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Research Report

Phospho-acetylation of histone H3 in the amygdala after acute lithium chloride

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ABSTRACT

Acute injection of a high dose of lithium chloride (LiCl) increases c-Fos expression in the central nucleus of the amygdala (CeA). We investigated if LiCl-induced c-Fos expression in the CeA is correlated with histone acetylation and phospho-acetylation. Chromatin modifications such as acetylation and phosphorylation are necessary for optimal gene expression, and gene expression may be increased by inhibiting the activity of histone deacetylases. LiCl (0.15 M, 12 ml/kg, i.p.) highly increased the levels of acetylation and phospho-acetylation of histone H3 in the CeA. The time course of these increases closely corresponded to and preceded the time course of c-Fos induction. Moreover, LiCl-induced c-Fos was co-localized with phospho-acetylated histone H3 in a majority of c-Fos-positive cells in the CeA. Systemic administration of a histone deacetylase inhibitor, sodium butyrate (NaB; 0.3 M, 0.4 g/kg, i.p.), significantly increased the levels of LiCl-induced c-Fos and phospho-acetylated histone H3 in the CeA. NaB also enhanced conditioned taste aversion learning induced by pairing saccharin consumption with LiCl injection, by making the conditioned taste aversion more resistant to extinction. These results suggest that LiCl-induced c-Fos expression may be regulated by modification of histone H3, especially phospho-acetylation, in the CeA. Furthermore, the level of phospho-acetylation of histone H3, c-Fos induction, and amygdalar-dependent taste aversion learning is constrained by endogenous histone deacetylase activity.

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1. Introduction

Acute lithium chloride (LiCl) toxicity evokes physiological malaise like nausea and diarrhea and it is often used as an unconditioned stimulus to produce conditioned taste aversion (CTA) memory in animals. Systemic administration of a high dose of LiCl increases c-Fos expression in several brain regions including the central nucleus of the amygdala (CeA) (Yamamoto et al., 1992; Spencer and Houpt, 2001; Kwon et al., 2008). LiCl-induced c-Fos expression in the brain results from transsynaptic and intracellular signaling. LiCl chemoreception is indirectly relayed to the amygdala from the area postrema

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Abbreviations: AcH3, acetylated histone H3; AcH4, acetylated histone H4; ANOVA, analysis of variance; BLA, basolateral amygdala; BSA, bovine serum albumin; cAMP, cyclic adenosine monophosphate; CeA, central nucleus of the amygdala; CTA, conditioned taste aversion; Cy3, indocarbocyanine; FITC, fluorescein isothiocyanate; HDAC, histone deacetylase; LA, lateral amygdala; LiCl, lithium chloride; Lys, lysine; NaB, sodium butyrate; NaCl, sodium chloride; pAcH3-positive, phospho-acetylated histone H3-positive; PBS, phosphate-buffered saline; Ser, serine; TBS, Tris-buffered saline; TBST, TBS-Tween 20; TSA, trichostatin A

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and the nucleus of the solitary tract via the release of neurotransmitters such as glutamate (Yasoshima et al., 2000; Miranda et al., 2002) and glucagon-like peptide-1 (Seeley et al., 2000; Kinzig et al., 2002). Previous studies have provided evidence that the pathway involving cyclic adenosine monophosphate (cAMP), protein kinase A, and cAMP response element-binding protein is activated within the amygdala after LiCl administration, perhaps upstream of the induction of transcription factors such as c-Fos, Fra-2, and ICER (Lamprecht et al., 1997; Koh et al., 2002, 2003; Kwon et al., 2008; Spencer and Houpt, 2001).

Gene expression is controlled not only by binding transcription factors on the DNA promoter but also by dynamic changes in chromatin structure and histone tails (Spencer and Davie, 1999; Chan and La Thangue, 2001; Ogryzko, 2001; Turner, 2002; Martin and Sun, 2004; Levenson and Sweatt, 2006; Clayton et al., 2006; Ito, 2007). About 147 bp DNA is wrapped with a histone complex composed of one histone H3/H4 tetramer and two histone H2A/H2B dimers to form a nucleosome. The N-terminal tail domains of histones are exposed on the nucleosome surface and undergo enzymatic modifications such as acetylation, phosphorylation or methylation at specific amino acid residues (Spencer and Davie, 1999; Turner, 2002; Peterson and Laniel, 2004; Watson et al., 2004; Clayton et al., 2006). The modifications on the tail domains of histones induce changes in chromatin structure. For example, acetylation and phosphorylation of the tail domains of histone H3 may destabilize compaction of chromatin folding and maintain the unfolded structure of transcribing nucleosomes (Spencer and Davie, 1999; Nowak and Corces, 2004; Ito, 2007). Moreover, this conformational change in chromatin structure allows greater accessibility for transcription factors and RNA polymerases to activate transcriptional initiation (Chan and La Thangue, 2001; Ogryzko, 2001; Turner, 2002). Conversely, histones are deacetylated and dephosphorylated by histone deacetylases (HDACs) and protein phosphatases, respectively (Spencer and Davie, 1999; Nowak and Corces, 2004).

The level of histone acetylation can be increased by inhibition of HDAC activity (Cress and Seto, 2000; Marks et al., 2001; Davie, 2003). Sodium butyrate (NaB) is known to inhibit the activity of HDAC (Boffa et al., 1978). NaB can regulate gene expression by remodeling chromatin structure subsequent to HDAC inhibition (Cress and Seto, 2000; Marks et al., 2001; Davie, 2003), and acute or chronic injections of NaB can increase the level of histone acetylation in the brain (e.g. in the hippocampus, Schroeder et al., 2007; Fischer et al., 2007; Dash et al., 2009).

LiCl-induced c-Fos expression in the brain may also be correlated with dynamic changes in chromatin structure. Others have shown that stimulus-induced c-Fos expression in the brain (e.g. after electroconvulsive seizures or cocaine; Tsankova et al., 2004; Kumar et al., 2005) or *in vitro* (e.g. after epidermal growth factor or anisomycin; Cheung et al., 2000; Clayton et al., 2000) is correlated with acetylation and phosphorylation of histones. Moreover, HDAC inhibitors that enhance histone acetylation increase stimulus-induced c-Fos expression in the brain. For example, i.p. injection of trichostatin A (TSA) intensified mRNA expression of c-fos and c-jun genes in the mouse hippocampus after kainic acid stimulation (Sng et al., 2005). Similarly, administration of NaB (1.2 g/kg, i.p.) increased cocaine-induced c-fos mRNA expression in the rat striatum (Kumar et al., 2005). These previous studies suggest that c-Fos induction in the brain after certain stimuli may be correlated with histone modifications, and that HDAC activity constrains the level of c-Fos expression.

Modification of histones has been demonstrated recently to be a critical factor in the consolidation of several models of learning and memory. For example, mutant mice with decreased levels of cAMP response element-binding protein-binding protein, a histone acetyltransferase, exhibited memory deficits in spatial learning, long-term memory of contextual fear conditioning, and in recognition memory (Wood et al., 2005; Alarcón et al., 2004; Korzus et al., 2004). Furthermore, the impaired memory of the acetyltransferase mutant mice was alleviated by treatment with HDAC inhibitors (Alarcón et al., 2004; Korzus et al., 2004). Another study showed increases in histone acetylation, phosphorylation and phospho-acetylation in hippocampal area CA1 during contextual fear conditioning in rats (Chwang et al., 2006). Conversely, when learning was decreased after extracellular signal-regulated kinase/mitogen-activated protein kinase inhibition, levels of histone acetylation, phosphorylation and phospho-acetylation were also decreased (Chwang et al., 2006). Swank and Sweatt (2001) reported that exposure to a novel food, but not a familiar food, induced histone acetylation in the insular cortex, which correlated with experience-dependent taste learning. These results indicate that histone modifications may play a critical role in gene expression for long-term memory formation, including learning about novel tastes.

Although LiCl-induced c-Fos expression has been investigated at the molecular and cellular levels in the brain, the correlation between LiCl-induced c-Fos expression and histone modifications is unknown. Moreover, the effect of enhanced histone acetylation by HDAC inhibitors on CTA learning is also unknown. Continuing our studies on gene expression in the amygdala after LiCl administration, we investigated

1) if LiCl induces histone acetylation, phospho-acetylation, and c-Fos expression with parallel time courses in the amygdala, and if phospho-acetylation of histone H3 co-localizes with c-Fos expression,
2) if inhibition of endogenous HDAC activity by systemic NaB treatment increases c-Fos expression and phospho-acetylation of histone H3 induced by LiCl in the amygdala, and
3) if HDAC inhibition by systemic NaB enhances CTA learning after the pairing of novel saccharin with LiCl.

In addition, while establishing the parameters for NaB treatment, we found that a high systemic dose of NaB alone induced phospho-acetylation of histone H3 and c-Fos in the amygdala; this effect could be attributed to osmotic stimulation by the hypertonic solution.

Our results revealed that LiCl highly increased the levels of acetylation and phospho-acetylation of histone H3 in the amygdala before and during c-Fos expression, and that c-Fos and phospho-acetylated histone H3 were co-localized in cells of the CeA. NaB treatment significantly increased the levels of LiCl-

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induced c-Fos expression and phospho-acetylated histone H3 in the CeA. Furthermore, NaB treatment also strengthened LiCl-induced CTA learning. Our data suggest that LiCl-induced c-Fos expression may be regulated by modification of histone H3, especially phospho-acetylation, in the CeA. Furthermore, the level of phospho-acetylation of histone H3, c-Fos induction, and amygdalar-dependent taste aversion learning is constrained by endogenous histone deacetylase activity.

2. Results

2.1. *Experiment 1. LiCl induces histone H3 acetylation and phospho-acetylation and c-Fos expression*

To investigate if LiCl increases the levels of histone acetylation, phospho-acetylation and c-Fos expression in the amygdala, rats were treated with LiCl or saline. We examined histone modifications with antibodies directed against histones H4 (acetylated at lysine [Lys] 5, 8, 12 and 16) and H3 (acetylated at Lys14, or acetylated at Lys9 and 14), and phospho-acetylated histone H3 (phospho-serine [Ser] 10 and acetyl-Lys14).

Levels of acetylation were measured in tissue punches of the CeA, basolateral amygdala (BLA), and lateral amygdala (LA) by western blotting at 0.5, 1 and 3 h after LiCl (0.15 M, 12 ml/kg) or 0.5 h after saline injections. The only difference was seen at 0.5 h after LiCl, at which time point LiCl significantly increased the level of acetylated histone H3 (Lys14), but not the levels of acetylated histone H3 (Lys9/Lys14) and acetylated histone H4 30 min after administration in the CeA (*t*-test, $p<0.05$; Fig. 1). There were no significant differences between LiCl and NaCl in the levels of acetylated histones H3 and H4 in the BLA and LA (data not shown).

The number of phospho-acetylated histone H3 (pAcH3)- and c-Fos-positive cells in the amygdala was quantified by immunohistochemistry 0.5, 1, 3 and 6 h after either LiCl (0.15 M, 12 ml/kg) or NaCl (0.15 M, 12 ml/kg) injections (see Fig. 2). In the CeA, two-way ANOVA showed that there was a significant interaction of treatment and time on the number of pAcH3-positive cells ($F(3,40)=26.25$, $p<0.0001$; see Fig. 2A). LiCl significantly increased the number of pAcH3-positive cells in the CeA at 0.5 and 1 h compared to the saline injection groups. The number of pAcH3-positive cells returned to the level of NaCl-injected rats at 3 h.

There was also a significant effect interaction of LiCl administration and time on the numbers of c-Fos-positive cells CeA ($F(3,40)=32.64$, $p<0.0001$). LiCl significantly increased the number of c-Fos-positive cells in the CeA at 1 and 3 h compared to the NaCl injection groups (Fig. 2B). The number of c-Fos-positive cells returned to the level of NaCl-injected rats at 6 h.

Thus, induction of pAcH3 at 0.5 h preceded c-Fos induction at 1 h and 3 h in the CeA. Furthermore, there was a significant positive correlation between the number of pAcH3-positive cells and c-Fos-positive cells within the CeA of the same animals at 1 h ($r(4)=0.84$, $p<0.05$).

To determine if LiCl-induced c-Fos is co-localized with phospho-acetylated histone H3 in the CeA, double immunofluorescence labeling of c-Fos and phospho-acetylated histone H3 was performed 1 h after LiCl injection (0.15 M, 12 ml/kg).

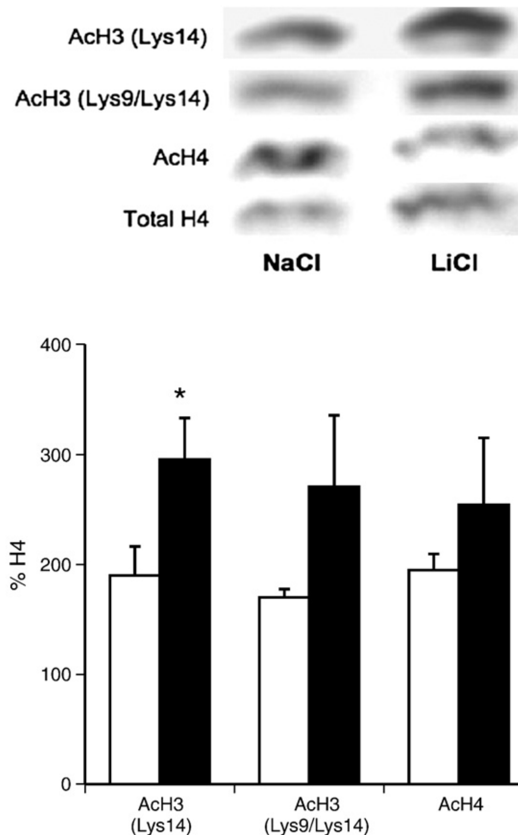

Fig. 1 – Quantification of immunoblot densities for acetylated histones H3 and H4 in the CeA 0.5 h following LiCl (black bars; 0.15 M, 12 ml/kg, i.p.) or NaCl (white bars). Representative immunoblots are shown above the graph. LiCl significantly increased the level of acetylated histone H3 (Lys14), but not the levels of acetylated histone H3 (Lys9/Lys14) and acetylated histone H4 in the CeA. Acetylation percentages were determined by comparing each band density of acetylated histone H3 or acetylated histone H4 with each band density of total H4 in the same blot. *$p<0.05$ vs. NaCl.

LiCl-induced c-Fos- and pAcH3-positive cells in the CeA were immunolabeled with fluorescent indocarbocyanine (Cy3) and fluorescein isothiocyanate (FITC) second antibodies (see Fig. 3). Roughly the same number of cells was labeled with anti-c-Fos and anti-pAcH3 (Fig. 4). 76% of Cy3-labeled c-Fos-positive cells were double-labeled with phospho-acetylated histone H3 antibodies, and 66% of FITC-labeled pAcH3-positive cells were double-labeled with c-Fos antibodies.

A greater number of c-Fos cells in the CeA were observed using immunofluorescence compared to chromogenic labeling with DAB above. This may reflect either greater sensitivity of the sheep anti-c-Fos antibody (Genosys) used for immunofluorescence vs. the rabbit anti-c-Fos antibody (Oncogene) used in the other experiments, or it may reflect greater sensitivity of the fluorescent labeling vs. chromogenic DAB labeling. Nonetheless, qualitatively similar results were found with both techniques.

The number of pAcH3-positive cells was very low in the BLA (<15 cells/section) and LA (<5 cells/section) at all time points. However, LiCl increased the number of pAcH3-positive

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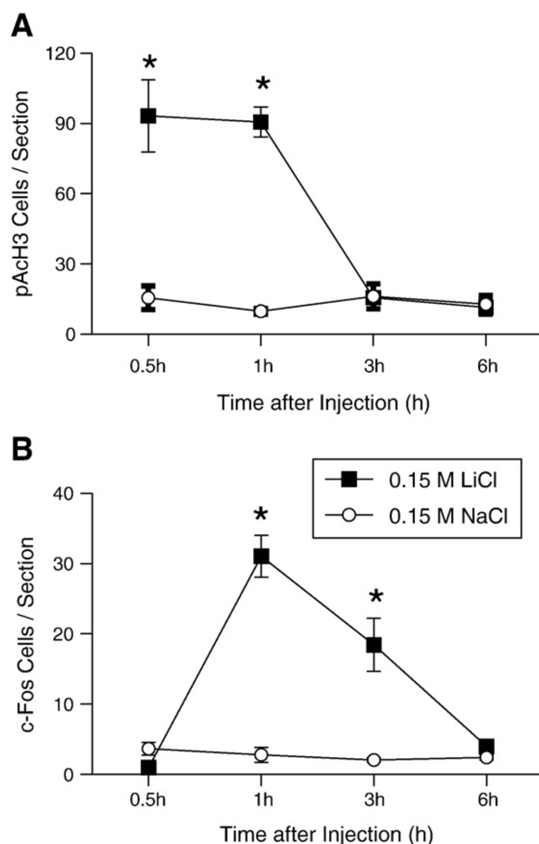

Fig. 2 – Quantification of pAcH3-positive (A) and c-Fos-positive cells (B) in the CeA 0.5, 1, 3 and 6 h following LiCl (0.15 M, 12 ml/kg, i.p.) or NaCl. LiCl increased the number of pAcH3-positive cells in the CeA at 0.5 and 1 h compared to the NaCl injection group (A). LiCl increased the number of c-Fos-positive cells in the CeA at 1 and 3 h compared to the NaCl injection group (B). *$p < 0.005$ vs. NaCl.

cells in the BLA (significant interaction of treatment and time, $F(3,40) = 16.09$, $p < 0.0001$) and in the LA (significant interaction of treatment and time, $F(3,40) = 10.05$, $p < 0.0001$), with increased pAcH3-positive cells at 0.5 and 1 h compared to the NaCl injection groups (data not shown).

There were also significant differences in the number of c-Fos-positive cells among some groups in the BLA (significant interaction of treatment and time $F(3,40) = 3.063$, $p < 0.05$) and LA (significant effect of treatment, $F(1,40) = 9.6$, $p < 0.005$, and time, $F(3,40) = 5.64$, $p < 0.005$) that paralleled responses in the CeA. However, the average numbers of c-Fos-positive cells were very low in the BLA (<3 cells/section) and LA (<1 cell/section) at any time point following LiCl or NaCl (data not shown).

2.2. *Experiment 2. NaB increases phospho-acetylation of histone H3*

To determine if inhibition of HDACs by systemic NaB would enhance basal histone acetylation, histone phospho-acetylation, and c-Fos expression in the amygdala, rats were treated with either 0.3 M or 0.9 M NaB, or iso-osmotic NaCl injections (see Fig. 5).

The number of pAcH3- and c-Fos-positive cells in the rat amygdala was quantified by immunohistochemistry 0.5, 1, 3, 6 and 9 h after NaB (0.3 or 0.9 M, 12 ml/kg) or 1 h after iso-osmotic NaCl injections. (Because NaCl injection was not found to induce significant pAcH3 or c-Fos labeling at other time points in Experiment 1 above, only a 1-h time point was included in this experiment).

One-way ANOVA results for 0.3 M NaB showed that there was a significant effect of treatment for the numbers of pAcH3-positive cells in the CeA ($F(4,29) = 11.79$, $p < 0.0001$; Fig. 5A). NaB (0.3 M) significantly increased the number of pAcH3-positive cells in the CeA at 0.5 h compared to the 0.3 M NaCl control. However, a low dose of NaB (0.3 M) did not significantly induce c-Fos expression in the CeA (Fig. 5B).

Administration of 0.9 M NaB also had a significant effect on the numbers of pAcH3-positive cells in the CeA ($F(6,41) = 25.41$, $p < 0.0001$; Fig. 5A). The high dose of NaB (0.9 M) significantly increased the number of pAcH3-positive cells in the CeA at 0.5 and 1 h compared with other time points including 0.3 and 0.9 M NaCl controls.

NaB (0.9 M) also had a significant effect of treatment for the numbers of c-Fos-positive cells in the CeA ($F(5,35) = 9.01$, $p < 0.0001$; Fig. 5B). NaB (0.9 M) significantly increased the number of c-Fos-positive cells in the CeA at 1 and 3 h compared with 0.3 M NaCl.

Interestingly, 0.9 M NaCl also increased the numbers of pAcH3- and c-Fos-positive cells in the CeA compared with 0.3 M NaCl (Figs. 5A and B). Thus, the enhanced phospho-acetylation and c-Fos expression seen after 0.9 M NaB could be accounted for largely by osmotic effects of the injection.

One-way ANOVA results for 0.3 M NaB showed that there was a significant effect of treatment for the numbers of pAcH3-positive cells in the BLA ($F(4,29) = 5.76$, $p = 0.002$) with increased number at 0.5 h compared to NaCl-injected controls, but not in LA ($F(4,29) = 1.57$, $p = 0.21$); 0.3 M NaB did not significantly enhance c-Fos expression in the BLA or LA (data not shown).

Administration of 0.9 M NaB also had a significant effect on the numbers of pAcH3-positive cells in the BLA ($F(6,41) = 24.06$, $p < 0.0001$), and LA ($F(6,41) = 12.63$, $p < 0.0001$). The high dose of NaB (0.9 M) significantly increased the number of pAcH3-positive cells in the BLA and LA at 0.5 and 1 h compared with other time points including 0.3 and 0.9 M NaCl controls (data not shown).

Amygdalar tissue punches were also examined by western blotting 0.5, 1 and 3 h after NaB (0.3 M only, 12 ml/kg) or 0.5 h after iso-osmotic NaCl. One-way ANOVA found no significant effect of treatment for the levels of acetylated histone H3 (Lys14 and Lys9/Lys14) and acetylated histone H4 (Lys5/Lys8/Lys12/Lys16) in the CeA, LA and BLA (data not shown).

2.3. *Experiment 3. NaB increases LiCl-induced c-Fos and phospho-acetylated histone H3*

To determine if cellular activation after LiCl is constrained by HDAC activity, rats were injected with both LiCl (0.15 M, 6 ml/kg) and NaB (0.3 M, 12 ml/kg). This dose of NaB did not induce c-Fos on its own (Experiment 2). One-way ANOVA results showed that there was a significant effect of treatment for the numbers of pAcH3-positive cells in the CeA ($F(3,22) = 99.93$,

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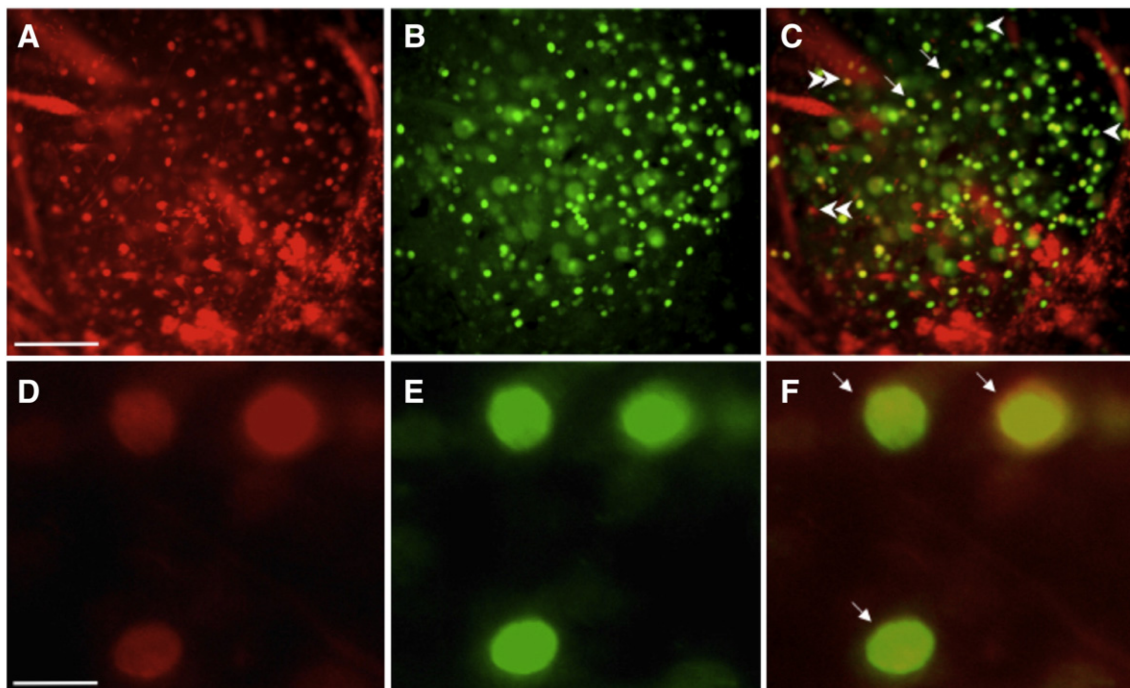

Fig. 3 – Photomicrographs of fluorescently-labeled c-Fos-positive (red, A,D) and pAcH3-positive cells (green, B,E) and merged images (C,F) from the CeA 1 h after a LiCl injection. Yellow cells indicate c-Fos/phospho-acetylated histone H3 double-labeled cells (C and F). Double arrowheads in (C) indicate cells labeled with only anti-c-Fos not pAcH3. Arrowheads in (C) indicate cells labeled with only anti-phospho-acetylated histone H3 not c-Fos. Arrows in (C) and (F) indicate c-Fos and phospho-acetylated histone H3 double-labeled cells. Scale bars, 200 μm in A and 20 μm in D.

$p<0.0001$; Fig. 7A). NaB significantly increased the number of pAcH3-positive cells in the CeA compared to the vehicle group between LiCl groups, but not between NaCl groups (Figs. 6 and 7A).

The numbers of c-Fos-positive cells in the CeA were significantly affected by LiCl and NaB treatments ($F(3,22)=47.12$, $p<0.0001$; Fig. 7B). NaB significantly increased the number of c-Fos-positive cells in the CeA induced by LiCl, but did not increase c-Fos in NaCl-injected rats (Figs. 6 and 7B).

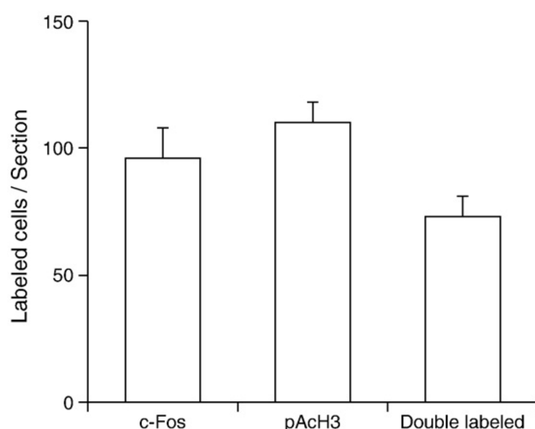

Fig. 4 – Quantification of c-Fos and phospho-acetylated histone H3 fluorescently-labeled cells in the CeA 1 h after LiCl injection.

There was no significant effect of treatment on the levels of phospho-acetylated histone H3 or c-Fos in the BLA and LA (data not shown).

2.4. *Experiment 4. NaB enhancement of LiCl-induced CTA learning*

Because NaB enhanced histone phospho-acetylation and c-Fos expression after LiCl, the effect of NaB on LiCl-induced CTA was tested by pairing saccharin (0.125%) with LiCl (0.15 M, 3 ml/kg) after NaB administration (0.3 M, 12 ml/kg). On the day of conditioning, average intake of saccharin was 6.2 ± 0.4 g and there was no difference in saccharin intake among the 4 groups (NaCl/NaCl, NaCl/NaB, LiCl/NaCl, LiCl/NaB). CTA magnitude was measured with 6 days of 2-bottle testing of saccharin vs. water divided into 48-h preference tests to minimize side preferences (see Fig. 8A). Although the mean saccharin preference of the LiCl/NaB group was lower than the mean saccharin preference of the other groups, a two-way repeated measures ANOVA with group and 2-bottle test days as factors found no effect of group ($F(3,40)=2.48$, $p=0.09$) and no effect of test day ($F(2,40)=2.34$, $p=0.11$). However, rats receiving LiCl alone extinguished faster than rats receiving LiCl followed by NaB (see Fig. 8B). Four of 6 rats in the LiCl/NaCl group extinguished within 6 day of 2-bottle preference testing, but none of the 6 rats in the LiCl/NaB group extinguished. This difference was significant by χ^2 test ($\chi^2=6.0$, $p<0.02$).

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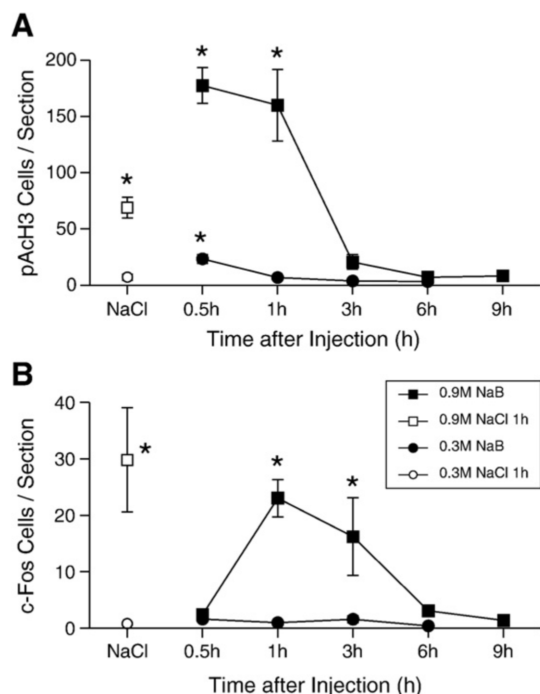

Fig. 5 – Quantification of pAcH3-positive cells (A) and c-Fos positive cells (B) in the CeA 0.5, 1, 3, 6 and 9 h following injections (12 ml/kg, i.p.) of NaB at either 0.3 M (black circle) or 0.9 M (black square) or 1 h following injections of NaCl at either 0.3 M (white circle) or 0.9 M (white squares). Compared to 0.3 M NaCl, the low dose NaB (0.3 M) transiently increased the number of pAcH3-positive cells in the CeA at 0.5 h (A), but did not increase c-Fos expression (B). Both the high dose of NaB (0.9 M) and NaCl (0.9 M) greatly increased the number of pAcH3-positive cells (A) and increased the number of c-Fos-positive cells (B), suggesting that hypertonic 0.9 M injections induced both c-Fos and pAcH3 in the CeA. *$p<0.01$ vs. 0.3 M NaCl.

3. Discussion

In the present study, we established that systemic LiCl increased the levels of acetylation and phospho-acetylation of histone H3 in the amygdala, especially in the CeA. The time courses of these increases closely corresponded to, but preceded, the LiCl-induced increase in c-Fos. Moreover, LiCl-induced c-Fos co-localized with phospho-acetylated histone H3 in a majority of LiCl-induced c-Fos-positive cells in the CeA. Systemic NaB treatment significantly increased the levels of LiCl-induced c-Fos expression and phospho-acetylated histone H3 in the CeA. Furthermore, NaB strengthened LiCl-induced CTA learning as seen in resistance to extinction.

3.1. *Histone modifications and c-Fos expression*

We first investigated the effect of LiCl administration on histone acetylation and phospho-acetylation in the amygdala and the temporal and spatial correlation with c-Fos expression. LiCl significantly increased the level of acetylated histone H3 (Lys14) compared to the control group in the CeA at 30 min as measured with western blotting. LiCl also increased the numbers of pAcH3 (Ser10-Lys14)-positive cells in the CeA, BLA and LA at 0.5 and 1 h as measured by immunohistochemistry. This increase in acetylation and phospho-acetylation of histone H3 occurs just before and during the expression of c-Fos in the CeA. Consistent with other reports, LiCl induced c-Fos protein in the CeA at 1 and 3 h, after the peak of acetylated and phospho-acetylated histone H3. Moreover, LiCl-induced histone acetylation (at Lys14 on histone H3) in the CeA at 0.5 h correlates with c-Fos transcription because c-Fos mRNA expression after LiCl was highest at this time point (Spencer and Houpt, 2001).

We further investigated the co-localization between LiCl-induced c-Fos and phospho-acetylated histone H3 in the CeA with double immunolabeling. This showed that LiCl-induced c-Fos co-localized with phospho-acetylated histone H3 in a majority of c-Fos-positive cells in the CeA. This result is similar to a previous report demonstrating that stress-induced c-Fos was co-localized with phospho-acetylated histone H3 in granule neurons of the rat dentate gyrus (Chandramohan et al., 2007). Thus, phospho-acetylation of histone H3 in the CeA may be an important factor for LiCl-induced c-Fos expression. (However, other factors than phospho-acetylated histone H3 must be also required to induce c-Fos expression in the cell, because not all pAcH3-positive cells expressed c-Fos after LiCl administration).

These results suggest that stimulus-induced c-Fos expression is triggered by dynamic changes in chromatin structure. Others have shown that stimulus-induced c-Fos expression is correlated with acetylation, phosphorylation or phospho-acetylation of histones (Crosio et al., 2003; Ho et al., 2007; Chandramohan et al., 2007, 2008; Collins et al., 2009). Studies using chromatin immunoprecipitation have also demonstrated that stimulus-induced c-Fos expression is correlated with histone modifications at the c-Fos promoter. For example, phosphorylated, acetylated or phospho-acetylated histone H3 was associated with the c-Fos promoter after follicle-stimulating hormone stimulation in cultured granulosa cells (Salvador et al., 2001). Electroconvulsive seizure treatment increased expression of c-Fos mRNA and the levels of acetylated histone H4 and phospho-acetylated histone H3, but not acetylated histone H3 on lysines 9 and 14, at the c-Fos promoter in the rat hippocampus (Tsankova et al., 2004). Cocaine stimulation increased the levels of acetylated histone H4, but not acetylated histone H3 on lysines 9 and 14, at the c-Fos promoter and phospho-acetylated histone H3 in the rat striatum (Kumar et al., 2005).

The pattern of histone modifications is variable across studies. Unlike electroconvulsive seizure or cocaine stimuli, in the present study LiCl did not increase acetylated histone H4 in the CeA, but did increase acetylation of histone H3 at lysine14 and phospho-acetylation of histone H3. Thus, the pattern of histone acetylation may be specific to the type of stimulation, the activated brain region, or the expressed genes.

3.2. *HDAC inhibition by NaB*

To establish a functional role for histone acetylation in regulation of c-Fos expression, we used systemic NaB as an

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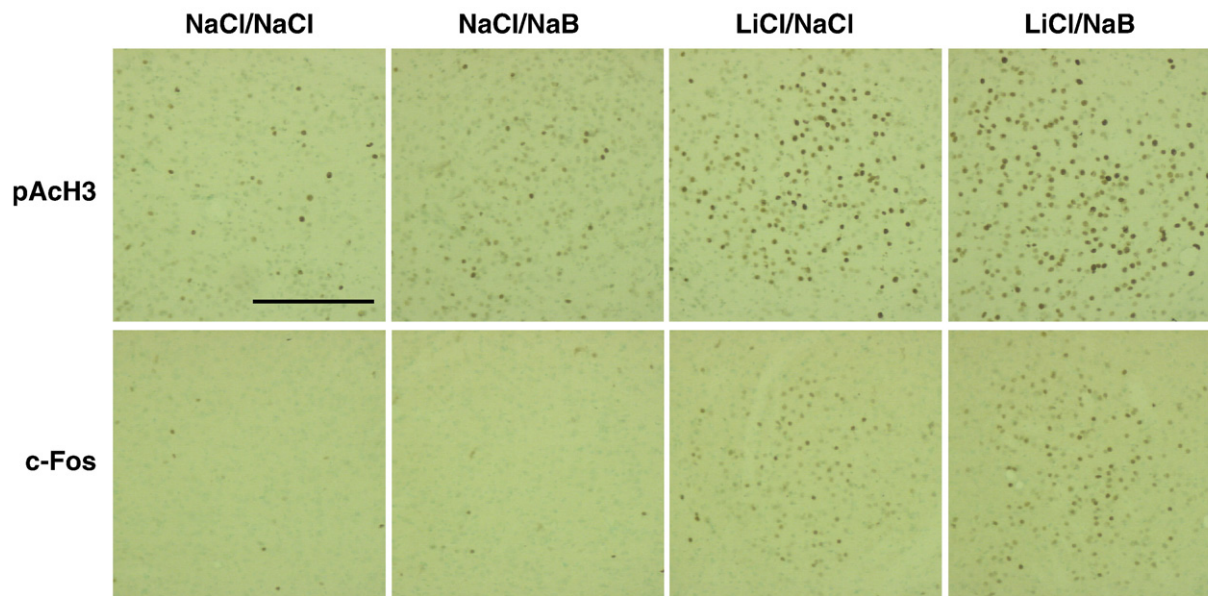

Fig. 6 – Photomicrographs of pAcH3 (top panels) and c-Fos (bottom panels) immunohistochemistry in the CeA after injection with LiCl, NaB or the combined LiCl/NaB. Rats were first injected with NaCl or LiCl (0.15 M, 6 ml/kg, i.p.). Ten minutes later, the rats were injected with NaB (0.3 M, 12 ml/kg, i.p.) or NaCl, then 50 min later processed for immunohistochemistry. LiCl induced both pAcH3 and c-Fos in the CeA (LiCl/NaCl vs. NaCl/NaCl). NaB alone did not induce pAcH3 or c-Fos (NaCl/NaB), but did increase the levels of LiCl-induced pAcH3- and c-Fos-positive cells in the CeA (LiCl/NaB vs. LiCl/NaCl). Scale bar, 500 μm.

HDAC inhibitor to increase acetylation. NaB increased the number of LiCl-induced pAcH3-positive cells in the CeA, and significantly enhanced the induction of c-Fos by LiCl. This result is similar to reports showing that histone acetylation enhanced by HDAC inhibition increased stimulus-induced c-Fos expression in the brain (Sng et al., 2005) or *in vitro* (Ho et al., 2007), and to a report showing that NaB increased the levels of cocaine-induced pAcH3-positive cells and c-Fos expression in rat striatum (Kumar et al., 2005). Thus it appears that HDAC activity acts as a constraint on acetylation and phosphorylation of histones, and thus may limit gene expression including stimulus-induced c-Fos expression.

Although NaB is normally used to increase histone acetylation, it also increased phospho-acetylation of histone H3 in this study. Similar to our result, it has been reported that TSA increased phospho-acetylation of histone H3 *in vitro* (Hauser et al., 2002). It may be that changes in chromatin structure, which occur as a result of increased histone acetylation subsequent to treatment of HDAC inhibitors, can increase the accessibility of the target substrate for histone kinases (Whitlock et al., 1983). Thus, NaB and TSA may indirectly increase phosphorylation of histone H3 along with its acetylation. However, it is not clear whether acetylation and phosphorylation of histone H3 occur synergistically (Cheung et al., 2000) or independently (Thomson et al., 2001).

Interestingly, a high dose of NaB (0.9 M, 1.2 g/kg) greatly increased phospho-acetylated histone H3 and c-Fos in the CeA. This effect could be explained by the hypertonicity of the treatment, however, because an iso-osmotic control (0.9 M NaCl) also increased phospho-acetylated histone H3 and c-Fos in the CeA. This high concentration of NaB has been used in several previously published studies that did not include osmotic controls (Schroeder et al., 2007; Fischer et al., 2007; Dash et al., 2009). To reduce the osmotic stress to the animals, we used a lower dose of NaB (0.3 M, 0.4 g/kg) and found that much less phospho-acetylation of histone H3 was induced in the CeA and BLA. Furthermore, unlike the high dose, the low dose of NaB alone did not increase basal levels of c-Fos expression in the amygdalar regions.

Although NaB increased pAcH3-positive cells using immunohistochemistry, no increase in the level of histone acetylation was observed by western blot in the amygdala following NaB treatment, although others have seen increased levels of histone acetylation in the brain after high doses of NaB (Schroeder et al., 2007; Fischer et al., 2007; Dash et al., 2009). The changes in histone acetylation caused by a low dose of NaB may be below the level of detection by western blot.

3.3. *Conditioned taste aversion after NaB*

Given the correlation of histone acetylation and phosphorylation with c-Fos expression, and the ability of the HDAC inhibitor NaB to enhance both the histone modifications and c-Fos induced by LiCl, we predicted that NaB would be able to enhance LiCl-induced CTA as well. Therefore, intake of a novel saccharin solution was paired with systemic injections of LiCl and NaB. In parallel with its minimal effects on cellular activation, NaB alone did not induce a CTA. Although the group receiving both LiCl and NaB appeared to have a slightly stronger CTA during extinction compared with the group receiving LiCl alone, there was no statistical enhancement of the CTA by NaB in terms of 2-bottle preference score. However, a significantly larger number of rats extinguished within 6 days after LiCl compared to rats treated with LiCl and NaB,

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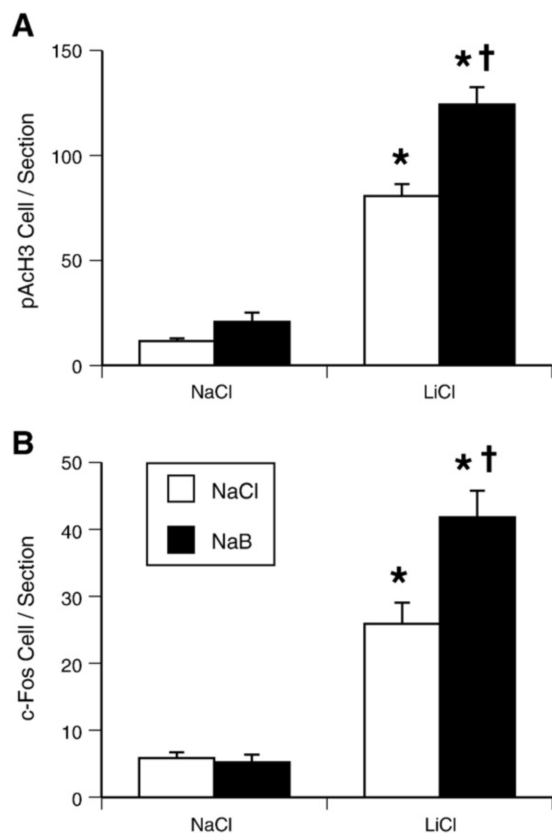

Fig. 7 – Quantification of pAcH3-positive cells (A) and c-Fos-positive cells (B) in the CeA induced by NaCl or LiCl injection (0.15 M, 6 ml/kg, i.p.) followed by injection of NaCl (white bars) or NaB (black bars; 0.3 M, 12 ml/kg, i.p.). NaB significantly increased LiCl-induced pAcH3-positive cells (A) and c-Fos-positive cells (B) in the CeA. $^*p<0.05$ vs. NaCl/NaCl, †$p<0.05$ vs. LiCl/NaCl control.

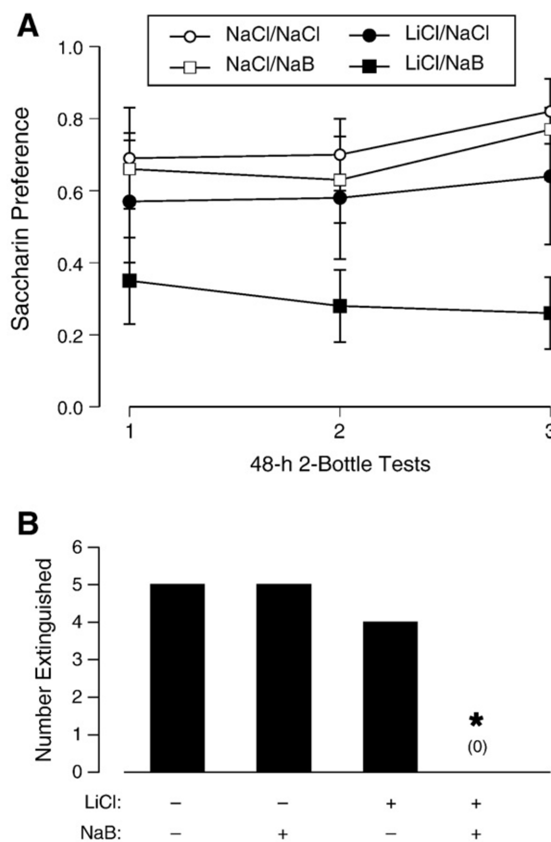

Fig. 8 – A. Mean saccharin preference scores measured by 48-h, 2-bottle preference tests across 6 days of CTA extinction. Although rats (black squares) injected with NaB (0.3 M, 12 ml/kg, i.p.) after LiCl (0.15 M, 3 ml/kg, i.p.) showed a lower saccharin preference than rats injected with vehicle alone (white circles), NaB alone (white squares), or LiCl followed by NaCl (black circles), this difference was not significant. B. Number of rats extinguished during the 6 days of preference testing. Rats were considered extinguished if they showed a 0.9 or higher preference for saccharin over water. While 4 of 6 rats injected with NaCl after LiCl extinguished, none of the rats injected with NaB after LiCl extinguished. $^*p<0.02$ by chi-squared test.

none of which extinguished. Thus, NaB appeared to enhance LiCl-induced CTA learning by making the CTA more resistant to extinction.

Consistent with our result that NaB enhanced CTA learning, it has been demonstrated that NaB treatment increased the memory of fear conditioning (Levenson et al., 2004; Fischer et al., 2007), extinction of conditioned fear (Bredy et al., 2007) and spatial learning in the water maze (Fischer et al., 2007; Dash et al., 2009). A larger effect was seen in some of these studies, perhaps because NaB was used with a much higher dose in these previous studies than the present study (1 or 1.2 vs. 0.4 g/kg). Alternatively, NaB may vary in its effectiveness across different types of learning.

3.4. Summary

We have shown a correlation between modification of histone H3 and LiCl-induced c-Fos expression in the amygdala. LiCl-induced chemoreceptive stimulation may induce c-Fos expression via dynamic changes in histone structure as well as activation of transcription factors in the amygdala. The effect of NaB on LiCl-induced c-Fos and modification of histone H3 suggests that c-Fos expression after LiCl stimulation may be regulated by HDAC modification of histone H3, especially phospho-acetylation, in the CeA. In addition, NaB enhanced LiCl-induced CTA learning, suggesting that histone deacetylation may serve as a constraint on amygdalar-dependent learning.

4. Experimental procedures

4.1. Animals

Adult male Sprague–Dawley rats (300–450 g, Charles River Laboratories, Wilmington, MA) were individually housed under a 12-h light–12-h dark cycle (lights on 07:00) at 25 °C with free access to Purina rodent chow and distilled water. All experiments and procedures were conducted in the first half of the lights on period. Anesthesia (sodium pentobarbital) was used to minimize pain and discomfort. All experiments were

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approved by the Florida State University institutional animal care and use committee.

4.2. Western blotting

Rats were anesthetized with sodium pentobarbitol and their brains were removed. The brains were immediately frozen in M-1 Embedding Matrix (Shandon, Pittsburgh, PA) with dry ice and kept in a −80 °C freezer until used. Frozen brain blocks including the lateral, basolateral and central nuclei of the amygdala were cut in the coronal plane between −2.40 mm and −3.10 mm from bregma. The individual nuclei of the amygdala were bilaterally dissected out from the brain blocks using Palkowitz tissue punches (0.53 mm diameter for LA and 0.75 mm diameter for BLA and CeA) at −20 °C. Individual tissue samples were homogenized by using a tissue grinder in extraction buffer (0.5 M Tris–HCl, pH 6.8; 10% glycerol; 2% SDS; 1% β-mercaptoethanol; 0.5% bromophenol blue) on ice. The tissue homogenate was boiled for 5 min, centrifuged at 15,000 ×*g* for 10 min, and then transferred into a new tube. The protein concentration was determined with Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA). Each protein sample was loaded in 15% SDS polyacrylamide gels and electrotransferred onto polyvinylidene-difluoride membranes. The membranes were blocked in 5% nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris pH 7.6, 0.8% NaCl) containing 0.1% Tween 20 (TBST), and then hybridized with primary antibodies in TBST for 3 h at room temperature. The primary antibodies used were: anti-acetylated Histone H3 (Lys14, 1:1000, Upstate, Lake Placid, NY), anti-acetylated Histone H3 (Lys9/Lys14, 1:20,000, Upstate), anti-acetylated Histone H4 (Lys5/Lys8/Lys12/Lys16, 1:5000 or 1:10,000, Upstate) and, for a loading control, anti-Histone H4 (1:500, Upstate). The membranes were washed in TBST, and then hybridized with a horseradish peroxidase conjugated secondary antibody (sc-2030, 1:5000, Santa Cruz Biotechnology) in TBST for 1 h at room temperature. The membranes were washed again in TBST, incubated in Enhanced Chemiluminescence Plus (Amersham ECL plus, GE Healthcare, UK) solution, and then exposed to film (Amersham Hyperfilm ECL). The film was digitized using the Gel Logic 100 (Kodak, Rochester, NY).

4.3. Immunohistochemistry

Rats were anesthetized with sodium pentobarbitol and perfused first with 100 ml of isotonic saline containing 0.5% sodium nitrate and 1000 U heparin, and then with 400 ml phosphate-buffered 4% paraformaldehyde. Brains were dissected out and post-fixed for 3 h, then cryoprotected in 30% sucrose for 1–2 days. Brain sections were cut at 40 μm on a −20 °C microtome, and washed twice in 0.1 M phosphate-buffered saline (PBS) for 10 min. After PBS washes, sections were washed twice in 0.2% Triton–1% bovine serum albumin (BSA)-PBS for 30 min, and washed in PBS-BSA for 10 min twice.

For chromogenic immunohistochemistry, sections were incubated overnight with primary antibodies in PBS-BSA at room temperature. The primary antisera used were anti-phospho-acetylated histone H3 (Ser10-Lys14, 1:200, Upstate) and anti-c-Fos (Ab-5, 1:20,000, Oncogene Research). (Preliminary tests found that the antisera against acetylated histones used for western blotting stained essentially all cell nuclei within tissue sections.) Sections were washed in PBS-BSA for 10 min twice, and incubated for 1 h with the biotinylated goat anti-rabbit antibody (Vector Laboratories) at a dilution of 1:200 in PBS-BSA. After washes in PBS-BSA for 10 min twice, antibody complexes were amplified using the Elite Vectastain ABC kit (Vector Laboratories), and visualized by a 5-min reaction in 0.05% 3,3-diaminobenzidine tetrahydrochloride. Sections were immediately washed twice in 0.1 M phosphate buffer and mounted on gelatin-coated slides. Sections on the slides were stained with Methyl Green Nuclear Counterstain (Vector Laboratories) and coverslipped with Permount.

For fluorescent immunohistochemistry, sections were incubated in the mixture of two primary antibodies (sheep anti-c-Fos, 1:1000, Genosys Biotechnologies Ltd., Cambridgeshire, UK and rabbit anti-phospho-acetylated histone H3, 1:200, Upstate) in PBS-BSA at room temperature overnight. Sections were washed in PBS-BSA for 10 min twice and incubated in the mixture of two secondary antibodies (Cy3-conjugated donkey anti-sheep and FITC-conjugated goat anti-rabbit, 1:100, Jackson ImmunoResearch) in PBS-BSA for 1.5 h at room temperature in darkness. Sections were washed in PBS for 10 min twice, mounted on gelatin-coated slides, and coverslipped with Vectashield mounting medium (Vector Laboratories) in darkness.

4.4. Quantification and statistical analysis

For the western blot results, band density was measured from the gels using the Gel Logic 100 system (Kodak, Rochester, NY). The density of each band was normalized to the density of total histone H4 band from the same blot.

For the chromogenic immunohistochemistry, cells expressing darkly-positive, nuclear staining were quantified with custom software (MindsEye, T. Houpt). Regions were digitally-captured at 40× magnification on a Macintosh computer using an Olympus Provis AX-70 microscope with a Dage-MTI DC-330 CCD camera and Scion LG-3 framegrabber. Counting was restricted to the BLA, CeA or LA as delineated by a hand-drawn outline. Bilateral cell counts were averaged for 6 sections of the amygdala for each rat. The individual mean counts for each region were then averaged across rats within experimental groups.

Double immunofluorescence-labeled sections were examined and photographed under a fluorescence microscope (Leica DML, Leica Microsystems, Wetzlar, Germany). Three sections of the CeA region from each rat were photographed, and the number of single- and double-labeled cells was counted. c-Fos- and pAcH3-positive cells were viewed with Leica filtercube sets: GFP (excitation filter: BP 470/40, dichromatic mirror 500, suppression filter BP 525/50) and Y3 (BP 545/30, 565, BP 610/75) for FITC and Cy3, respectively.

Significant effects across treatment groups were detected by one-way or two-way ANOVA and Neuman–Keuls post-hoc tests (Kaleidagraph, Synergy Software). The χ^2 test was used to compare the number of rats extinguished in Experiment 4. All data are presented as the mean ± standard error of the mean.

4.5. Experiment 1. Histone acetylation, phospho-acetylation and c-Fos expression after LiCl

In order to investigate whether LiCl increases the levels of acetylated histone H3, acetylated histone H4, phospho-

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acetylated histone H3 and c-Fos in the amygdala, rats (n=6 per group) were injected with LiCl (Sigma). Rats were sacrificed 0.5, 1 and 3 h after LiCl (0.15 M, 12 ml/kg, i.p.) or 0.5 h after saline injections (0.15 M NaCl, 12 ml/kg, i.p.). Their brains were processed for western blotting to measure levels of acetylated histones H3 and H4 as described above.

To quantify the number of cells in the amygdala activated by LiCl, another set of rats was sacrificed 0.5, 1, 3 and 6 h after LiCl (0.15 M, 12 ml/kg, i.p., n=6 per time point) or saline injections (0.15 M NaCl, 12 ml/kg, i.p., n=4 per time point). Their brains were processed for immunohistochemistry to measure the levels of c-Fos expression and phospho-acetylated histone H3 as described above.

In order to determine whether LiCl-induced c-Fos is co-localized with phospho-acetylated histone H3 in the CeA, double immunofluorescence labeling of c-Fos and phospho-acetylated histone H3 was performed. Rats (n=6) were anesthetized with sodium pentobarbitol 1 h after a LiCl injection (0.15 M, 12 ml/kg, i.p.) and perfused. Their brains were processed for fluorescent immunohistochemistry as described above.

4.6. *Experiment 2. Histone acetylation, phospho-acetylation and c-Fos expression after NaB*

In order to investigate whether NaB itself induces the levels of c-Fos expression and phospho-acetylated histone H3 in the amygdala, rats received injections of two different doses of NaB (Sigma) (0.3 M or 0.9 M, 12 ml/kg, i.p.). Rats were sacrificed 0.5, 1, 3, 6 and 9 h after NaB injections (n=6 per time point and treatment). For controls, rats were sacrificed 1 h after iso-osmotic saline injections (0.3 M or 0.9 M NaCl, 12 ml/kg, i.p., n=6 per group). Their brains were processed for immunohistochemistry to measure the levels of c-Fos expression and phospho-acetylated histone H3 as described above.

In order to investigate whether NaB induces the levels of acetylated histones H3 and H4 in the amygdala, another set of rats was sacrificed after NaB injections. Because both a high dose of NaB (0.9 M) and iso-osmotic control (0.9 M NaCl) increased the levels of c-Fos expression and phospho-acetylated histone H3 in the CeA (see the Experiment 2 results), the lower dose of NaB (0.3 M, 12 ml/kg) was employed for western blots. Rats (n=6 per group) were sacrificed 0.5, 1 and 3 h after NaB (0.3 M, 12 ml/kg, i.p.) or 0.5 h after iso-osmotic saline (0.3 M NaCl, 12 ml/kg, i.p.) injections. Their brains were processed for western blot to measure the levels of acetylated histones H3 and H4 as described above.

4.7. *Experiment 3. Effect of NaB on LiCl-induced c-Fos expression and phospho-acetylated histone H3*

In order to investigate whether NaB could increase the levels of LiCl-induced c-Fos and phospho-acetylated histone H3 in the amygdala, rats were injected with LiCl (0.15 M, 6 ml/kg, i.p.) or saline (0.15 M NaCl, 6 ml/kg, i.p.). To enhance the NaB effect and remain below the maximal level of LiCl-induced c-Fos, we used a lower dose of LiCl compared with the dose used in Experiment 1. Ten minutes after LiCl or saline injections, rats (n=6 per group) were injected with NaB (0.3 M, 12 ml/kg, i.p.) or iso-osmotic saline (0.3 M NaCl, 12 ml/kg, i.p.). Rats were sacrificed 50 min after NaB or iso-osmotic saline injections. Their brains were processed for immunohistochemistry to measure the levels of c-Fos expression and phospho-acetylated histone H3 as described above.

4.8. *Experiment 4. Effect of NaB on CTA learning*

Eight days prior to the CTA conditioning day, the rats were placed on a water deprivation schedule under which they received daily water access in one drinking session. The initial session was 3 h in length and the session times were diminished each day so that the day before conditioning the rats received their water in a 10-min session. On the conditioning day, rats were divided into four different groups (NaCl/NaCl, NaCl/NaB, LiCl/NaCl and LiCl/NaB; n=6 per group) and given a 10-min access to a bottle containing 0.125% saccharin solution. Twenty minutes after the end of saccharin intake, LiCl/NaCl and LiCl/NaB groups were injected with LiCl (0.15 M, 3 ml/kg, i.p.) and NaCl/NaCl and NaCl/NaB groups were injected with saline (0.15 M NaCl, 3 ml/kg, i.p.). To prevent acquisition of a maximal CTA, LiCl treated rats received a lower dose of LiCl than was used in Experiments 1 and 3. Ten minutes after LiCl or saline injections, LiCl/NaB and NaCl/NaB groups were injected with NaB (0.3 M, 12 ml/kg, i.p.) and LiCl/NaCl and NaCl/NaCl groups were injected with iso-osmotic saline (0.3 M NaCl, 12 ml/kg, i.p.). Three hours after conditioning, rats received ad libitum access to water overnight. The next day, CTA acquisition and extinction was measured with 48-h, 2-bottle preference tests. Rats were given 24-h free access to both saccharin and water bottles. Bottles were placed side by side, and the placement of the bottles was alternated each day to observe possible position bias. Consumption of each solution was measured by weighing the bottles daily for 6 days. The preference score was calculated for each rat for each 48-h period by dividing the saccharin consumed by the total fluid consumed (saccharin/(water+saccharin)). A score of 1.0 indicates that all fluid intake was saccharin. A low preference score indicates intake largely of water, and thus an aversion to saccharin. Rats were considered extinguished if their preference score was 0.9 or higher.

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