LiCl treatment alone (also marked with asterisks in Supplemental Table 1).

Table 3

	OXT	INS1	HLA-C	GLRA2
Non-Contingent LiCl Clamped	1.01 (0.12)	1.53 (0.91)	1.00 (0.07)	1.06 (0.27)
Contingent LiCl	4.27 (0.44)	5.29 (0.52)	2.65 (0.43)	1.23 (0.16)
Fold Change	4.2	3.4	2.6	1.1

Group mean expression ratio (relative to 18S rRNA) of oxytocin (OXT), insulin 1 (INS1), major histocompatibility complex, class I, C (HLA-C), and glycine receptor, alpha 2 subunit (GLRA2) mRNA in the amygdala from non-contingent LiCl/sucrose intake clamped and contingent LiCl groups. SE is indicated in parentheses below each mean expression value. Fold: change in CTA animals relative to pseudo-conditioned.