# $N^{\omega}$-nitro-L-arginine methyl ester attenuates lithium-induced c-Fos, but not conditioned taste aversion, in rats

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## Abstract

Lithium chloride (LiCl) at doses sufficient to induce conditioned taste aversion (CTA) causes c-Fos expression in the relevant brain regions and activates the hypothalamic-pituitary-adrenal (HPA) axis. It has been suggested that nitric oxide (NO) in the central nervous system may play a role not only in the activation of HPA axis but also in CTA learning, and that LiCl may activate the brain NO system. To determine the role of NO in lithium-induced CTA, we examined the lithium-induced CTA, brain c-Fos expression, and plasma corticosterone level with $N^{\omega}$-nitro-L-arginine methyl ester (L-NAME) pretreatment. Intraperitoneal L-NAME (30 mg/kg) given 30 min prior to LiCl significantly decreased lithium-induced c-Fos expression in the brain regions implicated in CTA learning, such as the hypothalamic paraventricular nucleus (PVN), central nucleus of amygdala (CeA), and nucleus tractus of solitarius. However, either the lithium-induced CTA acquisition or the increase in plasma corticosterone was not attenuated by L-NAME pretreatment. These results suggest that NO may be involved in lithium-induced neuronal activation of the brain regions, but not in the CTA acquisition or the HPA axis activation.
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## 1. Introduction

Lithium chloride (LiCl) is conventionally used as an unconditioned stimulus in the formation of conditioned taste aversion (CTA), a form of classical conditionings. Intraperitoneal lithium chloride at doses sufficient to mediate CTA also induce c-Fos expression in the brain regions, such as the hypothalamic paraventricular nucleus (PVN), the nucleus tractus of solitarius (NTS), and the central nucleus of amygdala (CeA), and c-Fos expression in these brain regions is considered to correlate with CTA learning (Yamamoto et al., 1992; Houpt et al., 1994; Lamprecht and Dudai, 1995; Schafe et al., 1995; Schafe and Bernstein, 1996; Swank et al.,

1996; Sakai and Yamamoto, 1997). It has been reported that nitric oxide (NO) may take an important role in CTA learning (Rabin, 1996; Prendergast et al., 1997; Wegener et al., 2001) and that lithium chloride increases both the synthesis and activity of nitric oxide synthase (NOS) in the brain (Bagetta et al., 1993) and nitric oxide modulates lithium-induced CTA learning (Wegener et al., 2001). Indeed, large populations of neuronal nitric oxide synthase (nNOS) containing cells and fibers, identified by NADPH-diaphorase (NADPH-d) staining, are distributed in the brain regions implicated in CTA learning such as the PVN (Vincent and Kimura, 1992), parabrachial nucleus, NTS, and various subdivisions of the ventrolateral medulla (Vincent and Kimura, 1992; Dun et al., 1994; Krukoff and Khalili, 1997). However, the previous reports regarding to the role of nitric oxide in CTA learning have been inconsistent. It was reported that nitric oxide donor, sodium nitroprusside or *N*-tert-butyl-alpha-phenyl nitrone produces a CTA in rats,

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which is prevented by pretreatment with a NOS inhibitor, $N^\omega$-nitro-L-arginine (Rabin, 1996). On the other hand, nitric oxide precursor, L-arginine, was reported to counteract the aversion produced by lithium chloride, furthermore, NOS inhibitors, methylene blue, 7-nitroindazole, and $N^\omega$-nitro-L-arginine methyl ester (L-NAME) all produced a CTA (Prendergast et al., 1997; Wegener et al., 2001) Overall, it is likely that nitric oxide may be involved in lithium-induced CTA learning, however, its mechanism is still unclear. In order to find a molecular mechanism of the nitric oxide involvement in lithium-induced CTA learning, we examined if L-NAME pretreatment modulates lithium-induced CTA acquisition as well as c-Fos expression in the brain regions.

Hypothalamic-pituitary-adrenal (HPA) axis activation also plays an important role in CTA learning. Intraperitoneal injection of lithium chloride induces adrenocorticotrophic hormone (ACTH) release (Sugawara et al., 1988), activates HPA axis (Hennessy et al., 1976), and adrenalectomy impairs the acquisition of lithium-induced taste aversion in mice (Peeters and Broekkamp, 1994). Pretreatment with dexamethasone on the conditioning day attenuates CTA expression (Smotherman et al., 1976; Revusky and Martin, 1988), while injection of ACTH enhances and prolongs CTA expression (Hennessy et al., 1980). It was reported that mRNA level of nNOS in the hypothalamic paraventricular nucleus, the center of the HPA axis, is increased in lithium-treated rats (Anai et al., 2001). Previous studies with NOS blockers done by others suggest that nitric oxide participates in the regulation of corticotropin-releasing factor (CRF) and arginine vasopressin release from the hypothalamic neurons (Costa et al., 1993; Ota et al., 1993) as well as in the stress-induced release of ACTH and corticosterone (Rivier, 1994) and c-Fos expression in the hypothalamus (Amir et al., 1997). Nitric oxide has also been reported to stimulate transcription of CRF and its receptor in the hypothalamus of intact rats (Lee et al., 1999). Overall, it is much likely that nitric oxide may take a role in the formation of lithium-induced CTA, at least partly, through a modulation of the HPA axis activation. In order to determine if nitric oxide is involved in lithium-induced activation of the HPA axis, we examined the hypothalamic c-Fos expression as well as the plasma corticosterone level with L-NAME treatment prior to lithium.

## 2. Materials and methods

### 2.1. Animals

Male Sprague–Dawley rats (250–300 g) were supplied from the Division of Laboratory Animal Medicine, Yonsei University College of Medicine. Rats were cared in a specific pathogen free barrier area where the temperature ($22 \pm 1$ °C) and humidity (55%) were controlled constantly with a 12 h light–dark cycle (light between 07:00 and 19:00). The rats had access to standard laboratory food (Purina Rodent Chow, Purina Co., Seoul, Korea) and tap water (membrane filtered purified water) ad libitum. Animals were cared according to The Guide for animal experiments, 2000, edited by The Korean Academy of Medical Sciences, which is consistent with NIH Guideline for the Care and Use of Laboratory Animals, 1996 revised. Animal experiments were approved by the Committee for the Care and Use of Laboratory Animals at Yonsei University (Project License No. # 090).

### 2.2. Drugs

$N^\omega$-nitro-L-arginine methyl ester (Sigma Co., MO, USA) was dissolved in 0.9% physiological saline and administered intraperitoneally at a dose of 30 mg/ml/kg 30 min prior to different doses of intraperitoneal lithium chloride [0,19, 38, 76 mg/kg of LiCl (Sigma Co., MO, USA) for c-Fos immunohistochemistry, and 19 or 76 mg/kg for either the CTA test or plasma corticosterone assay; $n = 6$/dose]. In the control groups, the same volume of sterile physiologic saline was injected instead of L-NAME prior to each dose of lithium chloride ($n = 6$/dose).

### 2.3. Conditioning procedure

Rats had free access to chow pellets, but had only 5 h of access to water daily (12:00–17:00) as the only source of fluid during days 1–6 as training period. On day 7, the conditioning day, rats were allowed to drink 5% sucrose as the only source of fluid for 15 min, and then immediately after sucrose, they received an intraperitoneal injection of L-NAME (30 mg/kg) or sterile saline followed by isotonic LiCl (19 or 76 mg/kg) with 30 min of interval. Water was supplied immediately after the conditioning until 5:00 p.m. On days 9–14, after 1 day of recovery with 5 h of water supply, rats had access to 5% sucrose for 15 min daily at 12:00 p.m and water was offered right after sucrose until 5:00 p.m. The weight of sucrose solution consumed was recorded and used to quantify the CTA. To determine if L-NAME alone produces a CTA, an additional 12 rats received only L-NAME ($n = 6$) or saline ($n = 6$) immediately after sucrose drinking on the conditioning day and water was provided until 5:00 p.m.

### 2.4. Tissue preparations for histologic staining

One hour after lithium chloride, rats were overdosed with sodium pentobarbital (Hallym Pharmaceutical Co., Seoul, Korea) and transcardially perfused first with 100 ml of heparinized isotonic saline containing 0.5% $NaNO_2$ (Sigma Co., MO, USA), followed by 400 ml of ice-cold 4% paraformaldehyde (Sigma Co., MO, USA) in 0.1 M sodium phosphate buffer (PB). The brains were immediately dissected out, blocked, post-fixed for 2 h, and transferred into 30% sucrose (Sigma Co., MO, USA) for cryoprotection. Forty micron coronal sections were cut on a freezing, sliding microtome (HM440E, Microm Co., Germany). Alternate

sections were collected through the rostral-caudal extent of the PVN (between bregma $-1.3$ mm and $-2.1$ mm), the CeA (between bregma $-2.2$ mm and $-2.8$ mm), and the NTS (between bregma $-12.8$ mm and $-14.3$ mm). All coordinates were based on Paxinos and Watson (1986).

## 2.5. NADPH-diaphorase histo/c-Fos immunohistochemistry

Free-floating tissue sections were treated with 0.1% Triton in 0.1 M Tris for 15 min at 37 °C, followed by a 15 min reaction in 0.1 M Tris, 0.1% Triton, 0.05% beta-NADPH (Sigma Co., MO, USA), 0.0125% nitroblue tetrazolium (Sigma Co., MO, USA) at 37 °C. The reaction was terminated with ice-cold 0.1 M Tris. Tissue sections were washed twice for 15 min in 0.1 M sodium phosphate buffered saline (PBS), then treated with 0.2% Triton, 1% bovine serum albumin (BSA) in PBS for 30 min. After washing twice in PBS-BSA, sections were incubated overnight with rabbit anti-c-Fos peptide antibodies (1:10,000 dilution, Oncogene Sciences, CA, USA). Sections were washed twice in PBS-BSA and incubated for 1 h with biotinylated anti-rabbit IgG (1:200 dilution, Vector Laboratories, CA, USA), and then bound secondary antibodies were amplified with the ABC kit (Vectastain Elite Kit, Vector Laboratories, CA, USA). Antibody complexes were visualized with 0.05% of diaminobenzidine (Sigma Co., MO, USA) for 5 min. Sections were mounted in anatomical order onto gelatin-coated slides from 0.05 M PB, air dried, dehydrated through a graded ethanol to xylene, and coverslipped.

## 2.6. Plasma corticosterone

One hour after lithium chloride (19 or 76 mg/kg, 0.15 M) with either L-NAME (30 mg/kg) or the same volume of saline pretreatment with 30 min interval, all rats were rapidly anesthetized by $CO_2$ gas and decapitated once unresponsive. As the control group, an additional six rats were decapitated 1 h after the second saline injection paired with the first saline injection given 30 min earlier. Trunk blood was collected into 1.5 ml microcentrifuge tubes containing 5 μl heparin, and the plasma was isolated by centrifugation at $3000 \times g$ for 10 min at 4 °C. The plasma was transferred into new tubes, frozen in liquid nitrogen and stored at $-80$ °C until the corticosterone levels were determined by radioimmunoassay (Coat-A-Count kit, DPC Co., CA, USA). To minimize diurnal variation in the plasma corticosterone levels, all blood was collected 2–3 h after lights-on.

## 2.7. Statistical analysis

Cells expressing NADPH-diaphorase and/or c-Fos in each brain region were hand-counted blind after digitizing 720 μm × 540 μm images of all consecutive sections using an Olympus BX-50 microscope (Olympus Co., Tokyo, Japan) and MCID image analysis system (M2, Imaging Research Inc., Ont., Canada). Cells containing only blue stain in the cytoplasm were counted as NADPH-d, only distinct brown dot as c-Fos positive cells. Cells containing both the brown dot and the blue stain in cytoplasm were considered as c-Fos/NADPH-d double stained cells. The number of cells in two sections from either the PVN (closest sections to bregma $-1.88$ mm) or the CeA (closest sections to bregma $-2.30$ mm) region from each brain was averaged. The NTS was divided into three subregions: caudal (ventral and caudal to the area postrema), intermediate (abutting the forth ventricle), and rostral (where the NTS separates from the forth ventricle). Each of these three subregions was represented by approximately six sections of the NTS sections collected from each rat. Cell counts for all sections within each region of each rat were averaged per section, and the individual mean counts for each region averaged across rats by region within experimental groups. All data were analyzed by one-way analysis of variance (ANOVA) and preplanned comparisons with the control were performed by post hoc Fisher's PLSD or Scheffe's test.

## 3. Results

### 3.1. NADPH-d/c-Fos double staining in the PVN, CeA and NTS

Intraperitoneal lithium chloride (LiCl; 0.15 M, 76 mg/kg) remarkably induced c-Fos expression in the paraventricular nucleus (Fig. 1a and b), the central nucleus of amygdala (Fig. 1c and d), and the intermediate nucleus tractus of solitarius (iNTS) (Fig. 1e and f), compared to the same volume of saline injection. Large population of NADPH-diaphorase stained cells in the PVN showed c-Fos immunoreactivity (-ir) 1 h after the lithium injection (Fig. 1b). Thirty-two percent of NADPH-d cells in the PVN of the saline injected rats, 75% in the lithium rats, exhibited c-Fos-ir (Fig. 2), which reveals a potential implication of nitric oxide in the lithium-induced c-Fos expression, the neuronal activation, in this brain region. Almost no c-Fos-ir neurons in the iNTS showed NADPH-d staining, however, NADPH-d stained cells and fibers appeared to be located closely to the neurons expressing c-Fos (Fig. 1f). In the CeA of the lithium treated rats, the cells and fibers containing NADPH-d were localized near by a group of neurons expressing c-Fos, although they were not inter-mingled (Fig. 1d), not like in the iNTS (Fig. 1f). This distribution allowed us to have an assumption of nitric oxide involvement in the lithium-induced neuronal activation in these brain regions as well, in recall of a highly diffusible property of the gaseous neurotransmitter, nitric oxide.

### 3.2. Dose dependent induction of c-Fos by LiCl and the effect of L-NAME pretreatment

One hour after the intraperitoneal injection of LiCl at different doses (0, 19, 38, 76 mg/kg 0.15 M), a dose-dependent increase in c-Fos induction was detected in all the
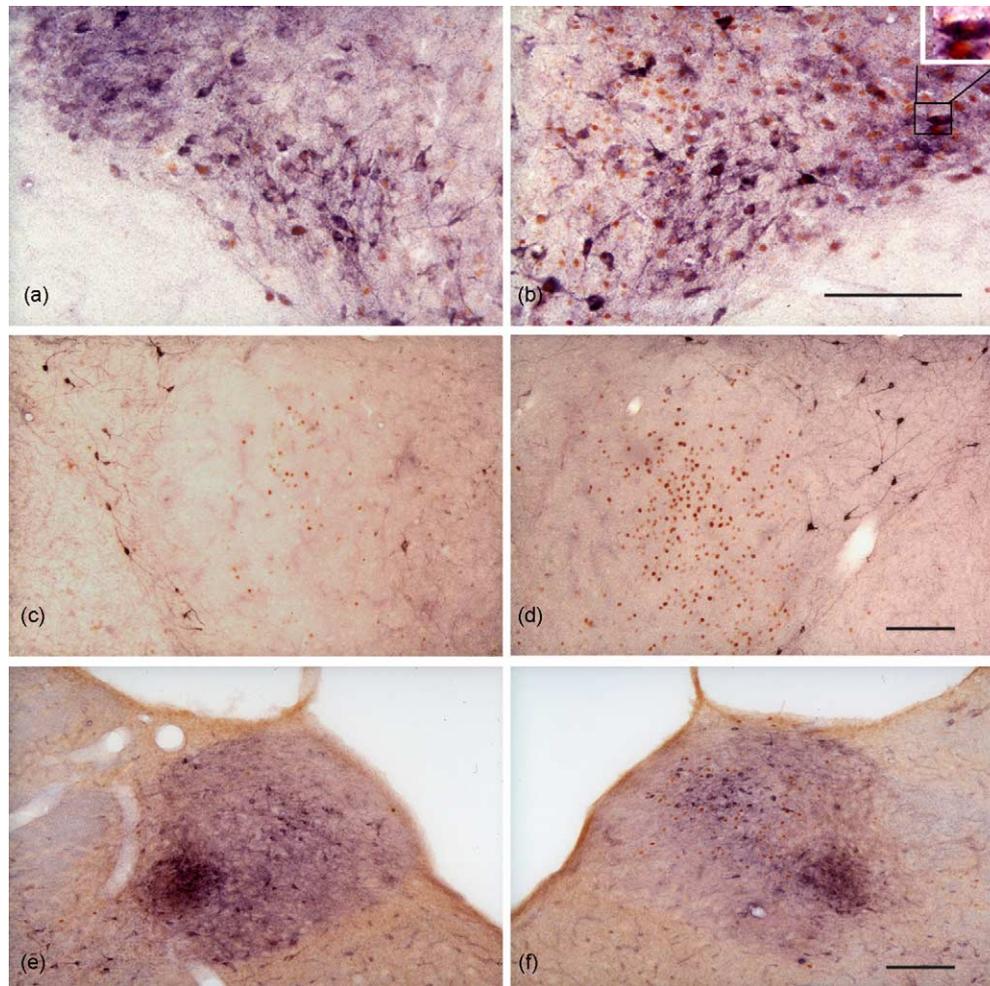
Fig. 1. Photographs of NADPH-diaphorase histostaining (blue) and c-Fos immunostaining (brown). The brain tissues were processed with NADPH-d histostaining followed by c-Fos immunohistochemistry 1 h after the intraperitoneal injection of LiCl (12 ml/kg of 0.15 M LiCl; 76 mg/kg) or saline (12 ml/kg of 0.15 M NaCl). Large population of c-Fos immunoreactive (-ir) neurons was detected in the paraventricular nucleus (PVN; b), the central nucleus of amygdala (CeA; d), and the nucleus tractus solitarius intermediate (iNTS; f) of the lithium treated rat, compared to the saline rat (a, c and e). c-Fos expressing neurons appeared to be located near by the NADPH-d stained cells and fibers in each brain region, particularly some of the PVN neurons exhibited both c-Fos-ir and NADPH-d staining (a, b). Scale bars: 100 μm.
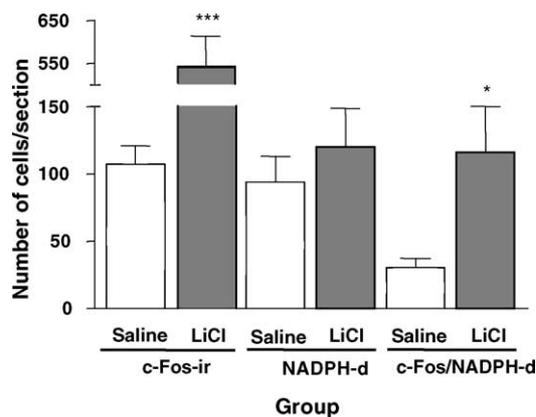


Fig. 2. Number of c-Fos-ir, NADPH-d, or double stained (c-Fos/NADPH-d) cells in the PVN 1 h after the intraperitoneal LiCl (76 mg/kg; solid bars) or saline (open bars). Number of doubly stained cells markedly increased by LiCl, i.e. ∼32% of NADPH-d cells in the saline control group, however, ∼75% in the LiCl group, exhibited c-Fos-ir as well. $^{*}P < 0.05$, $^{***}P < 0.001$ vs. each saline control.

brain regions examined, such as the PVN, the CeA, the NTS (Fig. 3). Intraperitoneal L-NAME (30 mg/kg) 30 min prior to LiCl significantly attenuated c-Fos expression induced by each dose of LiCl in all three regions. These results indicate that LiCl induces neuronal activation in these brain regions in a dose dependent manner, and suggest that nitric oxide maybe, at least partly, involved in the neuronal activation induced by LiCl.

### 3.3. L-NAME pretreatment on lithium-induced CTA

Intraperitoneal injection of isotonic LiCl at a dose of 76 mg/kg, but not 19 mg/kg, given 30 min after the 15 min of sucrose drinking session significantly induced a CTA to the novel sucrose taste (Fig. 4a and b). However, intraperitoneal injection of L-NAME (30 mg/kg) immediately after the sucrose session, 30 min prior to LiCl, did not attenuate the CTA induced by a 76 mg/kg of LiCl (Fig. 4b), in spite of its significant attenuation effect produced in the
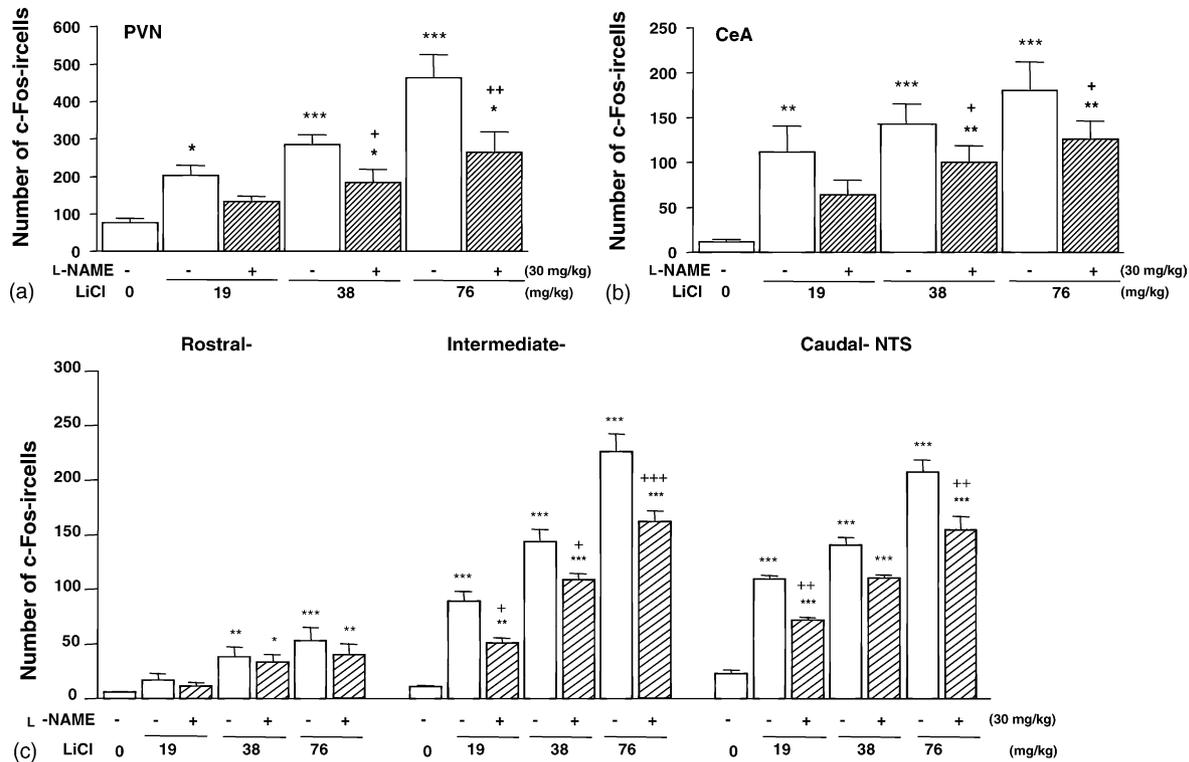
Fig. 3. Dose effect of LiCl on c-Fos induction and the effects of L-NAME administration on the lithium-induced c-Fos expression in each brain region. The number of c-Fos-ir neurons increased by LiCl (0, 19, 38, 76 mg/kg, 0.15 M; open bars) dose-dependently in the PVN (a), CeA (b), and NTS (c) 1 h after the intraperitoneal injection. Intraperitoneal L-NAME (30 mg/kg) 30 min prior to LiCl significantly attenuated the lithium-induced c-Fos expression at all LiCl doses (solid bars). $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. saline/saline; $^\dagger P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs. saline/LiCl at each dose.

neuronal activation of the brain regions implicated in CTA learning (Fig. 3). Moreover, a 19 mg/kg of LiCl produced a significant CTA when it was paired with L-NAME pretreatment (Fig. 4a). Interestingly, intraperitoneal L-NAME alone (30 mg/kg) did not produce a CTA (Fig. 4c), and furthermore L-NAME pretreatment significantly attenuated c-Fos expression induced by 19 mg/kg of LiCl as we recall (Fig. 3).

### 3.4. Plasma corticosterone

Plasma corticosterone level was examined by radio-immunoassay after the lithium injection with/without L-NAME pretreatment in order to determine the effect of L-NAME pretreatment on the lithium-induced activation of hypothalamic-pituitary-adrenal axis. A remarkable increase in the plasma corticosterone level was detected 1 h after the intraperitoneal injection of high dose LiCl (76 mg/kg, 0.15 M), revealing a significant activation of the HPA axis, but no changes were detected after the low dose LiCl (19 mg/kg, 0.15 M) (Fig. 5). L-NAME (30 mg/kg) administration 30 min prior to LiCl did not modulate the effect of LiCl at either dose (19 or 76 mg/kg, 0.15 M) on the plasma corticosterone level (Fig. 5). Blood samples of the low dose lithium groups were collected on a different experimental day from the high dose groups with their own saline/saline control group. Plasma corticosterone concentration of the

saline/saline control group was 74.742 ± 17.395 ng/ml in the high dose groups, and 200.952 ± 39.848 ng/ml in the low dose groups, respectively. Data were presented in percent ratio to make a relative comparison between the low and high dose lithium effects (Fig. 5).

### 4. Discussion

In the present study, the behavioral, hormonal, and neuronal effects of nitric oxide inhibition on the acquisition of lithium-induced conditioned taste aversion to the novel sucrose solution were examined. We firstly demonstrated that the intraperitoneal lithium chloride as an unconditioned stimulus has a dose effect on both the brain c-Fos expression and CTA acquisition. This result concurs with the conclusion made in previous reports in terms of a correlation between CTA learning and c-Fos expression, i.e. the molecular correlation between the learning behavior and neuronal activation per se (Yamamoto et al., 1992; Houpt et al., 1994; Lamprecht and Dudai, 1995; Schafe et al., 1995; Schafe and Bernstein, 1996; Swank et al., 1996; Sakai and Yamamoto, 1997). Nitric oxide has been considered as a neuromodulator in the central nervous system (Moncada et al., 1991; Snyder and Bredt, 1992), and reported to play a role in learning and memory (O'Dell et al., 1991; Schuman and Madison, 1991; Haley et al., 1992). In the present study,
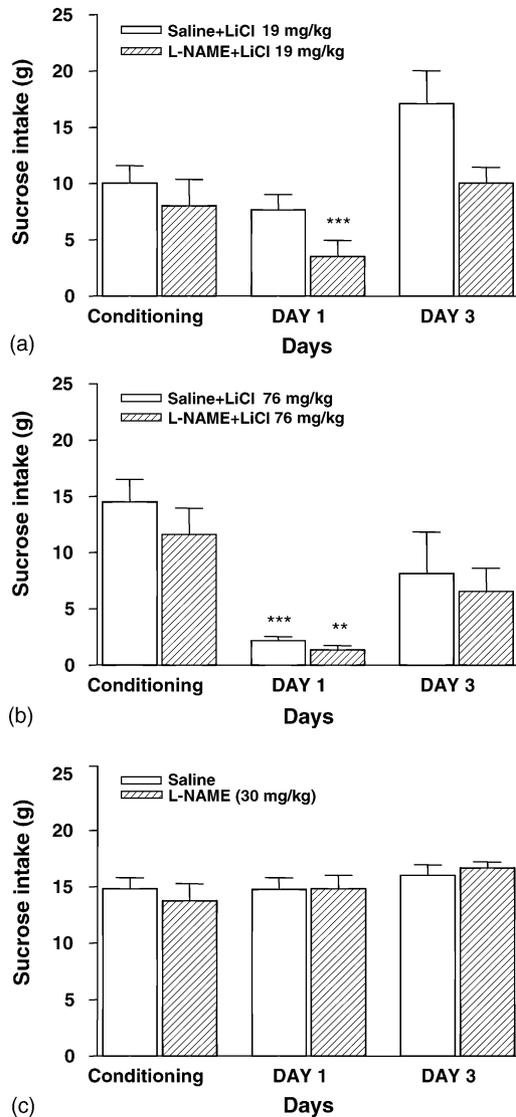
(a)

(b)

(c)

Fig. 4. Effects of L-NAME pretreatment on lithium-induced CTA. Isotonic LiCl at 76 mg/kg dose (b), but not 19 mg/kg (a), significantly induced a CTA to the novel sucrose taste (open bars). Intraperitoneal injection of L-NAME (30 mg/kg) immediately after sucrose intake and 30 min prior to LiCl at the conditioning did not modulate the effect of high dose LiCl on the CTA formation (b, solid bars), however, it augmented the effect of low dose LiCl (a, solid bars). L-NAME alone at a dose of 30 mg/kg did not produce a CTA (c). $^{**}P < 0.01$; $^{***}P < 0.001$ vs. the conditioning day.

fairly large population of NOS containing neurons ($\sim$75%) in the PVN exhibited c-Fos induction by intraperitoneal LiCl, and NADPH-diaphorase stained cells and fibers appeared to be located near by the c-Fos expressing neurons in the CeA and the NTS. This supported a possible implication of nitric oxide in both the lithium-induced CTA learning as reported elsewhere (Wegener et al., 2001) and the lithium-induced neuronal activation, referred by c-Fos expression, in those brain regions. Indeed, the pretreatment of $N^{\omega}$-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase, significantly attenuated the lithium-induced c-Fos expression in those brain regions. However, L-NAME administration prior to LiCl at the conditioning did not blunt,
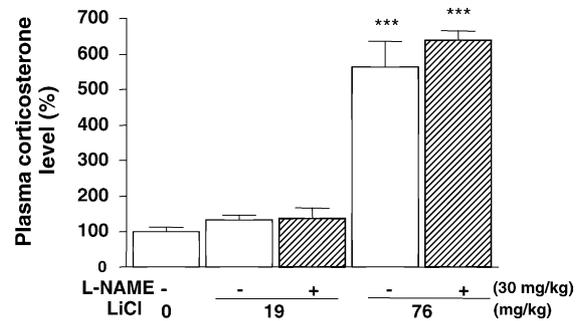


Fig. 5. Changes in the plasma corticosterone levels. Isotonic LiCl at 76 mg/kg dose, but not 19 mg/kg, increased the plasma corticosterone level significantly 1 h after the intraperitoneal injection (open bars). L-NAME (30 mg/kg) pretreatment (solid bars) did not modulate the effects of either doses (19 or 76 mg/kg) of LiCl on the plasma corticosterone level. $^{***}P < 0.001$ vs. saline injected control.

but rather augmented the acquisition of lithium-induced CTA, in spite of its significant attenuation effect on the lithium-induced c-Fos expression. In other words, the behavioral outcome, CTA learning, of L-NAME pretreatment was opposite to the molecular influence, i.e. the neuronal activation in the brain regions. These results suggest that the amount of neuronal activation, referred by the number of c-Fos expressing neurons, in the brain regions at the conditioning may not be strongly correlated with the strength of CTA acquisition. Moreover, c-Fos expression, at least in the PVN, CeA and NTS, could not always be used as a reliable molecular index of CTA learning. Overall, the number of c-Fos expressing neurons in the brain regions may not be directly correlated with the acquisition of an aversive memory, because LiCl-like c-Fos patterns of neuronal activation were observed in the absence of behaviorally evident aversive consequences (Benoit et al., 2000).

Intraperitoneal LiCl activates the hypothalamic-pituitary-adrenal axis (Hennessy et al., 1976), and induces adrenocorticotrophic hormone release (Sugawara et al., 1988). The activation of HPA axis, ACTH and corticosterone releases are involved in the lithium-induced CTA learning (Smotherman et al., 1976; Hennessy et al., 1980; Revusky and Martin, 1988; Peeters and Broekkamp, 1994). In this study, intraperitoneal injection of isotonic LiCl at a dose of 76 mg/kg induced a remarkable increase in the plasma corticosterone level as well as a strong CTA, but 19 mg/kg of LiCl had no effect on either the CTA learning or the plasma corticosterone level. These results concur with previous reports showing that the HPA axis activation may correlate with the lithium-induced CTA learning (Smotherman et al., 1976; Hennessy et al., 1980; Revusky and Martin, 1988; Peeters and Broekkamp, 1994).

There is some evidence previously reported revealing a regulatory role of NO in the HPA axis. For examples, L-NAME enhances the plasma corticosterone and ACTH level (Giordano et al., 1996), augments the stimulatory effect on the HPA axis by various agents (Budziszewska et al., 1998, 1999; Bugajski et al., 1998; Kwon Kim and Rivier, 1998).

On the contrary, it also has been reported that L-NAME blunts the stress-induced neuronal activation of the hypothalamus (Amir et al., 1997) and ACTH release (Rivier, 1994), and NO stimulates the transcription of corticotropin-releasing factor and its receptor in the hypothalamus (Lee et al., 1999). It has been reported that LiCl increases both the NOS activity and synthesis in the brain (Bagetta et al., 1993; Anai et al., 2001), and activates the HPA axis as it was mentioned above (Hennessy et al., 1976; Smotherman et al., 1976; Hennessy et al., 1980; Revusky and Martin, 1988; Sugawara et al., 1988; Peeters and Broekkamp, 1994). Taken together, it can be hypothesized that NO may exert a modulatory effect on the lithium-induced CTA learning, at least in part, through the activation process of the HPA axis. In this study, L-NAME pretreatment did not modulate the effect of LiCl on the plasma corticosterone level or the CTA learning. In terms of NOS effect on CTA learning, our result concurs with previous report that both specific NOS inhibitor 7-nitroindazole and non-specific NOS inhibitor methylene blue failed to influence the aversion produced by LiCl (Wegener et al., 2001). Regarding L-NAME effect on the HPA axis, the present result suggests that NO may not be involved at least in the lithium-induced activation of the HPA axis.

Interestingly, L-NAME (30 mg/kg) pretreatment produced a significant attenuation effect on the neuronal activation induced by all doses of LiCl (19, 38, or 76 mg/kg), referred by c-Fos expression, in the hypothalamic paraventricular nucleus. In other words, L-NAME exerted its inhibitory effect on the lithium-induced neuronal activation in the PVN, the center of the HPA axis, however, the plasma corticostrone level, the final product of the HPA axis activation, was not affected. This suggests that the reduced activation of the hypothalamic neurons by L-NAME pretreatment might still be big enough to activate the HPA axis and increase the plasma corticosterone level consequently. A higher dose of L-NAME was not used, because it was reported that L-NAME alone activates the HPA axis when it is administered at higher doses than we used (Giordano et al., 1996; Budziszewska et al., 1998, 1999; Bugajski et al., 1998), and L-NAME alone at a dose of 30 mg/kg did not induce either CTA (Fig. 4c) or c-Fos expression in the PVN (data not shown). However, the behavioral effect of low dose (19 mg/kg) LiCl on CTA learning was augmented by L-NAME pretreatment at 30 mg/kg without increasing the plasma corticosterone level. Moreover, the low dose lithium-induced c-Fos expression, the neuronal activation, in all the brain regions examined, including the PVN, was even lowered by the L-NAME pretreatment. We conclude that this enhancement in the CTA acquisition by the L-NAME (30 mg/kg) paired with the low dose LiCl (19 mg/kg) might be due to its peripheral effect.

It was suggested that the systemic administration of L-NAME may produce a possible effect of peripheral NOS inhibition on the development of a CTA (Prendergast et al., 1997). It was reported that NOS inhibitors such as L-NAME, *N*-monomethyl-L-arginine, and $N^\omega$-nitro-L-arginine prevent the relaxation of the gastrointestinal (GI) smooth muscles induced by electrical stimulation (Desai et al., 1991; Tottrup et al., 1991). It is possible that L-NAME may induce GI constriction and/or peristaltic dysregulation, either of which may serve as a salient aversive GI cue in a CTA trial. It is likely that the GI effect produced, if any, by 30 mg/kg of L-NAME might have been too small to induce a CTA by itself. However, it could be enhanced by pairing with a low dose LiCl, as was done in our experimental paradigm. And perhaps this enhancing effect of L-NAME on the acquisition of an aversive memory might have been hidden when it was paired with high dose LiCl, which known to produce alone a strong aversive effect. Central administration of L-NAME to the experimental system is currently under our consideration in order to define the role of the brain NO in lithium-induced CTA learning.

In summary, intraperitoneal LiCl increases c-Fos expression in the brain regions such as the PVN, CeA, and NTS, activates the HPA axis, and induces a CTA to novel sucrose solution, in a dose-dependent manner. Systemic L-NAME at a dose of 30 mg/kg attenuates lithium-induced brain c-Fos expression, but not the HPA axis activation or the CTA formation induced by high dose lithium (76 mg/kg). Interestingly, the systemic L-NAME augmented the CTA acquisition when it was paired with low does lithium (19 mg/kg). We conclude that NO may be involved, at least partly, in the brain c-Fos expression, but not likely in the HPA axis activation, during lithium-induced CTA formation. Additionally, the number of c-Fos expressing neurons in the brain regions such as the PVN, CeA or NTS may not be a direct index for the CTA acquisition.

## Acknowledgements

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