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Area postrema lesions attenuate LiCl-induced c-Fos expression correlated with conditioned taste aversion learning

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ABSTRACT

Lesions of the area postrema (AP) block many of the behavioral and physiological effects of lithium chloride (LiCl) in rats, including formation of conditioned taste aversions (CTAs). Systemic administration of LiCl induces c-Fos immunoreactivity in several brain regions, including the AP, nucleus of the solitary tract (NTS), lateral parabrachial nucleus (latPBN), supraoptic nucleus (SON), paraventricular nucleus (PVN), and central nucleus of the amygdala (CeA). To determine which of these brain regions may be activated in parallel with the acquisition of LiCl-induced CTAs, we disrupted CTA learning in rats by ablating the AP and then quantified c-Fos-positive cells in these brain regions in sham- and AP-lesioned rats 1 h following LiCl or saline injection. Significant c-Fos induction after LiCl was observed in the CeA and SON of AP-lesioned rats, demonstrating activation independent of an intact AP. LiCl-induced c-Fos was significantly attenuated in the NTS, latPBN, PVN and CeA of AP-lesioned rats, suggesting that these regions are dependent on AP activation. Almost all of the lesioned rats showed some damage to the subpostremal NTS, and some rats also had damage to the dorsal motor nucleus of the vagus; this collateral damage in the brainstem may have contributed to the deficits in c-Fos response. Because c-Fos induction in several regions was correlated with magnitude of CTA acquisition, these regions are implicated in the central mediation of lithium effects during CTA learning.

1. Introduction

Conditioned taste aversion (CTA) is a robust form of associative learning with unique single-trial and long-delay characteristics. A single pairing of a novel taste with a toxic substance such as lithium chloride (LiCl) produces a robust and persistent avoidance of substances containing that taste [1]. Furthermore, CTAs can be formed with a delay of minutes to several hours between the taste and the toxin [2–4]. These unique features make CTA learning a useful experimental model for studying learning and memory at the neuroanatomical level.

c-Fos immunohistochemistry can be used to identify sites of neuronal activation involved in the processing of both taste and toxic stimuli. LiCl is widely used as a toxin to produce CTA in rats, and several investigators have shown that systemic administration of LiCl increases c-Fos protein or mRNA in many brain regions, including the nucleus of the solitary tract (NTS), area postrema (AP), lateral parabrachial nucleus (latPBN), central nucleus of the amygdala (CeA), and the supraoptic nucleus (SON) and paraventricular nucleus (PVN) of the hypothalamus [5–11]. Several of these regions also receive input from gustatory pathways, so that they may be important for the integration and association of taste and toxic information during acquisition of a CTA.

Lesion studies have identified some brain regions that are sensitive to LiCl and mediate CTA learning. The acute, central effects of LiCl are thought to require the detection of lithium by the AP, a highly vascularized circumventricular organ in the brainstem. AP neurons are activated by blood-borne toxins, and are sensitive to LiCl [12]. Several studies have demonstrated that lesions of the area postrema block the acquisition of LiCl-induced CTA in rats [13–20] and monkeys [21]. This loss of function is not due to deficits in taste perception or CTA learning because rats with AP lesions are able to express CTAs acquired prior to AP ablation [22], and AP-lesioned rats can form CTAs produced by other drugs such as amphetamine [13,23] or apomorphine [24] or by motion sickness [18,21]. These studies show that the AP is necessary for the central detection of LiCl leading to the acquisition of LiCl-induced CTA. Other behavioral and physiological responses to systemic LiCl such as lying-on-belly, delayed gastric emptying and hypothermia are also blocked by AP ablation [16]. However, not all responses to LiCl are lost in AP-lesioned rats [17,19,25].

The study of c-Fos expression after LiCl administration in AP-lesioned rats would be useful in defining brain regions that may be important for the acquisition of LiCl-induced CTAs. We hypothesized that brain regions involved in the central processing of CTA learning

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would fulfill two complementary criteria: 1) the site should be activated by a stimulus that induces CTA learning (e.g. LiCl), and 2) the site should not be activated under conditions when CTA learning fails (e.g. following AP ablation). Furthermore, because AP lesions do not block all the behavioral and physiological effects of LiCl, we also expected that AP lesions would not attenuate LiCl-induced c-Fos in all brain regions. Thus, in the present study we ablated the AP in rats and verified functional disruption of LiCl-induced CTA learning. We then quantified c-Fos by immunohistochemistry in several brain regions in sham- and AP-lesioned rats 1 h following LiCl or saline injection. We examined the NTS and latPBN which receive direct projections from the AP, and which are critical for CTA learning. We examined the SON and PVN, which mediate the neuroendocrine responses to systemic LiCl (e.g. oxytocin, vasopressin, or CRH release). We also examined the CeA, which receives brainstem projections and has been implicated in CTA learning. Our results demonstrate that LiCl-induced c-Fos expression is attenuated in the NTS, latPBN, PVN and CeA, but not the SON, of AP-lesioned rats, implicating these brain regions in central mechanisms of CTA learning and other effects of LiCl.

2. Materials and methods

2.1. Animals

Forty-two adult male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were housed individually under a 12-h light, 12-h dark cycle at 25 °C. At the start of the experiment the rats weighed between 250 and 300 g. Purina rodent chow and water were provided ad libitum, except as noted below. All experiments were approved by the Institutional Animal Care and Use Committee of Florida State University.

2.2. Lesions of the area postrema

Twenty-six rats received AP lesions and 16 rats received sham lesions. Rats were anesthetized with a cocktail of magnesium sulfate (75 mg/kg), chloral hydrate (153 mg/kg) and pentobarbital (35 mg/kg) and placed in a stereotaxic apparatus with the head in a ventroflexed position. A midline incision was made on the back of the neck and the neck muscles were retracted. With the aid of a dissecting microscope, the atlanto-occipital membrane was punctured and removed to expose the dorsal surface of the medulla. To permit clear visualization of the AP, a small portion of the base of the skull was removed with rongeurs to enlarge the foramen magnum. The membrane covering the AP was carefully removed and a thermal lesion of the AP was made by briefly touching the structure with the tip of a small cautery. Sham-lesioned rats were treated in an identical fashion, but the AP was not touched and left intact. Immediately following the lesion, rats invariably stopped breathing and some required resuscitation by manual compression of the rib cage. Rats were given antibiotics (16 mg sulfamethoxazole/3.2 mg trimethoprim s.c.) and were maintained on sweetened condensed milk (diluted 1:2 with water) with vitamin supplements for 1 week after surgery. Seven lesioned rats did not survive the post-operative period.

2.3. CTA acquisition and expression

Once the rats had surpassed their pre-operative weights and were steadily gaining weight (2–3 weeks following surgery), they were placed on an 18-h water-deprivation schedule. After 3 days, all rats ($n=19$ lesioned rats, $n=16$ sham rats) were given access for 30 min to 5% sucrose and then injected with LiCl (i.p., 0.15 M, 12 ml/kg). Sucrose intake was measured by weighing sucrose bottles before and after the 30 min access. Water was returned for an additional 5.5 h and then rats continued on an 18-h water-deprivation schedule. Forty-eight hours after the pairing of sucrose and LiCl, all rats were again given access to 5% sucrose for 30 min in a 1-bottle test of CTA expression. For each rat, CTA magnitude was calculated from the sucrose intake 48 h after LiCl injection as a percentage of the rat's sucrose intake prior to LiCl injection (% suppression). A positive % suppression indicates a decrease in sucrose intake. The water deprivation schedule was discontinued following behavioral testing. Based on CTA magnitude, lesioned rats were subsequently divided into two groups: an APX group ($n=13$) that failed to acquire a CTA, and a "failed-APX" group ($n=6$) that showed significant CTA expression comparable to sham rats despite their surgical lesion (see Results section).

2.4. Tissue collection and immunohistochemistry

Approximately 1 week after behavioral testing, rats were given an injection of either LiCl or NaCl (i.p., 0.15 M, 12 ml/kg). Three groups of rats received LiCl injections: sham-lesioned rats ($n=8$), APX rats ($n=6$), and failed-APX rats ($n=6$). Two groups of rats received NaCl injections: APX rats ($n=7$) and sham-lesioned rats ($n=8$). Rats were overdosed 1 h later with sodium pentobarbital. When completely unresponsive, the rats were perfused transcardially, first with 100 ml of isotonic saline/0.5% sodium nitrite/1000 U heparin, and then with 400 ml phosphate-buffered 4% paraformaldehyde. The brains were removed, blocked, post-fixed for 2 h and then transferred to 0.1 M phosphate buffer (PB) for storage at 4 °C. Individual blocks of hindbrain and forebrain tissue were transferred into 30% sucrose 24 h to 1 week prior to sectioning. Rats were perfused in 2 cohorts, each containing half the rats from each treatment group. Hindbrain and forebrain tissue from each cohort were processed separately within 1–2 weeks after perfusion. Tissue from all treatment groups was processed in parallel.

Forty micron coronal sections were cut on a freezing, sliding microtome. Alternate sections were processed from the medulla at the level of the NTS (bregma −12.8 mm to −14.3 mm) and the pons at the level of the PBN (bregma −9.16 mm to −10.3 mm). Every fourth section was processed from the forebrain through the hypothalamus and amygdala (bregma −0.8 mm to −3.6 mm). Coordinates were based on Paxinos and Watson's atlas [26]. Sections were immediately processed after cutting for c-Fos immunohistochemistry.

Free-floating tissue sections were washed twice for 15 min in 0.1 M phosphate-buffered saline (PBS) and then incubated for 30 min in 0.2% Triton X-100/1% bovine serum albumin (BSA)/PBS. After two washes in PBS/BSA for 15 min each, sections were incubated overnight with a rabbit anti-c-Fos antiserum (Ab-5, Oncogene Research) at a dilution of 1:20,000. After two 15-min washes in PBS/BSA, sections were then incubated for 1 h with a biotinylated goat anti-rabbit antibody (Vector Laboratories) at a dilution of 1:200. 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 (DAB). Sections were stored in 0.1 M PB until mounted onto gelatin-coated glass slides and coverslipped using Permount. Alternate coronal sections through the caudal hindbrain were stained with cresyl violet to evaluate AP lesions.

Cells expressing darkly-positive, nuclear c-Fos immunoreactivity were quantified using a custom software program (MindsEye 1.19b, T. Houpt). Images were digitally captured in a 0.72 mm × 0.54 mm counting frame (0.87 mm × 0.65 mm for latPBN). For the NTS, latPBN and SON, counting was restricted to the area delineated by a hand-drawn outline. Outlining was not necessary for the PVN and CeA because these regions mostly filled the counting frame or had little c-Fos outside the area of interest. Bilateral cell counts were averaged for 3 sections of the PVN (approximately bregma −1.8 mm to −2.12 mm), 4 sections of the SON (bregma −1.3 mm to −1.8 mm), 6 sections of the CeA (bregma −2.3 mm to −3.14 mm),

8 sections of the latPBN, and 12 sections of the NTS for each rat. (c-Fos was not counted in the NTS of 2 failed-APX rats, due to the extent of their lesions.) The individual mean counts for each region were then averaged across rats within experimental groups.

2.5. Statistical analysis

Data are presented as the mean ± standard error of the mean. t-Test, one- and two-factor analysis of variance (ANOVA), Tukey's HSD post-hoc test, and correlation analyses were performed using commercially available software (StatView 4.5, Abacus Concepts, Berkeley, CA; BMDP-SOLO V.6.0, Chicago, IL). Two-way ANOVA (drug × surgery) was used to compare c-Fos counts in NaCl and LiCl-injected sham-lesioned and APX rats. One-way ANOVA was used to compare c-Fos counts in LiCl-injected sham-lesioned, APX, and failed-APX rats. Significant results of post-hoc analyses are presented in the figure legends.

3. Results

3.1. Confirmation of CTA deficit and AP lesion

Approximately 2–3 weeks after surgery, rats were screened for the ability to acquire a CTA to sucrose after a single pairing with LiCl. The range of CTA magnitude expressed as % suppression is shown for all rats in Fig. 1A. An independent t-test of sham-lesioned rats vs. all lesioned rats indicated a significant effect of surgery ($t(33) = 3.1$, $p < 0.001$). Sham-lesioned rats showed a % suppression from pre-conditioning sucrose intake ranging from 20% to 75% (mean = 51 ± 4%; median = 55%). All of the responses observed in the sham group (n = 16) fell within two standard deviations of the mean for that group and were indicative of the acquisition of a mild to strong CTA. The relatively low suppression of some sham-lesioned rats may reflect the use of a single-bottle expression test.

CTA acquisition of the AP-lesioned rats was variable, with a range of suppression from 69% to −129% (i.e., an increase in intake). Lesioned rats expressing a CTA with % suppression more than two standard deviations below the mean suppression of the sham group (i.e. falling below the dotted line in Fig. 1A) were designated as having behaviorally-confirmed lesions ("APX rats", n = 13, mean suppression = −39 ± 13%). Those rats with % suppression falling within two standard deviations of the mean of the sham group (i.e. falling above the dotted line in Fig. 1A) were considered to have functionally incomplete lesions and were assigned to the "failed-APX" group (n = 6; mean suppression = 47 ± 8%).

The mean sucrose intakes for the 3 groups before and after pairing with LiCl injection are shown in Fig. 1B. The mean sucrose intakes for the 3 groups before and after pairing with LiCl injection are shown in Fig. 1B. There was no significant difference in sucrose intake between the groups prior to LiCl pairing. After conditioning, however, sucrose intake was significantly different ($(F2,32) = 23.5$, $p < 0.0001$) such that sham rats and failed-APX rats significantly reduced their sucrose intake compared to APX rats.

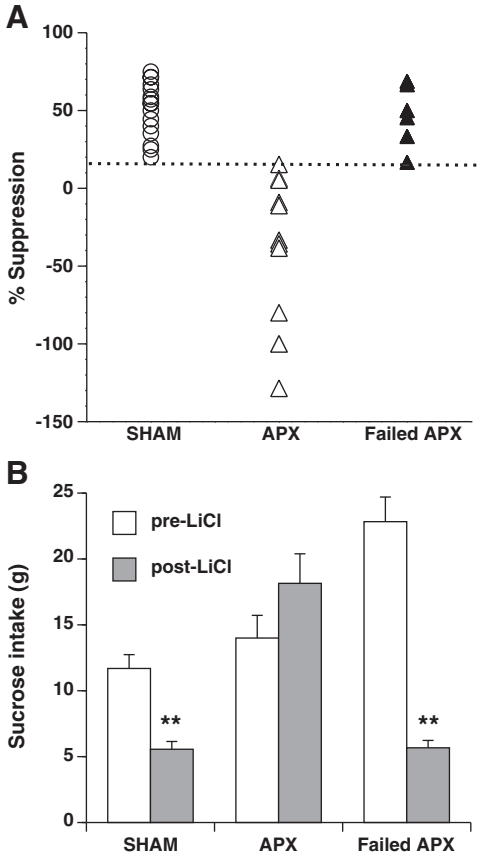

Fig. 1. Behavioral test of CTA acquisition. A. Percent suppression, expressing the difference between sucrose intake on conditioning day and sucrose intake on the test day, for individual sham-lesioned rats (circles, n = 16) and lesioned rats (triangles, n = 19). Lesioned rats falling within 2 standard deviations of the mean sham value (i.e. black triangles above the dotted line, n = 6) were designated "failed-APX" rats. Lesioned rats below the line were designated APX rats (white triangles, n = 13). B. Average sucrose intakes before (pre-LiCl) and after (post-LiCl) pairing with LiCl for sham-lesioned rats, APX rats that showed impaired CTA learning, and failed-APX rats that showed intact CTA learning. ** $p < 0.005$ compared to pre-LiCl intake.

3.2. c-Fos immunohistochemistry

c-Fos induction was quantified in the brainstem and forebrain of sham-lesioned rats and APX rats after either NaCl or LiCl injection, and in failed-APX rats after LiCl injection.

3.3. c-Fos in the NTS and latPBN

c-Fos expression in the NTS and latPBN is shown in Figs. 2 and 3. In both the NTS and latPBN there was a significant interaction between surgery and drug ($F(1,25) = 8.3$ and 12.0, respectively, $p < 0.005$). In both regions, LiCl induced significantly more c-Fos compared to NaCl controls in sham-lesioned rats but not in APX rats. Failed-APX rats showed less c-Fos after LiCl-injection than sham-lesioned rats in the NTS ($F(2,17) = 5.62$, $p < 0.05$), but were not different from either sham-lesioned or APX rats in the latPBN.

3.4. c-Fos in the hypothalamus and amygdala

LiCl treatment significantly induced c-Fos in the SON of both sham and APX rats compared to NaCl-treated rats (Figs. 4 and 5A), such that there was a main effect of drug ($F(1,25) = 31.7$, $p < 0.0001$), but no effect of surgery and no interaction. There was no significant difference in c-Fos in the SON of failed-APX vs. APX or sham LiCl-injected rats.

In contrast to the SON, in the PVN there was a significant interaction between surgery and drug ($F(1,25) = 6.0$, $p < 0.05$) (Figs. 4 and 5B). LiCl significantly increased c-Fos in sham-lesioned rats, but there was no increase in the number of c-Fos-positive cells in the PVN of APX-LiCl rats compared to sham-NaCl or APX-NaCl rats. There was no significant difference in c-Fos in the PVN of failed-APX vs. either APX or sham LiCl-injected rats.

In the CeA (Figs. 6 and 7), there was a significant interaction between surgery and drug ($F(1,25) = 9.7$, $p < 0.005$). Acute LiCl administration induced significantly greater numbers of c-Fos-positive cells in both sham-LiCl and APX-LiCl rats compared to sham-NaCl and APX-NaCl rats. However, the number of c-Fos-positive cells was significantly lower in APX-LiCl rats than in sham-LiCl rats. Failed-APX

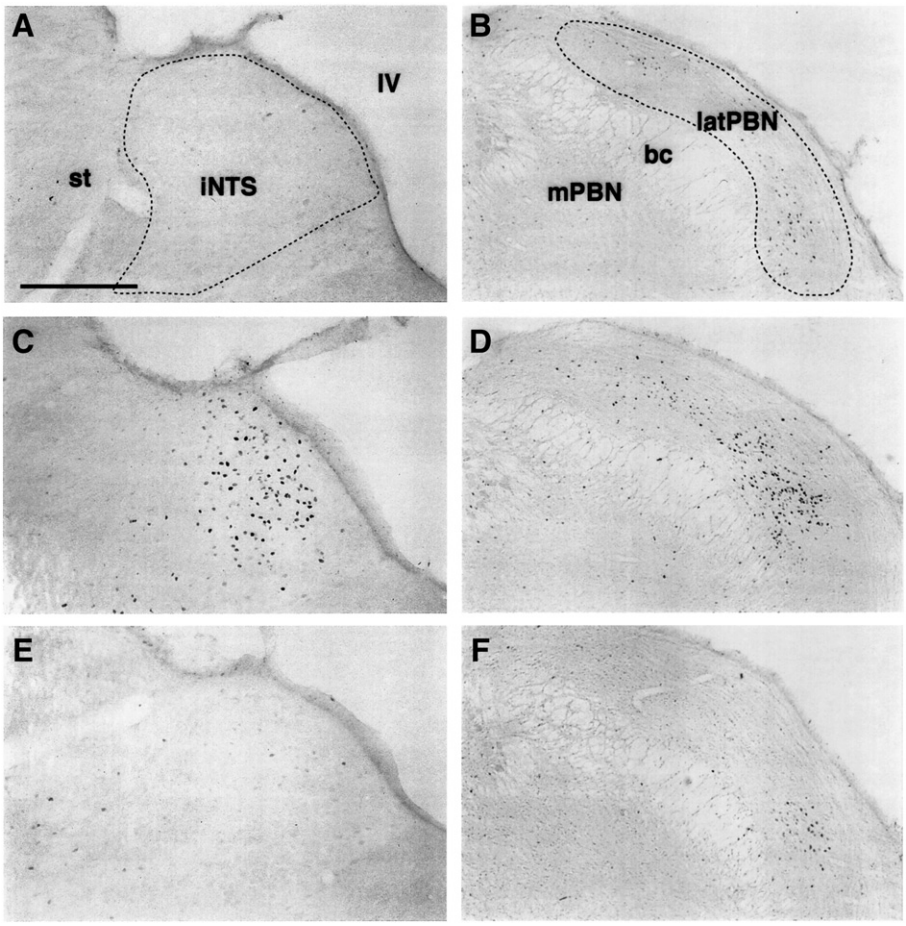

Fig. 2. Representative photomicrographs of c-Fos immunoreactivity in the intermediate NTS (iNTS; A, C, E) and lateral PBN (latPBN; B, D, F) in sham-NaCl (A, B), sham-LiCl (C, D), and APX-LiCl (E, F) rats 1 h following NaCl or LiCl injection. Photomicrographs of sham-NaCl rats are also representative of APX-NaCl rats (not shown). Dashed lines indicate representative region for c-Fos quantification. Abbreviations: IV, fourth ventricle; st, solitary tract; mPBN, medial PBN; bc, brachium conjunctivum. Scale bar in (A), 500 μm.

rats showed more c-Fos than APX rats in the CeA after LiCl injection ($F(2,17) = 8.83$, $p < 0.005$).

3.5. Correlations of CTA acquisition and c-Fos expression

LiCl induced variable levels of intake suppression in the CTA test and also induced variable numbers of c-Fos-positive cells in each brain region in individual rats of the sham-LiCl, APX-LiCl, and failed-APX group. Therefore, the number of c-Fos-positive cells in each region was correlated with the percentage suppression for each individual rat ($n = 20$; Fig. 8). There were significant correlations in the NTS ($r = 0.510$, $p < 0.05$), latPBN ($r = 0.539$, $p < 0.05$), SON ($r = 0.455$, $p < 0.05$), and CeA ($r = 0.709$, $p < 0.001$). There was no significant correlation between LiCl-induced c-Fos and CTA expression in the PVN ($r = 0.341$, $p > 0.10$).

3.6. Histological evaluation of AP lesion

Representative photomicrographs of cresyl violet-stained sections at the level of the AP are shown in Fig. 9. There was no apparent correlation between the extent of the AP lesion and performance in a taste aversion test. While all of the APX rats showed extensive AP damage, 4 subjects had incomplete lesions (one example in Fig. 9B).

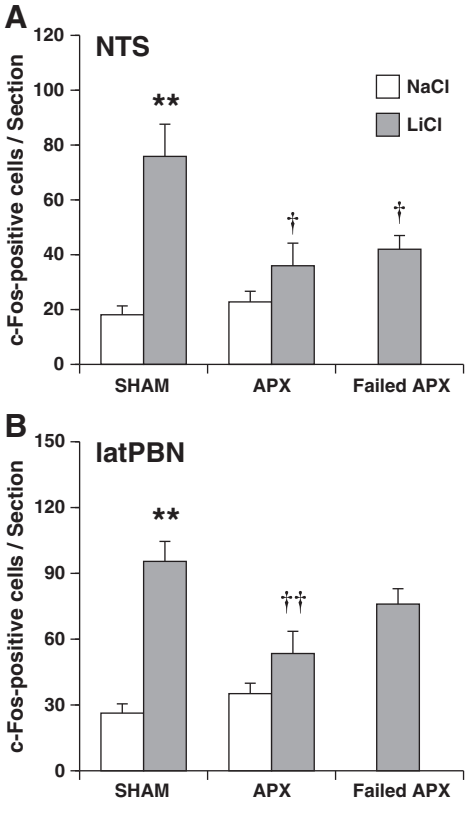

Fig. 3. Quantification of c-Fos induced in the NTS (A) and lateral PBN (B) of AP-lesioned (APX) and sham-lesioned (SHAM) rats 1 h following NaCl or LiCl injection, and for failed-APX rats after LiCl injection. $^{**}p < 0.01$ compared to sham-NaCl rats, $^{††}p < 0.01$ compared to sham-LiCl rats.

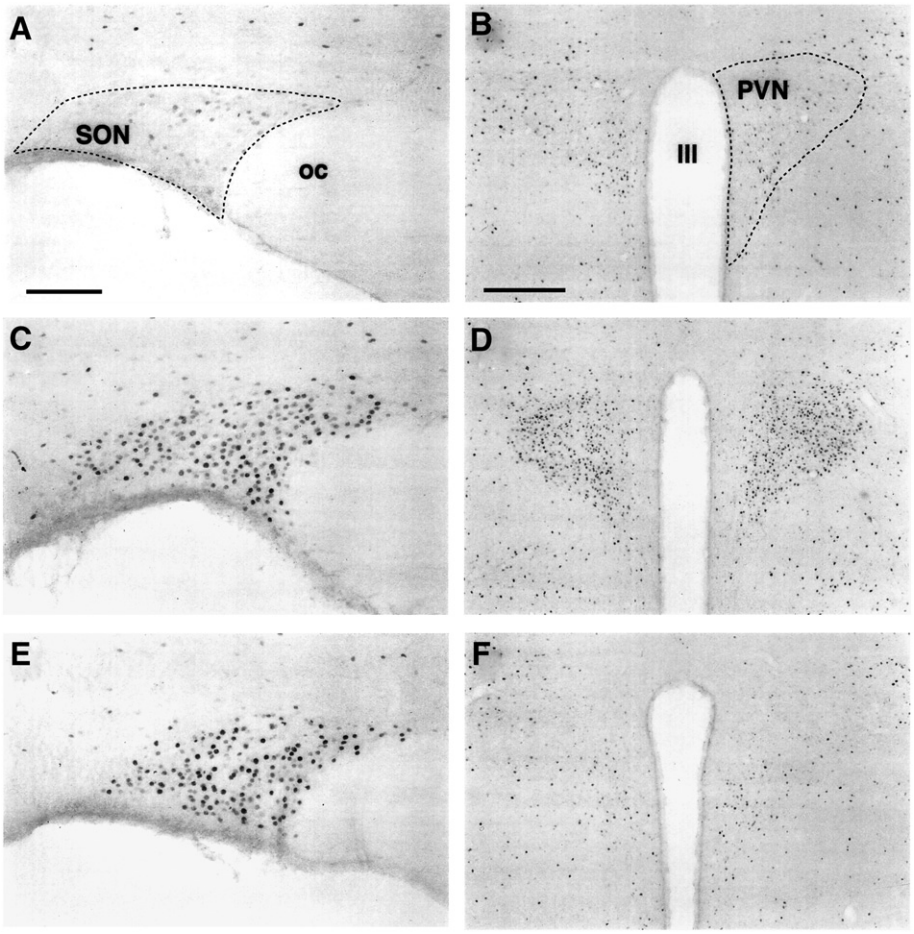

Fig. 4. Representative photomicrographs of c-Fos immunohistochemistry in the supraoptic (SON; A, C, E) and paraventricular (PVN; B, D, F) nuclei in sham-NaCl (A, B), sham-LiCl (C, D), and APX-LiCl (E, F) rats 1 h following NaCl or LiCl injection. Photomicrographs of sham-NaCl rats are also representative of APX-NaCl rats (not shown). Dashed lines indicate representative region for c-Fos quantification. Abbreviations: oc, optic chiasm; III, third ventricle. Scale bar in (A), 100 μm; scale bar in (B), 400 μm.

Furthermore, all of the failed-APX rats had some damage to the AP, and 3 of the 6 failed-APX rats appeared to have complete lesions of the AP (Fig. 9C). Although one failed-APX rat with a complete lesion fell just within our stringent exclusion criterion of 2 standard deviations of the mean suppression score of sham rats, the other two failed-APX rats with complete lesions formed strong taste aversions (45% and 67% suppression scores). The difference between complete lesions in the APX groups that were effective in disrupting CTA learning and complete yet ineffective lesions in failed-APX rats was not apparent. Virtually all of the lesioned rats in this study showed evidence of damage to the underlying NTS, and many rats (11 of 19) also showed damage to the dorsal motor nucleus of the vagus (Fig. 9D).

4. Discussion

In summary, lesions of the AP that blocked single-trial, long-delay CTA learning 1) abolished LiCl-induced c-Fos expression in the latPBN (confirming Yamamoto et al. [5]) and in the PVN; 2) significantly attenuated LiCl-induced c-Fos in the CeA and the NTS; and 3) had no effect on LiCl-induced c-Fos in the SON. Graded responses in c-Fos proportional to CTA acquisition were observed in the NTS, latPBN, CeA, and SON, but not in the PVN. The results of this study support two conclusions. First, the use of c-Fos as a marker of neuronal activation shows that some brain sites are still responsive to LiCl in APX rats despite the failure of these rats to acquire a LiCl-mediated CTA. Second, the attenuation of c-Fos expression with the deficit in CTA acquisition implicates specific areas (NTS, latPBN, PVN and CeA) in the central mediation of CTA learning.

4.1. Behavioral confirmation of AP lesion

Because we were interested in identifying brain regions required for CTA learning, we used AP ablation to disrupt CTA learning and then examined changes in c-Fos expression patterns following LiCl administration. Thus APX subjects were selected after verifying lesions with a behavioral test of AP function, i.e. by the inability to learn a sucrose-LiCl CTA, rather than by histological characteristics. There was considerable variability in the extent of the lesions generated in this study due to unavoidable damage to brainstem regions ventral to the AP. Despite this variability, we found that lesion size was not a reliable predictor of behavioral CTA expression. Although loss of LiCl-induced CTA learning was related to partial damage to the AP, histologically-complete AP lesions were not necessary to disrupt CTA learning (Fig. 9B) and, furthermore, were not always sufficient to disrupt CTA learning (Fig. 9D). Similar observations were reported by Rabin, Hunt, and Lee [14]. Our observations are also consistent with the results of Wang and Edwards (1997) which showed no difference in CTA expression between APX rats with small and large dorsal brainstem lesions [27]. Thus, behavioral CTA expression may be a more sensitive test of the functional completeness of AP lesion than histological evaluation.

4.2. AP-dependent induction of c-Fos

Lesions of the AP have been shown to block many of the acute behavioral and physiological responses to LiCl, such as hypothermia, delayed gastric emptying and lying-on-belly [16], decreased

locomotor activity [15,19], anorexia induced by chronic infusion of LiCl [28], vomiting in squirrel monkeys [21], and acquisition of LiCl-induced CTAs [13–21]. Conversely, AP lesions do not block LiCl-stimulated oxytocin or vasopressin release [19,25], anorexia induced by acute LiCl injection [19] or decreased heart rate response to LiCl [17]. Thus, AP lesions block some, but not all, of the behavioral and physiological effects of LiCl. Therefore, we predicted that AP lesions would block some, but not all, of the c-Fos responses to LiCl. In fact, we found in APX rats that c-Fos induction by LiCl was blocked in the NTS, PBN, PVN (and attenuated in the CeA), while the SON and CeA still showed a significant response to LiCl.

There was no significant difference in the number of c-Fos-positive cells in the NTS of APX-NaCl and APX-LiCl rats. While these data suggest that AP ablation blocked the c-Fos induction by LiCl seen in the sham rats, it is important to keep in mind that this effect could be the result of direct damage to the NTS rather than the loss of input from the AP. Furthermore, because vagal afferents contribute to LiCl-induced c-Fos in the brainstem [5], collateral NTS damage may contribute to the deficits of AP-lesioned rats. Variability in the lesion size complicated interpretation of the NTS data since it may not be appropriate to directly compare sham and APX groups. However, the amount of damage to the NTS was equivalent between APX-LiCl and APX-NaCl groups, allowing a reasonable comparison of data within surgical groups. More discrete lesions might not circumvent this problem because damage to the dendrites of neurons in the NTS that extend into the AP may result in retrograde cell death of NTS neurons.

APX lesion also blocked the LiCl-induced c-Fos expression in the latPBN. This is consistent with an earlier report that examined only the PBN in AP-lesioned rats after systemic LiCl [5]. Likewise, c-Fos induction was significantly reduced in the CeA (although still greater than vehicle controls), and blocked in the PVN. The reduced activation in the NTS, latPBN, PVN and CeA of APX rats is consistent with downstream activation by the AP of first-order (NTS, latPBN) and second-order (PVN, CeA) projection sites in intact rats. This is consistent with studies in APX rats stimulated with other agents which are thought to stimulate the AP, such as amylin [29], exendin-4 [30], or PYY [31].

Because all rats in this study underwent CTA acquisition, there is the additional possibility that prior exposure to LiCl or acquisition of a LiCl-induced CTA might alter the sensitivity of various brain regions to subsequent LiCl exposures. Although there are no reports of changes in c-Fos expression after repeated LiCl injections or CTA acquisition, there have been reported changes in c-Fos responses to taste stimuli after CTA [7,8] or to LiCl after prior taste exposures [32]. Because the AP-lesioned rats failed to acquire a CTA, it is therefore possible that the deficit in LiCl-induced c-Fos represents a deficit in CTA-induced sensitization, rather than a deficit in chemosensitive transduction by the AP.

4.3. AP-independent activation

Both the SON and CeA showed significant induction of c-Fos in APX rats after LiCl. In particular, the number of c-Fos-positive cells induced by LiCl in the SON was unaffected by AP ablation. It has been shown that the SON responds normally after AP ablation to other stimuli as well, both physiologically and at the level of c-Fos induction. For example, hypertonic saline stimulates release of oxytocin and vasopressin from the SON [33,34] and induces c-Fos expression in many of the same brain regions as LiCl, including the AP, NTS, latPBN, SON, and PVN [35–37]. Following AP ablation, the c-Fos response to hypertonic saline was abolished in the PVN but was unaltered in the SON [38]. This evidence, in addition to functional evidence showing that AP ablation does not affect LiCl-stimulated oxytocin and vasopressin release [25], implicates the SON as a brain region that is sensitive to LiCl by an AP-independent pathway.

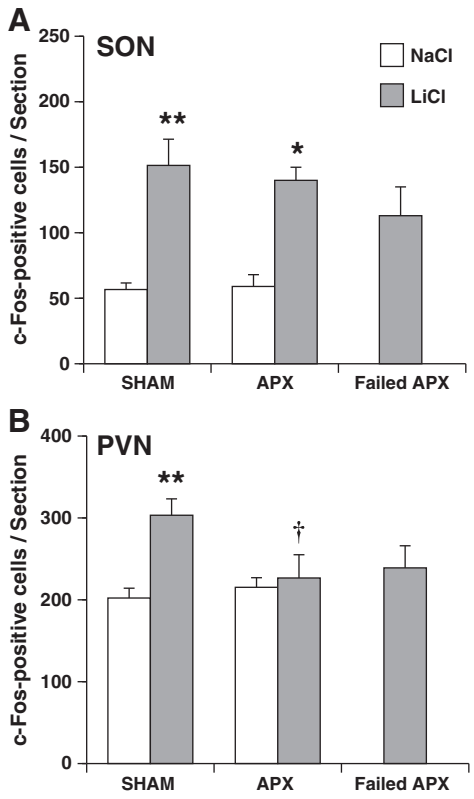

Fig. 5. Quantification of c-Fos induced in the SON (A) and PVN (B) of AP-lesioned (APX) and sham-lesioned (SHAM) rats 1 h following NaCl or LiCl injection, and for "failed-APX" rats after LiCl injection. $^{*}p<0.05$ compared to APX-NaCl rats, $^{**}p<0.01$ compared to sham-NaCl rats, $^{\dagger}p<0.05$ compared to sham-LiCl rats.

It is possible that the SON and CeA can be activated by LiCl through vagal or spinal afferents [5,39] or by secondary endocrine responses. The NTS, for example, is not only reciprocally connected to the AP, but also receives visceral afferent input directly from vagal fibers and indirectly from spinal afferents [24,40,41]. Subdiaphragmatic vagotomy attenuates c-Fos induction by systemic LiCl in the NTS, AP, and PBN [5]. In addition to its major projection to the PBN, the NTS also projects to the SON, PVN and CeA [42–44]. The PBN in turn sends projections to the CeA, NTS, PVN, and SON [43,45,46]. The amygdala and hypothalamus send projections back to the PBN and NTS [47,48]. Thus there are extensive and reciprocal connections among several of the regions examined in this report which could permit activation by LiCl in an AP-independent manner. The results from our failed-APX rats also indicate that AP-independent pathways can mediate both LiCl-induced CTA and c-Fos after acute LiCl.

Direct effects of LiCl on the brain also cannot be ruled out. Lithium ions are physiologically similar to sodium ions and are able to penetrate the blood–brain barrier [49]. Because all neurons rely on sodium for signaling, all neurons are potentially sensitive to the consequences of lithium substituting for sodium.

4.4. Correlation of c-Fos expression and CTA acquisition

In intact rats, LiCl has been shown to induce c-Fos expression in many brain areas, including the AP, NTS, latPBN, SON, PVN and CeA [5–11]. As described above, these brain regions are well-connected to each other, and this connectivity makes it challenging to assess the dependency of central pathways activated by LiCl and involved in CTA learning. When combined with lesions of nodes within the central network, c-Fos data can reveal necessary and sufficient patterns of activation. For example, the induction of c-Fos by LiCl in the SON of APX rats suggests that activation of the SON is not sufficient

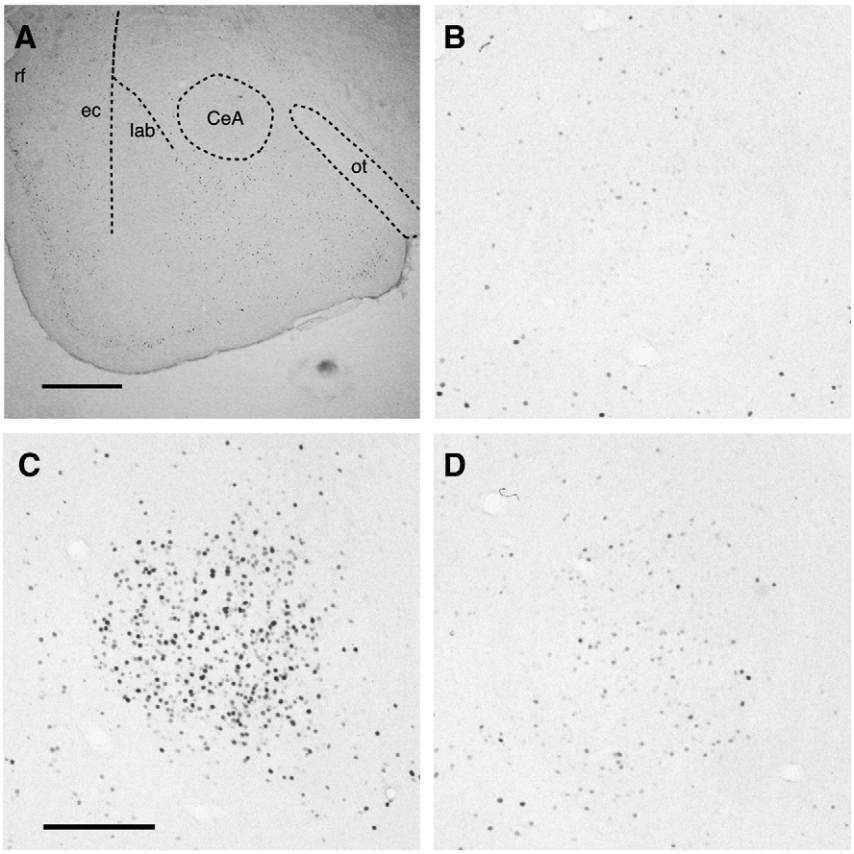

Fig. 6. Representative photomicrographs of c-Fos immunohistochemistry in the central nucleus of the amygdala (CeA) in sham-NaCl (A, B), sham-LiCl (C), and APX-LiCl (D) rats 1 h following NaCl or LiCl injection. The photomicrograph of the sham-NaCl rats is also representative of APX-NaCl rats (not shown). Abbreviations: rf, rhinal fissure; ec, external capsule; lab, longitudinal association bundle; ot, optic tract. Scale bar in (A), 1 mm; scale bar in (C), 500 μm.

for the acquisition of a CTA. Conversely, those regions which displayed an attenuated c-Fos response to LiCl in APX rats may be important in the central mechanisms of CTA learning. Thus, our results suggest that the PVN, NTS, latPBN and CeA may be involved in mediating responses to the unconditioned stimulus during CTA acquisition. Consistent with this interpretation, the "failed-APX" rats which were capable of acquiring a LiCl-induced CTA showed levels of c-Fos intermediate to sham and APX levels in most regions.

The number of c-Fos-positive cells induced by LiCl in the CeA, latPBN, and SON showed significant correlations with the magnitude of behavioral CTA expression in both lesioned and intact rats. This suggests that the amount of cellular activation in these regions may reflect the relative potency of LiCl in lesioned rats, either as an acute toxin or as the US for CTA. (This interpretation must be tempered, of course, because of the inability to examine c-Fos in the same animal before and after AP ablation, or simultaneously with the behavioral CTA test.)

There is behavioral evidence showing that the dose of LiCl used to produce a CTA determines the strength of the subsequent aversion [1]. Sakai et al. have also shown a significant correlation of c-Fos induction and CTA acquisition in the AP, NTS, and latPBN of intact rats using 13 different unconditioned stimuli and a range of LiCl doses [50]. An increase in c-Fos-positive cell counts may reflect an increase in the number of cells activated or an increase in the c-Fos levels within each cell such that a greater number of cells reach the threshold of sensitivity for detection by immunohistochemistry. Our data suggest that the amount of c-Fos protein synthesized or the number of cells activated by LiCl in the CeA, latPBN and SON may be correlated with the strength of the taste–toxin association.

There is considerable evidence supporting the involvement of the NTS, latPBN and CeA in CTA learning. Lesions of the latPBN block CTA learning in rats [51], and the PBN has projections to both the central and basolateral nuclei of the amygdala [52]. The expression of c-Fos and other genes in the brainstem and CeA is also critical for CTA learning. Injection of c-Fos antisense oligonucleotides into the fourth ventricle both significantly reduced LiCl-induced c-Fos in the NTS and latPBN and blocked CTA acquisition in mice [53]. Similarly, microinjection into the CeA of protein synthesis inhibitors or c-Fos or CREB antisense oligonucleotides blocked long-term CTA memory [54,55].

Although CeA activation is induced by LiCl and correlated with CTA acquisition, lesion studies do not unambiguously support a necessary role for the CeA. CTA acquisition can be attenuated by large amygdalar lesions [56–61] or lesions of the fiber pathways passing

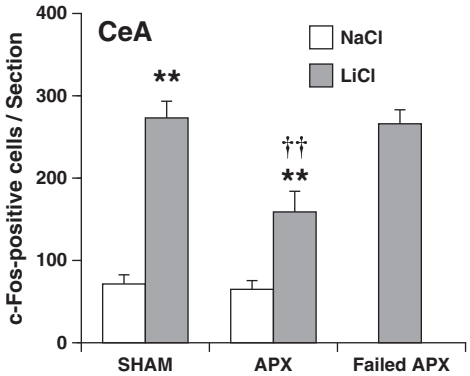

Fig. 7. Quantification of c-Fos induced in the CeA of AP-lesioned (APX) and sham-lesioned (SHAM) rats 1 h following NaCl or LiCl injection, and for "failed-APX" rats after LiCl injection. $^{**}p<0.01$ compared to NaCl-treated rats, $^{††}p<0.01$ compared to sham-LiCl.

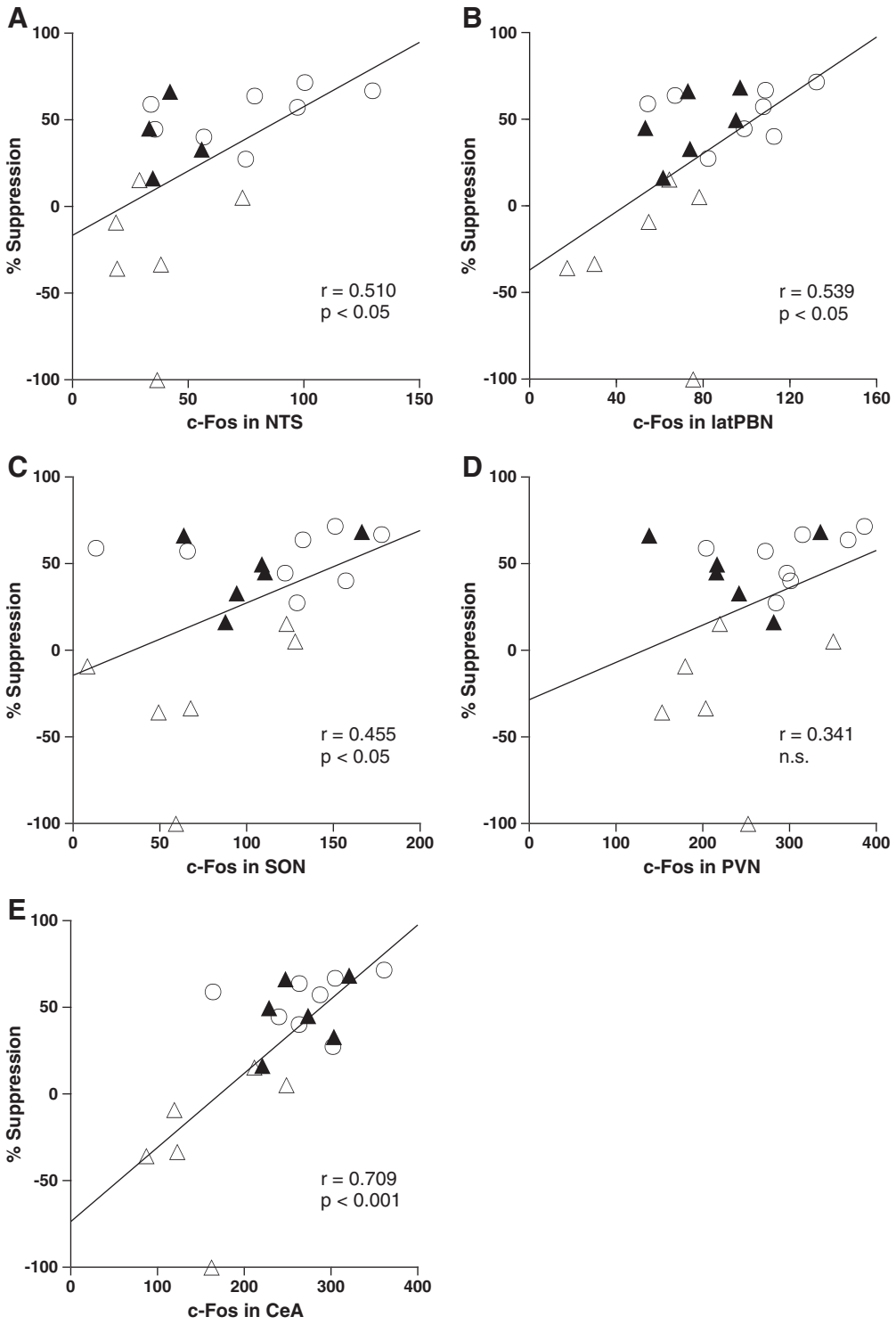

Fig. 8. Correlation plots of c-Fos 1 h following LiCl injection (number of c-Fos-positive cells/section) and CTA expression (% suppression) in the NTS (A), latPBN (B), SON (C), PVN (D), and CeA (E) of sham-lesioned rats (circles), AP-lesioned rats (white triangles), and "failed-APX" rats (black triangles).

through or connecting the amygdala with the gustatory cortex [56,62–64]. While lesions of the basolateral amygdala appear to reliably decrease the rate of CTA acquisition [57–59,65], lesions of the CeA do not always attenuate CTA acquisition [65–67]. However, the contribution of the CeA may be dependent on the method of CTA testing, such that CeA lesions block CTA tested with intraoral infusions but not with ad lib bottle intake [68].

In conclusion, c-Fos responses to LiCl were examined in several brain regions of rats with behaviorally-confirmed lesions of the AP. The SON and CeA were still partially responsive to LiCl in APX rats, suggesting that these regions are sensitive to LiCl by a pathway not dependent on the AP. LiCl-induced c-Fos expression in the NTS, latPBN, PVN and CeA was significantly attenuated in APX rats compared to sham-lesioned rats. Thus, these brain regions fulfill two complementary criteria for identifying a brain site with the neural processing of CTA learning. First, the site should be activated by a stimulus that can induce CTA acquisition. Because the NTS, latPBN, PVN and CeA express c-Fos after LiCl injection, they are activated by

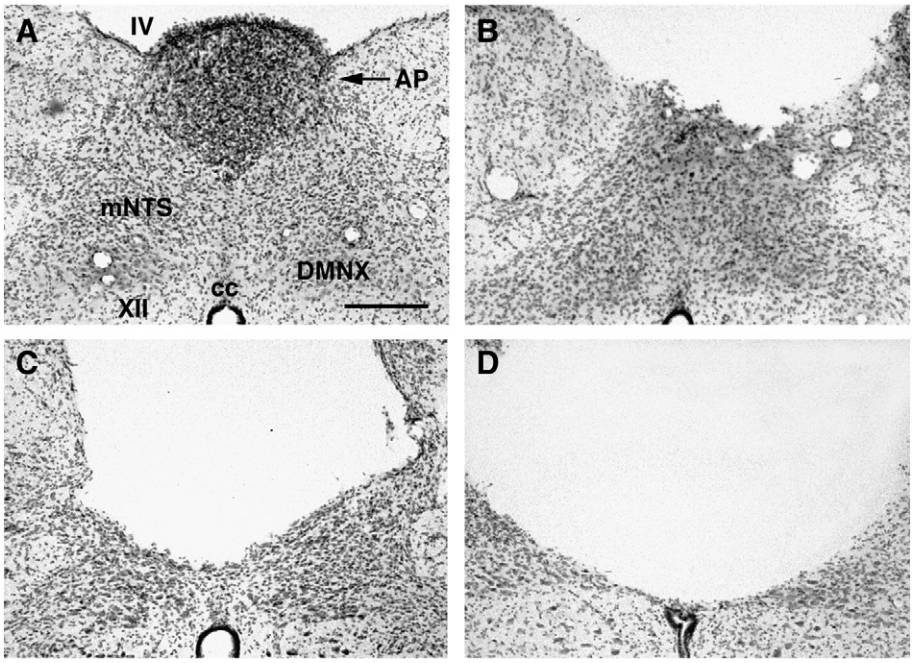

Fig. 9. Photomicrographs of brainstem sections at the level of the area postrema (AP). A. Sham-lesioned control rat showing an intact AP. B. Example of the smallest lesion that was effective at disrupting CTA learning. C. Example of a complete lesion from a rat that exhibited normal CTA learning. D. Example of the largest lesion that disrupted CTA acquisition. Note extensive damage to the medial NTS (mNTS) and dorsal motor nucleus of the vagus (DMNX). Abbreviations: IV, fourth ventricle; XII, hypoglossal nucleus; cc, central canal. Scale bar in (A), 500 μm.

the unconditioned stimulus. Second, under conditions when CTA acquisition fails to occur, critical brain sites should not be activated. Thus, c-Fos activation in the NTS, latPBN, PVN and CeA is significantly attenuated in APX rats that fail to learn single-trial, long-delay CTA after LiCl administration. Other sites do not meet both of these criteria: the SON, for example, meets the first criterion but not the second. Thus, research into the neuronal and molecular processes can now be focused on the cells whose activity is best correlated with the presence or absence of CTA learning.

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