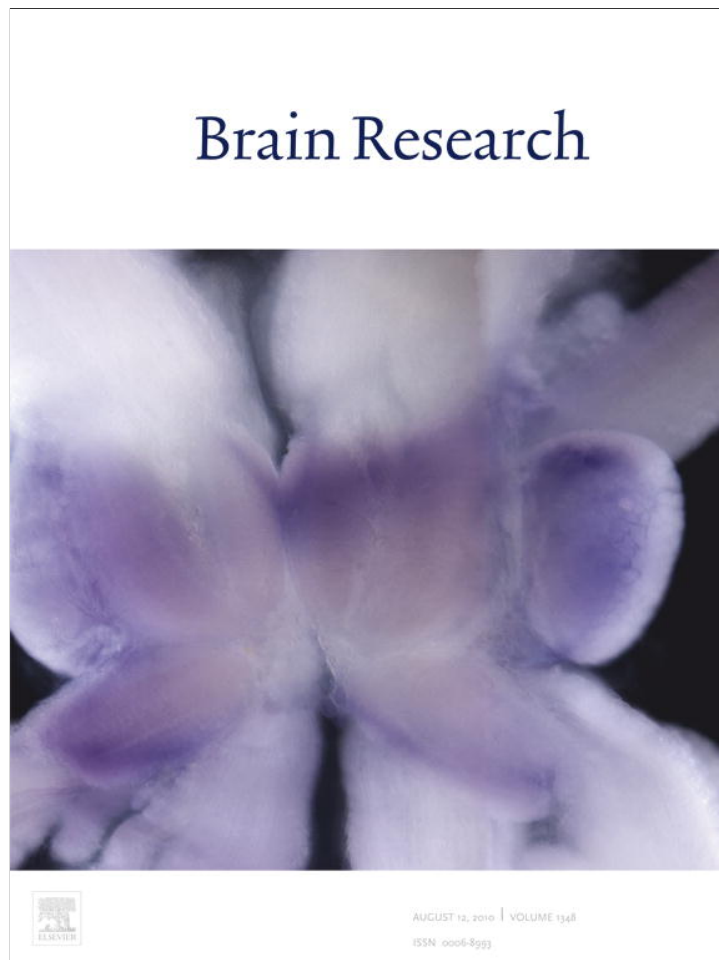


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Research Report

Intra-amygdalar okadaic acid enhances conditioned taste aversion learning and CREB phosphorylation in rats

Denesa L. Oberbeck, Stefanie McCormack, Thomas A. Houpt*

Department of Biological Science, Program in Neuroscience, The Florida State University, Tallahassee, FL 32306, USA

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ABSTRACT

Protein phosphatases (PPs) regulate many substrates implicated in learning and memory. Conditioned taste aversion (CTA) learning, in which animals associate a novel taste paired with a toxin and subsequently avoid the taste, is dependent on several serine/threonine phosphatase substrates and the PP1-binding protein spinophilin. In order to examine the effects of PP1/2A blockade on CTA acquisition and extinction, rats received bilateral infusions of okadaic acid (OA) (100 nM, 1 μ l/hemisphere) or vehicle (0.15 M NaCl) into the amygdala either 5 min prior to, or 5 min after, a single pairing of sodium saccharin (0.125%, 10-min access) and LiCl or NaCl (0.15 M, 3 ml/kg i.p.). Two-bottle, 24-h preference tests were conducted for 13 days to measure CTA expression and extinction. Rats conditioned with saccharin and LiCl showed a decreased preference for saccharin, and OA administered before (but not after) the pairing of saccharin and LiCl resulted in a significantly stronger CTA that did not extinguish over 13 days. The enhancement of the CTA was not due to aversive effects of OA, because rats given OA and a pairing of saccharin and NaCl did not acquire a CTA. Finally, OA administration increased levels of phosphorylated CREB immunoreactivity following a CTA trial. Together, these results suggest a critical role for PP1/2A during normal CTA learning. Because CTA learning was enhanced only when OA was given prior to conditioning, phosphatase activity may be a constraint on learning during the taste-toxin interval but not during acquisition and consolidation processes that occur after toxin administration.

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* Corresponding author. Department of Biological Science, King Life Sciences Bldg., 319 Stadium Drive, The Florida State University, Tallahassee, FL 32306-4295, USA.

E-mail address: houpt@neuro.fsu.edu (T.A. Houpt).

Abbreviations: AMPA, α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate; ANOVA, analysis of variance; APV, (dl)-amino-5-phosphonovaleric acid; BLA, basolateral amygdala; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CaN, calcineurin; CeA, central nucleus of the amygdala; CREB, cAMP-response element binding protein; CTA, conditioned taste aversion; DAB, diaminobenzidine tetrahydrochloride; DARPP-32, dopamine- and cAMP-regulated phosphoprotein MW 32; GLP-1, glucagon-like peptide 1; IC, insular cortex; LA, lateral amygdala; LiCl, lithium chloride; MAPK, mitogen-activated protein kinase; NaCl, sodium chloride; NIP1, nuclear inhibitor of PP1; NMDA, N-methyl-d-aspartate; NR, NMDA receptor; OA, okadaic acid; PB, phosphate-buffer; PBS, phosphate-buffered saline; pCREB, phosphorylated CREB; PKA, protein kinase A; PKC, protein kinase C; PP, protein phosphatase; Ser, serine; Thr, threonine

1. Introduction

The formation of a conditioned taste aversion (CTA) is a type of associative learning in which the pairing of a novel taste with a toxin results in a persistent change in the animal's behavior such that the animal subsequently avoids and rejects the taste. CTA learning has several unique features that make it an advantageous paradigm for examining behavioral and neural characteristics of learning and memory. Aversions are rapidly acquired after a single pairing (Garcia et al., 1961), which allows for the correlation of molecular events with acquisition; they can persist for months or years (Houpt et al., 1996), allowing the processes of consolidation and retrieval to be examined; and learning occurs even if an extended delay occurs between the taste and toxin (Garcia et al., 1966; Smith and Roll, 1967). Additionally, the anatomical circuits underlying CTA are relatively well defined, allowing region-specific manipulations and measurements. While a variety of neurochemicals has been implicated in CTA, the specific intracellular mechanisms mediating CTA acquisition are largely unknown. Although it is generally accepted that protein synthesis, gene expression and synaptic remodeling might underlie long term CTA memory, processes such as post-translational modification of existing proteins have been examined only recently (Berman et al., 1998; Desmedt et al., 2003; Jimenez and Tapia, 2004; Rosenblum et al., 1997; Selcher et al., 2002).

As in many forms of neuronal plasticity, phosphorylation of intracellular substrates is a key signaling step in CTA acquisition. At the postsynaptic membrane, tyrosine and serine phosphorylation of the NR2B subunit of the NMDA receptor (NR) increases in the rat insular cortex (IC) in response to novel tastants (Jimenez and Tapia, 2004; Rosenblum et al., 1997). Within the cytoplasm, tyrosine and threonine phosphorylation of mitogen-activated protein kinase (MAPK) increases in the IC in response to novel taste stimuli (Berman et al., 1998) and in the ventrolateral and central nuclei of the amygdala (CeA) in response to novel taste or toxic LiCl, respectively (Swank, 2000). And in the nucleus of the neuron, serine residues of the transcription factor cAMP response element binding protein (CREB) are phosphorylated in response to novel taste in the lateral amygdala (LA), in response to LiCl in the CeA (Swank, 2000), and in response to paired taste and LiCl in the IC (Desmedt et al., 2003). Several candidate kinases have been implicated in CTA learning such as protein kinase C (Yasoshima and Yamamoto, 1997), protein kinase A (Koh et al., 2002; Koh and Bernstein, 2003), and MAPK (Berman et al., 1998).

While research has focused on activation by phosphorylation, study of the termination of activation by dephosphorylation has been neglected. Serine/threonine protein phosphatases (PP) make up the majority of phosphatase activity in the brain, and both PP1 and PP2A have been implicated in learning and memory (Bollen and Stalmans, 1992; Cohen, 1989; Hubbard and Cohen, 1993; Shenolikar, 1994). A large body of in vitro evidence shows PP1/2A to be necessary for long-term depression (Mulkey et al., 1993), and long-term potentiation is promoted by PP1 inhibition (Blitzer et al., 1995). In vivo, transient genetic inhibition of PP1 in the

hippocampus before training enhances object recognition learning in mice (Genoux et al., 2002). Similarly, knockdown of cytoplasmic PP1-alpha in the hippocampus of mice using siRNA enhanced both contextual and cued fear conditioning (Peters et al., 2009). Inhibition of nuclear PP1 by overexpression of the nuclear inhibitor of PP1 (NIPP1) in mice at the behavioral level enhanced long-term memory for object recognition and spatial (Morris water maze) memory, and at the cellular level enhanced CREB phosphorylation and histone H3 phosphorylation (Koshibu et al., 2009). These results suggest phosphatases might negatively control the efficacy of neuronal transmission and memory formation.

Conversely, pharmacological inhibition of PP1/2A with OA has been shown to impair single-trial passive avoidance learning in neonatal chicks (Bennett et al., 2001) and to impair the spatial learning of rats during radial arm maze training (He et al., 2001, 2005). These results suggest phosphatase activity to be necessary at the time of training, implicating it in the acquisition and consolidation of memories.

Phosphatases are most likely active during CTA learning, and there is some direct evidence delineating their role (Baumgärtel et al., 2008). Our laboratory has demonstrated a critical role for spinophilin, a postsynaptic scaffolding protein that binds PP1, in normal CTA learning. Spinophilin knockout mice were unable to form a CTA to sucrose or salt taste solutions after pairing with low doses of LiCl. Higher concentrations of LiCl could induce a CTA in the spinophilin knockout mice, but one that extinguished more rapidly than wild type mice (Stafstrom-Davis et al., 2001). The attenuated CTA seen in the knockout mice may be attributed to a decrease in PP1 efficacy; in the absence of spinophilin, PP1 may be improperly targeted to dendritic spines. Electrophysiological evidence supports this hypothesis: the knockout mice did not show the typical PP1-mediated rundown of AMPA receptor currents and the enhancement of NMDA currents by PP1 inhibitors was attenuated (Feng et al., 2000). Due to their chronic and global deficit, however, it is impossible to use the spinophilin knockout mice to determine which neuroanatomical locations or time points during CTA acquisition or expression require normal PP1 function.

To test more directly the role of serine/threonine phosphatases in the amygdala during CTA learning, we administered OA into the amygdala before or after saccharin-LiCl conditioning and measured CTA acquisition and extinction. OA, a potent inhibitor of two major serine/threonine phosphatases, PP1 and 2A, has been used in a number of paradigms to affect learning and memory. Both enzymes are logical candidates for mediating dephosphorylation of important molecules in CTA learning. PP2A has been shown to bind to and dephosphorylate NMDA receptors (Chan and Sucher, 2001) and is localized within the amygdala and cortex (Abe et al., 1994). PP1 is highly expressed in many areas critical to CTA; including the amygdala (da Cruz e Silva et al., 1995). Substrates of PP1 that have been implicated in CTA learning include NMDA receptors (Blank et al., 1997; Wang et al., 1994; Yan et al., 1999), MAPK (Jiang et al., 2000), CREB (Bito et al., 1996; Hagiwara et al., 1992) and phospho-acetylated histones (Kwon and Houpt, 2010).

In the present experiments, OA was administered directly into the amygdala, targeting the CeA and/or the basolateral

amygdala (BLA). Because phosphatases regulate dynamic signaling cascades, we tested the effects of OA when injected at one of two time points; immediately before or immediately after the pairing of taste and toxin. These two time points probed the role of phosphatases in, respectively, 1) basal phosphorylation levels and phosphorylation changes stimulated by taste stimuli during the taste-toxin interval and 2) phosphorylation states during the immediate post-conditioning period. Dephosphorylation terminates the activity of many signaling molecules, and thus phosphatase inhibition might result in prolonged phosphorylation of substrates critical to CTA acquisition. Therefore, we predicted that OA administration would enhance CTA learning.

In addition, we predicted elevated levels of serine/threonine phosphorylation following OA pretreatment, in parallel with its behavioral effects. Therefore, we examined OA-mediated phosphorylation of CREB, an intracellular substrate of PP1 implicated in CTA learning (Josselyn et al., 2004; Lamprecht et al., 1997).

2. Results

2.1. Experiment 1: OA administration before conditioning

In order to test the effects of OA administered prior to the CS, four groups of rats received intra-amygdalar infusions 5 min prior to saccharin access: vehicle-treated rats receiving saccharin paired with NaCl or LiCl (vehicle–NaCl and vehicle–LiCl groups), and OA-treated rats receiving saccharin paired with NaCl or LiCl (OA–NaCl and OA–LiCl groups; see schematic in Fig. 1A). There was no difference in the mean intake of saccharin by OA-treated rats (14.5 ± 4.5 g) vs. vehicle-treated rats (14.0 ± 2.9 g) during conditioning.

During the 2-bottle extinction trials NaCl-injected rats had high preference scores for saccharin, while LiCl-treated rats acquired significant CTAs as seen by lower preference scores for saccharin. Furthermore, OA pretreatment enhanced CTA acquisition. One-way ANOVA of preference scores across treatment groups after the first day of 2-bottle pre-

ference testing showed a significant main effect for treatment group ($F(3,24)=10.43$, $p<0.01$; see Fig. 2A). Post hoc analyses showed that the saccharin preference of the vehicle–LiCl group was lower than the vehicle–NaCl and OA–NaCl groups and the saccharin preference of the OA–LiCl group was significantly lower than all other groups.

Two-way, repeated measures ANOVA across the 13 days of extinction testing revealed a significant main effect for treatment group ($F(3,24)=16.33$, $p<0.01$), and for day of testing ($F(12,228)=2.95$, $p<0.01$), but no interaction (see Fig. 2B). Post hoc analyses showed that the saccharin preference of the vehicle–LiCl group increased across days and was significantly different from the vehicle–NaCl and OA–NaCl-treated groups only on test days 1 and 2. In contrast, the saccharin preference of the OA–LiCl group remained significantly lower than all groups for all 13 days. Thus, the CTA of the vehicle–LiCl group extinguished by test day 3, but the CTA of the OA–LiCl group was persistent and significantly greater than that of all groups.

In rats with cannulae placement outside of the amygdala (see Fig. 6, white circles, $n=4$), preference scores after OA–LiCl treatment were not significantly different from the scores of vehicle–LiCl treated rats, ($F(1,120)=0.03$, $p>0.05$, data not shown). Thus, inhibition of phosphatases in areas adjacent to the amygdala was not sufficient to enhance CTA acquisition.

2.2. Experiment 2: OA administration after conditioning

In order to test the effects of OA administered immediately after acquisition, four groups of rats received intra-amygdalar infusions 5 min after LiCl or NaCl injection: rats receiving saccharin paired with NaCl and infused with OA or vehicle (NaCl–OA and NaCl–vehicle groups), and rats receiving saccharin paired with LiCl infused with OA or vehicle (LiCl–OA and LiCl–vehicle groups; see schematic in Fig. 1B).

As in Experiment 1, NaCl-injected rats had high preference scores for saccharin, while LiCl-injected rats acquired a CTA, as shown by a lower preference for saccharin. However, OA treatment after LiCl did not enhance the magnitude of the CTA or prolong extinction. One-way ANOVA across treatment

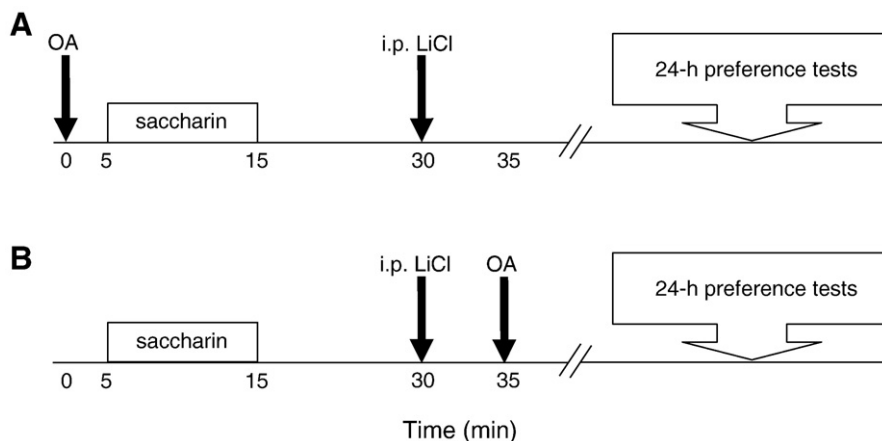


Fig. 1 – A) Schematic of CTA behavioral procedures for pretraining infusions of OA used in Experiment 1. B) Schematic of CTA training for posttraining infusions of OA used in Experiment 2.

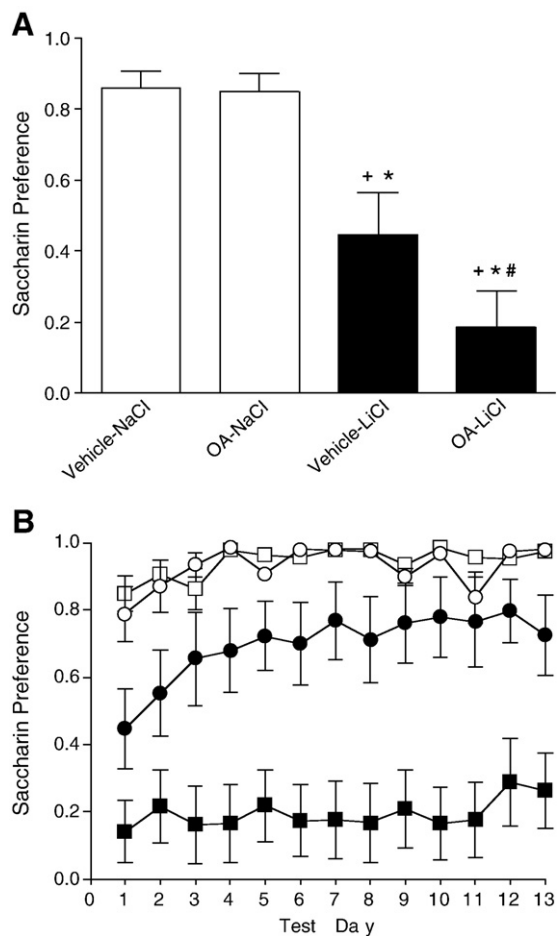


Fig. 2 – A) Expression of CTA on the first day of 2-bottle preference tests after rats received a single pairing of saccharin and LiCl with OA or vehicle injected into the amygdala 5 min before saccharin access. A significantly enhanced CTA was seen in the OA–LiCl group. $*p \leq 0.05$ vs. vehicle–NaCl; $\pm p \leq 0.05$ vs. OA–NaCl; $\#p \leq 0.05$ vs. vehicle–LiCl. **B)** Expression of CTA during extinction tests. The CTA of the OA–LiCl group (black squares) persisted across all test days and was significantly lower than the vehicle–LiCl group (black circles) after day 2, and significantly lower than vehicle–NaCl (white circles) and OA–NaCl (white squares) groups on all days.

groups after the first day of 2-bottle preference testing revealed a significant effect for treatment group ($F(3,18) = 10.75, p < 0.01$; see Fig. 3A). Post hoc analyses showed that both LiCl-treated groups had significantly lower preferences than the NaCl-treated groups ($p < 0.01$), but that intra-amygdalar infusion of OA did not alter the effect of NaCl or LiCl injection.

Two-way repeated measures ANOVA across the 13 extinction days revealed a significant main effect of treatment group ($F(3,18) = 4.32, p < 0.05$), and for test day ($F(12,216) = 4.43, p < 0.01$), but no interaction (see Fig. 3B). Post hoc analyses showed that the saccharin preferences of the LiCl–vehicle and LiCl–OA groups were both significantly lower than the preferences of the NaCl–OA and NaCl–vehicle groups on test days 1–8 and 10. The LiCl–vehicle and LiCl–OA groups were not significantly different from each other on any test day.

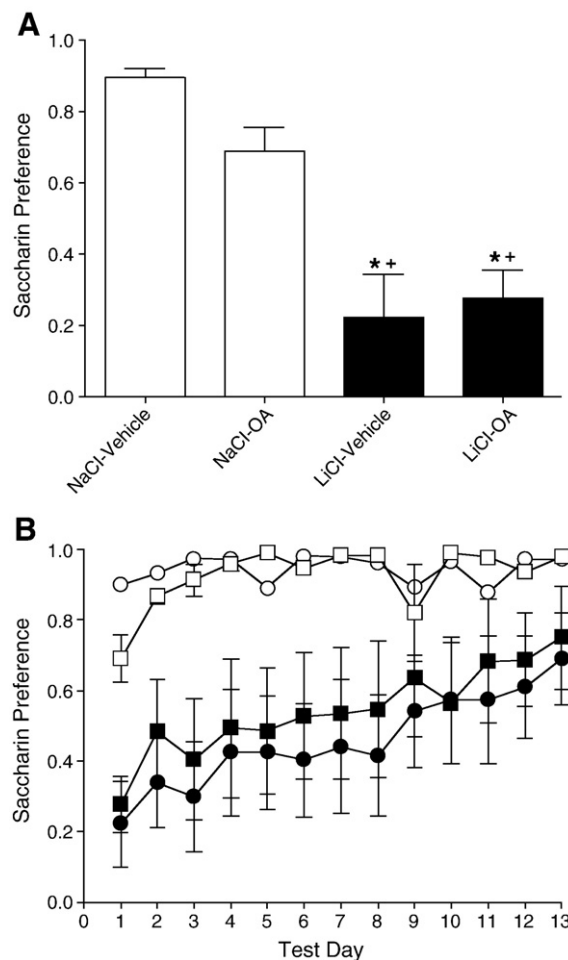


Fig. 3 – A) Expression of CTA on the first day of 2-bottle preference testing after rats received a single pairing of saccharin and LiCl with OA or vehicle injected into the amygdala 5 min after LiCl injection. A significant CTA compared to the NaCl–vehicle group was seen in both the LiCl-injected groups but OA injection after LiCl did not enhance the CTA. $*p \leq 0.05$ vs. NaCl–vehicle; $\pm p \leq 0.05$ vs. NaCl–OA; **B)** Expression of CTA during extinction tests. The CTA of the LiCl–OA group (black squares) extinguished after 4 test days, while the CTA of the LiCl–vehicle group (black circles) persisted for 8 test days. The NaCl–vehicle (white circles) and NaCl–OA (white squares) groups were not significantly different on any day.

2.3. Experiment 3: modulation of CTA-induced phosphorylation by okadaic acid

In order to examine the intracellular effects of OA pretreatment on phosphorylation of CREB during CTA training, pCREB levels were measured by immunohistochemistry in the BLA, LA, and CeA. pCREB was examined with and without OA administration before a single pairing of saccharin and LiCl (a CTA conditioning trial). Both the number of positive cells and the mean pixel density of positive cells were analyzed.

pCREB immunoreactivity was widespread in the amygdala but exclusively nuclear (see Fig. 4). There was no detectable difference in the density of immunostaining within cells

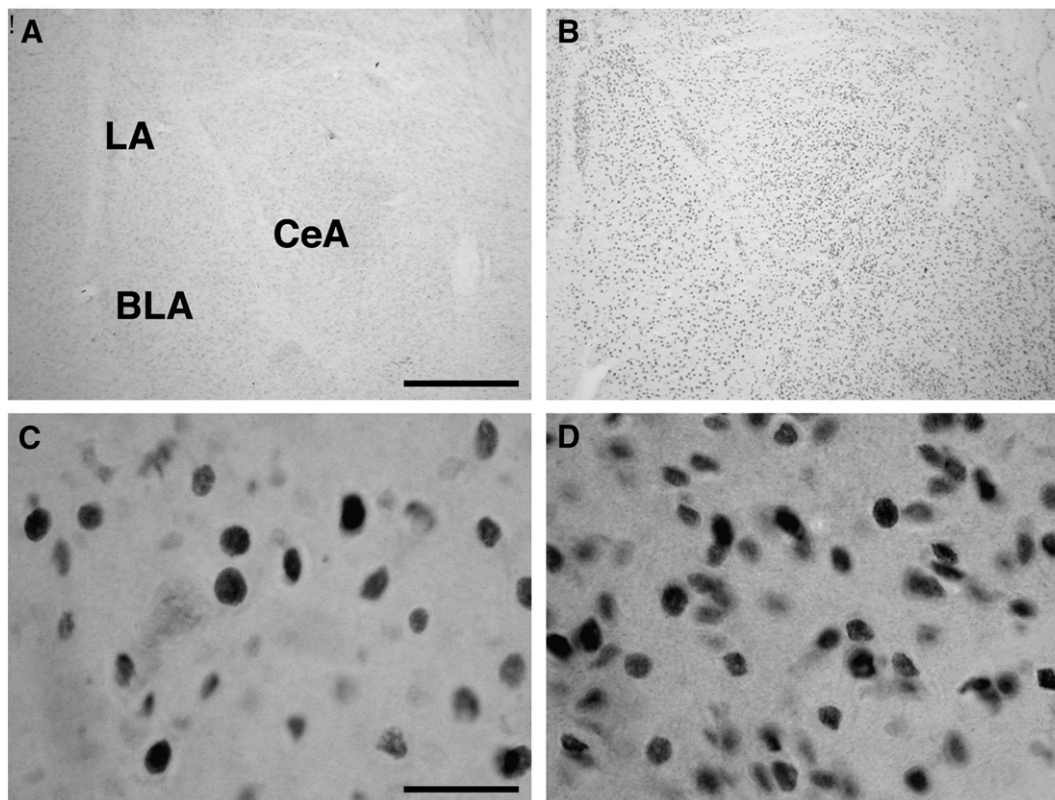


Fig. 4 – Photomicrographs of pCREB immunoreactivity in the amygdala after a single pairing of novel saccharin and LiCl in rats pretreated with vehicle (A, C) or OA (B, D). BLA, basolateral amygdala; LA, lateral amygdala; CeA; central nucleus of the amygdala. Scale bar in (A), 500 μm ; in (C), 30 μm .

(*t*-test, $p > 0.05$ vs. vehicle-treated rats), but an increase in the number of pCREB-immunopositive cells was apparent. Thus, cell counting was used to analyze the brain sections stained for pCREB. Pretreatment with OA significantly increased the number of pCREB-positive cells in the 3 amygdalar subnuclei examined compared to vehicle-treated rats (by *t*-test, $p < 0.01$ for each nuclei, Fig. 5).

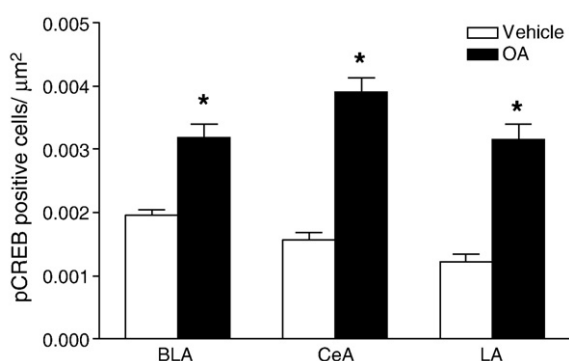


Fig. 5 – Quantification of pCREB immunoreactivity 15 min after saccharin-LiCl pairing in rats pre-treated with vehicle (white bars) or OA (black bars). In all 3 regions of the amygdala, OA pretreatment induced a significantly greater number of pCREB positive cells than vehicle pretreatment. * $p \leq 0.01$ vs. vehicle.

2.4. Verification of cannula placement

Fig. 6 shows the placement of all cannulae. Data was only included from rats with bilateral injector placements within or just above the dorsal amygdalar complex defined, relative to bregma, as lying within the boundaries of 2.1 to 4.2 mm anteroposterior, 3.8 to 5.6 mm mediolateral, and 7.4 to 8.4 mm dorsoventral. Data from 26 rats with bilateral or unilateral placements outside the amygdala were excluded; 66 rats had bilateral placements directed at the amygdala; the majority of cannulae were within or just above the CeA or BLA. Additionally, data from 5 rats (4 from Experiment 1, 1 from Experiment 2) were excluded for having intakes of less than 2 ml on conditioning day.

3. Discussion

These results are the first evidence of a functional role for PP1/2A activity in the amygdala during CTA learning in the rat. Intra-amygdalar administration of 100 nM OA 5 min before CTA acquisition enhanced CTA expression; rats treated with OA-LiCl exhibited a CTA of stronger magnitude compared to vehicle-LiCl treated rats, and failed to extinguish over 13 test days. The effect of OA was temporally specific, because intra-amygdalar administration of OA 5 min after CTA acquisition had no effect on subsequent CTA expression. The effect of OA

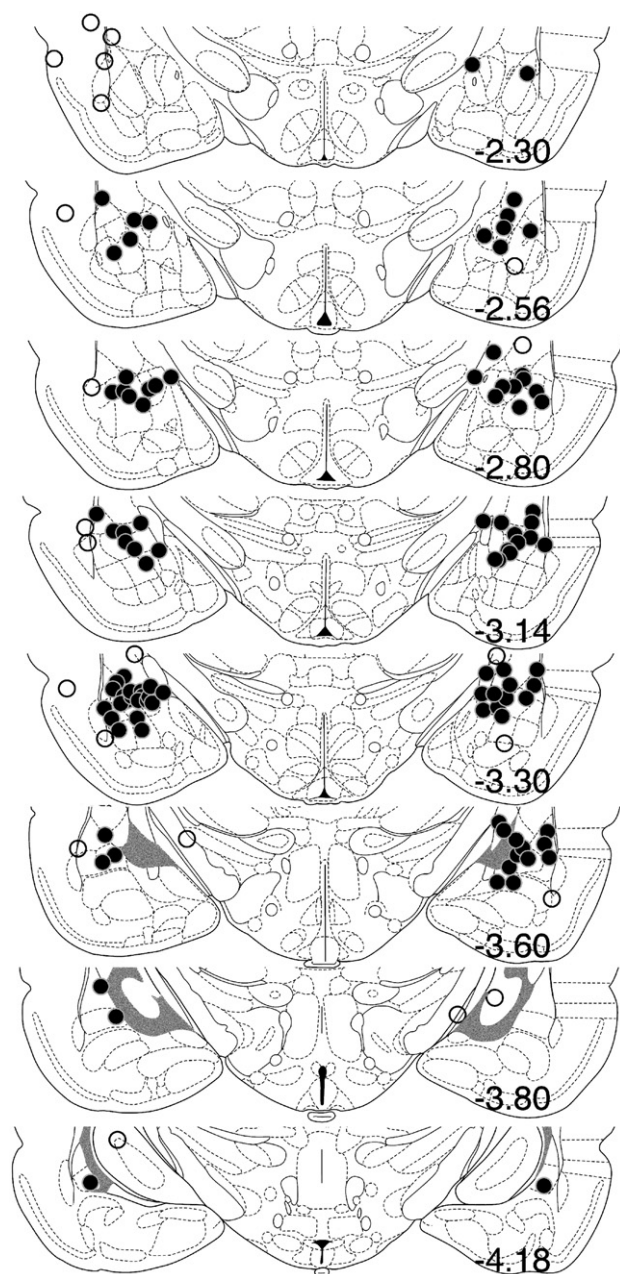


Fig. 6 – Coronal rat brain sections showing the location of injection sites in and around the amygdala. Each symbol shows the location of the tip of the guide cannulae. Black circles indicate correct, included placements; white circles indicate placements that were excluded. Images, spanning -2.30 mm through -4.18 mm relative to bregma, top to bottom; modified from Paxinos and Watson, 1997).

also appeared to be mediated specifically by the amygdala, because OA-LiCl treated rats with cannulae that fell outside of the amygdala did not show any enhancement of CTA expression. Because the major biological action of OA is inhibition of PP1/2A (among other phosphatases), we conclude that the enhanced CTA memory induced by OA administration resulted from inhibition of either one or both of these phosphatases. Our results suggest that the phosphatases are a

constraint on normal CTA acquisition, acting in response to the taste stimulus or during the taste-toxin interval.

The effects of OA on CTA learning do not appear to be an artifact of OA toxicity, as determined by the data from several control groups. Administration of OA alone was not aversive: infusion of OA prior to saccharin access on conditioning day had no acute effect on saccharin intake (Experiment 1). Furthermore, in the absence of LiCl, rats treated with OA before saccharin consumption (OA-NaCl group of Experiment 1) or after saccharin consumption (NaCl-OA group of Experiment 2) did not acquire an aversion to saccharin. (Because we used 24-h 2-bottle tests, we cannot rule out the possibility that OA might have induced a mild or transient CTA in the NaCl-OA group that extinguished in less than 24-h, and therefore was not detected. Such a transient effect would not account for the magnitude of CTA enhancement by OA observed in Experiment 1, however.) Therefore, it seems unlikely that OA enhanced CTA acquisition due to an innate aversive property or neurotoxic effects.

3.1. Temporal sensitivity

Because OA administration was only effective when given before the saccharin, there appears to be a temporal window when phosphatase activity in the amygdala is a critical limit on CTA learning. Findings from other laboratories are consistent with a limited and critical period of phosphatase activity during learning. For example, OA administration into the neonatal chick forebrain inhibited memory for a single-trial passive avoidance task only if given within a span of 10 min immediately before or after training, with the most pronounced inhibition seen when OA was administered 5 min before or after training (Zhao et al., 1995). Additionally, blockade of the phosphatase calcineurin (CaN) with cyclosporin A in the same paradigm inhibited memory only when administered -10 to $+40$ min relative to training (Bennett et al., 1996). Conditional transgenic inhibition of PP1 in mice both during and after water maze training prolonged memory for the task (Genoux et al., 2002). However, if PP1 inhibition was limited to only the training phase, memory decayed as in control animals, while if PP1 inhibition was limited to the post-training period, memory was prolonged. Thus, although the temporal specificity of PP1 inhibition on learning differs across paradigms, there appears to be a commonality in that PP1 activity mediates learning within a discrete critical period. The critical period may differ with the species and learning paradigm examined, or may even be stimulus-specific.

3.2. Enhancement of taste vs. toxin

From our present results, it cannot be determined whether the enhanced CTA results from signaling associated with either the taste or toxin stimuli, or changes mediated by associative processes. It is possible that OA in the amygdala is enhancing some quality of the taste (e.g. novelty, intensity, or palatability) or enhancing the visceral effects of the LiCl. Enhancement of the neural processing of either taste (Bahar et al., 2004) or toxin (Nachman and Ashe, 1973) could result in a stronger, more persistent CTA. Additional psychophysical testing would be required to determine if OA enhances a specific

sensory modality that is detectable in behaviors other than CTA learning.

OA could affect neurochemical and intracellular signaling pathways that mediate taste or toxin processing. Others have demonstrated neurochemical manipulations that appear to interact with the toxin pathway specifically. Systemic levels of LiCl are detected by the area postrema and relayed to the amygdala via projection neurons of the nucleus of the solitary tract and parabrachial nucleus. Two transmitters implicated in the hindbrain–amygdala pathway are glucagon-like-peptide-1 (GLP-1) and glutamate. Both are sufficient to mediate CTA, because a CTA can be induced by pairing a novel flavor with a microinjection of glutamate (Tucci et al., 1998) or saccharin with GLP-1 (Kinzig et al., 2002) into the amygdala as the US in place of the LiCl. Both glutamate and GLP-1 are also necessary for acquisition of a LiCl-mediated CTA, because the GLP-1 antagonist exendin (Thiele et al., 1998) and the NMDA receptor antagonist APV (Rosenblum et al., 1997) can block CTA induced by LiCl. Because both GLP-1 receptors and glutamate receptors can activate serine/threonine kinases, OA may disinhibit the postsynaptic consequences of LiCl administration.

Although taste processing also occurs within the amygdala (e.g. Kemble et al., 1979; Norgren, 1976; Reilly and Bornova, 2005; Schafe and Bernstein, 1996) with gustatory information relayed directly from the hindbrain (e.g. (Spray and Bernstein, 2004; Yamamoto et al., 1997)) and indirectly from the gustatory cortex (e.g. (Koh and Bernstein, 2005; Sakai and Yamamoto, 1999; Schafe and Bernstein, 1998; Yamamoto et al., 1984)), there has been little work on taste-specific neurochemistry within the amygdala. Novel tastes have been shown to induce immediate early gene expression in the amygdala (Koh et al., 2003), however, and OA might potentiate this intracellular activity.

3.3. Intracellular effects of OA

Although OA is widely used as a potent, membrane-permeable phosphatase inhibitor, it is semi-selective and can have non-specific effects at toxic doses. OA has been shown to inhibit both PP1 and PP2A in a concentration-dependent manner. OA can also inhibit other phosphatases such as PP3, but the distribution of PP3 in the amygdala has not been characterized. Thus, it is not possible to determine if OA enhances CTA learning by inhibition of PP1 or PP2A alone or in combination. Both enzymes are localized in the amygdala, although each has a different pattern of regulation, substrate specificity and subcellular localization (Abe et al., 1994; Strack et al., 1999). The concentration of OA used in the present experiment effectively blocks both phosphatases (IC_{50} is <1 nM for PP2A and 10–15 nM for PP1 (Honkanen et al., 1994)). Thus, a more specific method of phosphatase inhibition would be useful in deriving specific conclusions from future studies. Because OA inhibits PP1 only at much higher concentrations than PP2, while calyculin A inhibits PP1 and PP2A with similar potency at low concentrations (Honkanen et al., 1994), calyculin A could be used in concert with OA data to differentiate between the phosphatases. Alternatively, antisense oligonucleotide administration or transgenic manipulations of phosphatases and endogenous phosphatase inhibitors, such as Inhibitor-1 or DARPP-32 (both selective for PP1), could also be used for greater specificity.

Our results are consistent with others who have suggested a constraining role for phosphatases on learning (Baumgärtel et al., 2008; Genoux et al., 2002; Koshibu et al., 2009; Peters et al., 2009). Of particular relevance is the work of Baumgärtel and colleagues, who demonstrated a role in CTA for CaN, a calcium-activated serine/threonine phosphatase (Baumgärtel et al., 2008). They observed a decrease in CaN phosphatase activity in the mouse amygdala at 3 days after saccharin–LiCl pairing, which was correlated with an increase in Zif268 expression. Using inducible overexpression of the CaN auto-inhibitory domain in the forebrain, they also demonstrated that inhibition of CaN during acquisition resulted in a more persistent CTA. Similar to our results with OA, the enhanced CTA after CaN inhibition was markedly resistant to extinction. Conversely, overexpression of active CaN resulted in more rapid extinction of a CTA.

While establishing a role for CaN in CTA, however, Baumgärtel et al. found that PP1 activity was not increased after CTA conditioning or extinction. Furthermore, inhibition of PP1 by transgenic expression of a PP1 inhibitor did not enhance CTA acquisition or diminish extinction. Because we observed enhancement of CTA in rats with the PP1/2A inhibitor OA, this suggests either a species difference in the role of PP1, a difference in the efficacy or pharmacological vs. transgenic inhibition, or a greater contribution of PP2A than PP1 during CTA learning.

While phosphatase inhibition can enhance learning, there are also others who have shown attenuation of learning following phosphatase inhibition (e.g. He et al., 2001; Zhao et al., 1995). Thus, it seems that the effects of interfering with phosphatase function may be complex. In vivo, serine/threonine phosphorylation is reversible, dynamic, and governed by the opposing activities of kinases and phosphatases. Phosphatases can exert both positive and negative effects on signaling pathways and play crucial physiological roles in a variety of tissues and cells. Thus, the effect of phosphatase inhibitors on learning may be bidirectional (either enhancement or attenuation), depending not only on the substrate specificity of the phosphatase, but also on the dynamic state of phosphorylation within particular brain regions during particular phases of memory acquisition. Additional differences in species, learning model, or method of phosphatase inhibition may also play a role in contrasting behavioral results.

3.4. CREB and other phosphatase substrates

While our data suggest that PP1 and/or PP2A may play a role in CTA learning processes, the exact role and underlying mechanisms are unknown. However, several candidate substrates known to be critical in CTA learning are modulated by changes in phosphorylation state, including CREB. Pretreatment with OA before a single saccharin–LiCl pairing resulted in robust increases in pCREB in amygdalar subnuclei (Experiment 3). Thus, endogenous phosphatase activity serves as a constraint on the phosphorylation of CREB during CTA acquisition. Our results suggest increased pCREB levels as the intermediary by which OA administration enhances CTA learning.

It is notable that OA pretreatment enhance pCREB levels throughout adjacent subnuclei, so that the CeA, BLA, and LA all showed increased pCREB. It is possible that the microinjection of

OA diffused throughout these areas resulting in PP1 inhibition within in the BLA and LA that normally do not show particularly high pCREB levels. Another possibility is that PP1/2A in one nucleus (e.g. BLA) resulted in transynaptic activation of the other nuclei.

Two studies have found increased pCREB levels following a CTA conditioning trial in the amygdala (Desmedt et al., 2003; Swank, 2000). A necessary role for CREB in the formation of long-term CTA memory has been demonstrated using antisense oligonucleotides and CREB-deficient transgenic mice (Josselyn et al., 2004; Lamprecht et al., 1997).

Phosphorylation is necessary in order for CREB to bind to the cAMP response element in the promoter of several early response genes; thus the expression of these genes is essentially regulated by the phosphorylation state of CREB. OA has been shown to modulate CREB phosphorylation and CRE-dependent gene expression *in vitro*. For example, dephosphorylation of CREB resulted in decreased transcription of somatostatin in PC12 cells; OA treatment blocked the dephosphorylation of CREB and the decrease in somatostatin expression (Hagiwara et al., 1992). OA treatment also retarded the decay of pCREB levels in cultured hippocampal cells (Bitto et al., 1996). *In vivo*, inhibition of nuclear PP1 by overexpression of NIPP1 in mice enhanced CREB phosphorylation in the hippocampus (Koshibu et al., 2009). Thus the duration of CREB phosphorylation and subsequent CREB-dependent gene expression is regulated by phosphatase activity, and can be modulated by exogenous OA.

In addition to CREB, many other substrates may contribute to OA-mediated enhanced CTA learning. The NMDA receptor, for example, is essential in the amygdala for CTA learning as demonstrated by antagonist studies (Escobar et al., 1998; Tucci et al., 1998; Yasoshima et al., 2000). Increased tyrosine and serine NR2B phosphorylation has been reported in the IC of rats exposed to a novel taste (Jimenez and Tapia, 2004; Rosenblum et al., 1997). Thus hyperphosphorylation of the NMDA receptor in the amygdala during CTA learning might also enhance CTA learning, and future studies should be directed towards examining these possibilities.

4. Experimental procedure

4.1. Subjects

Adult male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing between 300 and 500 gm were used. Rats were housed individually in plastic cages in a temperature-controlled colony room at the Florida State University, with access to pelleted rat chow (Purina St. Louis, MO) and deionized–distilled water *ad libitum* except where specified otherwise. Rats were maintained on a 12-h light, 12-h dark cycle with lights on at 0700 h, and all experimental procedures took place during the light cycle.

4.2. Intracranial cannulation

Under halothane anesthesia, rats were placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA) and 26-gauge guide cannulae (model C315G; Plastics One, Inc.,

Roanoke, VA) were implanted bilaterally. Cannulae were directed at the stereotaxic coordinates of the BLA based on (Paxinos and Watson, 1997), with the tip of the guide cannula positioned 2.8 mm anteroposterior, 5.0 mm mediolateral from bregma and 9.0 mm dorsoventral from the skull surface. When verified histologically, cannulae were found to be clustered across both the BLA and the CeA (see Fig. 6). Dummy cannulae (model C315DC; Plastics One, Inc.) extending 1.0 mm beyond the guide cannula were inserted to prevent clogging. Screws were anchored to the skull and the assembly was fixed in place using dental acrylic. As an analgesic, all rats received ketofen intraoperatively (2 mg/0.2 ml). After at least 7 days recovery, rats were placed on a water-restriction schedule as below.

4.3. Intra-amygdala infusions

OA (Sigma-Aldrich, St. Louis, MO) was freshly dissolved in 0.15 M NaCl to a concentration of 100 nM and injected bilaterally through 28 gauge injection cannulae into the amygdala (1 μ l/hemisphere) at 1 μ l/min. The injector cannulae were left in place for 1 min before being withdrawn in order to avoid backflow along the injection tract. The dose of OA was based on previous behavioral studies (Bennett et al., 2001; Zhao et al., 1995), and preliminary analyses in our own laboratory. The timing of OA injections varied in each experiment as described below.

4.4. Conditioning procedure

Eight days prior to the conditioning day, rats were placed on a water-restriction schedule under which they received daily water access in one drinking session. An empty bottle was presented simultaneously with a water bottle to accustom the rats to a 2-bottle choice. The initial session was 3 h long, and the session times were diminished each day so that the day before conditioning the rats received their water in a single, 10 min session.

On the conditioning day, rats were given access to 0.125% sodium saccharin (conditioned stimulus) for 10 min. Fifteen minutes after the end of saccharin access, rats were injected intraperitoneally (i.p.) with LiCl or NaCl (0.15 M, 3 ml/kg) (unconditioned stimulus). Approximately 1 h after the injections, *ad lib* water bottles were returned overnight.

In order to test for CTA, a series of 24-h, 2-bottle preference tests was initiated on the day after the conditioning trial. Two bottles were placed on the cages; one containing the saccharin solution and the other containing distilled water. Fluid consumption was measured every 24 h and a preference score was calculated as follows:

$$(\text{saccharin consumption}) / (\text{saccharin consumption} + \text{water consumption})$$

The preference tests were continued for 13 post-conditioning days and the left/right position of saccharin and water bottles on the rats' cages was reversed each day. Because saccharin access during the preference tests was not paired with LiCl, the preference tests constituted extinction trials. A CTA was considered extinguished when the average saccharin preference was not different from the average preference of vehicle- and NaCl-injected rats.

4.5. Experiment 1: OA administration before conditioning

Following 8 days of water restriction as above, rats received bilateral injections of OA (100 nM, 1 μ l/hemisphere) or vehicle (0.15 M NaCl) into the amygdala. Five minutes later, all rats received 10-min access to saccharin. Fifteen minutes after the end of saccharin access, rats were injected with 0.15 M LiCl or NaCl (3 ml/kg, i.p.). Thus, there were four groups: vehicle-treated rats receiving saccharin paired with NaCl or LiCl (vehicle-NaCl and vehicle-LiCl, $n=5$ and 9, respectively), and OA-treated rats receiving saccharin paired with NaCl or LiCl (OA-NaCl and OA-LiCl, $n=6$ and 8). Approximately 1 h after the injections, ad lib water bottles were returned overnight. The next day, 24-h, 2-bottle preference tests were started and continued for 13 days (see Fig. 1A).

4.6. Experiment 2: OA administration after conditioning

Following 8 days of water restriction as above, rats received 10-min access to saccharin. Fifteen minutes after the end of saccharin access, rats were injected with LiCl or NaCl (0.15 M, 3 ml/kg, i.p.). Five minutes after the LiCl or NaCl injections, rats received bilateral injections of OA (100 nM, 1 μ l/hemisphere) or vehicle (0.15 M NaCl) into the amygdala. There were four groups: rats receiving saccharin paired with NaCl and injected with vehicle or OA (NaCl-vehicle and NaCl-OA, $n=4$ and 5 respectively), and rats receiving saccharin paired with LiCl injected with vehicle or OA (LiCl-vehicle and LiCl-OA, $n=7$ and 6). Approximately 1 h after the injections, ad lib water bottles were returned overnight. The next day, 24-h, 2-bottle preference tests were started and continued for 13 days (see Fig. 1B).

4.7. Experiment 3: modulation of CREB phosphorylation by okadaic acid

In order to determine if phosphatase activity constrained levels of serine133-pCREB induced by the pairing of saccharin and LiCl, rats were pretreated with intra-amygdalar injections of OA. PP1/2A inhibition by OA was predicted to increase serine phosphorylation of pCREB.

At least 7 days after surgery, rats were placed on water restriction schedule as described above. On conditioning day, rats received intra-amygdalar infusions of either OA (100 nM, $n=6$) or vehicle (0.15 M NaCl, $n=5$), 1 μ l/hemisphere at 1 μ l/min. Five minutes after the intra-amygdalar infusions of OA or vehicle, all rats were given 10-min access to 0.125% saccharin. Fifteen minutes after the end of saccharin access, all rats were injected with LiCl (0.15 M, 3 ml/kg, i.p.). Fifteen minutes after LiCl injection, all rats were overdosed with sodium pentobarbital and perfused as above. The bilateral placement of cannulae directed at the amygdala was verified as described below.

4.8. Immunocytochemistry and histology

At the conclusion of each experiment, rats were overdosed with sodium pentobarbital (Sleepaway, Fort Dodge Laboratories, Fort Dodge, IA) and transcardially perfused with heparinized phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. The brains were removed and fixed overnight in 4% parafor-

maldehyde and then transferred to 30% sucrose until sectioning. Forty-micron coronal sections were cut on a freezing, sliding microtome through the caudal-rostral extent of the brain. Every fourth section through the amygdala (bregma -2.30 to -4.18 ; coordinates taken from (Paxinos and Watson, 1997)) were processed for histology (Experiments 1–3) or immunohistochemistry (Experiment 4). Free-floating tissue sections were then washed twice for 10 min in 0.1 M PBS and incubated for 30 min in 0.1 M PBS, 1% bovine serum antibody (BSA), 0.2% Triton X-100. After two 10-min washes of 0.1 M PBS, 0.5% BSA, sections were incubated overnight at room temperature in anti-pCREB (rabbit polyclonal, 1:1000, Upstate Biotechnology) in PBS/BSA. After two 10-min washes in PBS/BSA, tissue was incubated for 1 h in biotinylated IgG secondary antibody (1:200, Vector Laboratories, Burlingame, CA). Antibody complexes were amplified with a 1-h incubation in the Vectastain Elite ABC kit solution (Vector Laboratories), and visualized with a 5-min, 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB) reaction. After two 5-min washes in 0.1 M phosphate buffer (PB), tissue was mounted onto gelatin-coated glass and dehydrated with a series of ethanol and xylene washes. Slides were counterstained using methyl green and coverslipped using Permount.

Slides were examined under a light microscope; the site of injection was determined as the most ventral point of the injector tip penetration tract. Data was included from rats with bilateral injector placements within or just above the dorsal amygdalar complex defined, relative to bregma, as lying within the boundaries of 2.1 to 4.2 mm anteroposterior, 3.8 to 5.6 mm mediolateral, and 7.4 to 8.4 mm dorsoventral, relative to bregma.

4.9. Image analysis

Sections containing brain regions of interest were photographed in a 0.72 \times 0.54 mm frame using a digital camera (Olympus) attached to an Olympus light microscope (Olympus AX70). Areas of examination were restricted to hand-drawn outlines of each nuclei. Positive cells, determined by pixel density, were counted using image analysis software (MindsEye, T. Houpt). Once determined as positive cells, the absolute pixel density of positive pCREB cells was calculated. Bilateral cell counts were averaged from the 5–7 sections from the LA, CeA, and BLA. The individual mean counts for each region were averaged across rats within each experimental group.

4.10. Statistical analysis

Data was analyzed using Statistica software (Statsoft, Tulsa, OK). Preference scores were analyzed using either one-way or two-way repeated measure analyses of variance (ANOVAs) (with extinction day as the repeated factor); cell counts in Experiment 4 were analyzed by t-test. All post-hoc comparisons were made by Neuman–Keuls test. Data are represented in all figures as mean \pm standard error of the mean.

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