

Central N^ω-nitro-L-arginine Methyl Ester Does not Influence Lithium-induced c-Fos and Conditioned Taste Aversion

Jeong Won Jahng¹, Si Ho Choi¹, Dong Goo Kim¹, and Thomas A Houpt²

¹Department of Pharmacology and Yonsei Brain Research Institute, BK21 Project for Medical Science, Yonsei University College of Medicine, Seoul, Korea;

²Department of Biological Science, Florida State University, Tallahassee, FL, USA.

LiCl at doses sufficient to induce conditioned taste aversion (CTA) causes c-Fos expression in the brain regions implicated in CTA formation. It has been reported that nitric oxide (NO) may play a role in CTA learning and LiCl increases both the synthesis and activity of NO synthase (NOS) in the brain. In this study, we examined the effect of central N^ω-nitro-L-arginine methyl ester (L-NAME) on the brain c-Fos expression and CTA learning induced by lithium in rats. In the results, intracerebroventricular L-NAME given prior to lithium did not change either the lithium-induced CTA or c-Fos in the relevant brain regions. This suggests that the brain NO system may not be involved in the neuronal activation during lithium-induced CTA formation.

Key Words: Conditioned taste aversion, lithium chloride, c-Fos, nitric oxide, rat, intracerebroventricular injection.

INTRODUCTION

Lithium chloride 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 be correlated with CTA learning.¹⁻⁷ Large populations of neuronal nitric oxide synthase containing cells and fibers, identified by NADPH-diaphorase staining, are distributed in the brain regions implicated in CTA learning.⁸⁻¹⁰ It has been reported that manipulation of nitric oxide level can produce a CTA,¹¹⁻¹³ modulate lithium-induced CTA learning,¹³ and the synthesis and activity of nitric oxide synthase is increased in the brain treated with lithium.^{14,15} We previously found that the neuronal activation in brain regions, referred by c-Fos expression, by lithium chloride was significantly attenuated with the systemic pretreatment of a nitric oxide synthase inhibitor, N^ω-nitro-L-arginine methyl ester (L-NAME).¹⁶ Previous reports together with our finding suggest that nitric oxide may be involved, at least partly, in the neuronal activation induced by lithium chloride in the brain regions during the CTA formation. However, it is still not clear whether the brain or the peripheral nitric oxide plays a role in lithium-induced CTA, because the previous studies regarding on the nitric oxide involvement in CTA learning all has been done with a systemic injection of the manipulating agents.¹¹⁻¹³

In the present study, we examined the lithium-induced c-Fos expression and CTA formation to novel sucrose taste in rats after an intracerebro-

Received March 29, 2003

Accepted June 2, 2003

This work was supported by the Neurobiology Research Program from the Korea Ministry of Science and Technology (# M1-01-08-00-0021) to JWJ.

Reprint address: requests to Dr. Dong Goo Kim, Department of Pharmacology, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea. Tel: 82-2-361-5277, Fax: 82-2-313-1894, Email: dgkimpharm@yumc.yonsei.ac.kr

ventricular administration of L-NAME, in order to define the role of the brain nitric oxide in lithium-induced CTA learning.

MATERIALS AND METHODS

Animals

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Florida State University. Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA) weighing 300-400g were individually housed in polycarbonate cages with wood-chip bedding with *ad libitum* access to tap water and standard rodent chow under 12h/12h light-dark cycle.

Under chloral hydrate (153 mg/kg) and pentobarbital (35 mg/kg) anesthesia, rats were stereotaxically implanted with a 22 gauge, stainless steel guide cannula (Plastics One, Roanoke, VA, USA) aimed towards the lateral cerebral ventricle (1.2 mm caudal to bregma, 1.5 mm lateral to the midline, and 4 mm below the skull surface). Guide cannulas were held in place with dental acrylic bonded to stainless steel screws anchored to the skull. An obturator was inserted into each guide cannula and remained in place except during injections when it was removed and replaced with an injector that extended 1.0 mm beyond the tip of the guide cannula. After 1 week of post-operational recovery, the patency and placement of the cannula was verified by injection of 100 ng human angiotensin II (Sigma Chemical Co., St Louis, MO, USA) dissolved in 0.15 M NaCl; rats with cannulas projecting into the lateral ventricle responded to the angiotensin injection by drinking water within 2 min, while rats that failed to drink were dropped from the study. Cannula placements were also verified postmortem by sectioning through the brain.

Drug treatment

N^ω-nitro-L-arginine methyl ester (L-NAME, Sigma Co.) was dissolved in aseptic physiological saline. Rats received an intracerebroventricular infusion of either L-NAME (250 μg) or isotonic

NaCl (180 μg) in a volume of 5 μl each through the guide cannulas, delivered over 30 s with a handheld 50 μl syringe (Hamilton Co., Reno, NV, USA). The injector was left in place for 30 s after the solution delivery. All rats received an intraperitoneal injection of LiCl (76 mg/kg, 12 ml/kg of 0.15 M) 30 min after L-NAME or aseptic physiologic saline administration.

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 (n=7) received an intracerebroventricular infusion of L-NAME (250 μg) or sterile saline (180 μg) followed by isotonic LiCl (76 mg/kg) with 30 min of interval. Water was supplied immediately after the conditioning until 5:00 PM. On day 9, after 1 day of recovery with 5 h of water supply, rats had access to 5% sucrose for 15 min at 12:00 PM. The weight of sucrose solution consumed was recorded and used to quantify the CTA.

c-Fos immunohistochemistry

Rats (n=6) were overdosed with sodium pentobarbital 1 h after lithium chloride following the intracerebroventricular injection of L-NAME or saline, and transcardially perfused first with 100 ml of heparinized isotonic saline containing 0.5% NaNO₂, followed by 400 ml of ice-cold 4% paraformaldehyde 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 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 paraventricular nucleus (PVN) (between bregma - 1.3 mm and - 2.1 mm), the central nucleus of amygdala (CeA) (between bregma -2.2 mm and -2.8 mm), and the nucleus tractus of solitarius (NTS) (between bregma - 12.8 mm and - 14.3 mm). All coordinates were based

on Paxinos and Watson.¹⁷

Free-floating tissue sections were washed twice for 15 min in 0.1M sodium phosphate buffered saline (PBS), then permeabilized 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), then bound secondary antibodies were amplified with the ABC kit (Vectastain Elite Kit, Vector Laboratories, CA, USA). Antibody complexes were visualized with 0.0% of diaminobenzidine 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.

Statistical analysis

c-Fos immunoreactive (-ir) cells in each brain regions were hand-counted after digitizing 720 × 540 micron images of all consecutive sections using an Olympus BX-50 microscope (Olympus Co., Tokyo, Japan) and MCID image analysis system (M2, Imaging Research Inc., Ontario, Canada). The number of c-Fos-ir cells in three sections from the PVN (closest sections to bregma -1.88 mm), or two sections from the CeA (closest sections to bregma -2.30 mm) region from each brain were averaged, respectively. 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 unpaired or paired t-test and expressed as mean ± s.e.m. A standard level of $p < 0.05$ (two-tailed) was used for statistical significance.

RESULTS

1 h after the intraperitoneal injection of lithium chloride, large numbers of c-Fos-ir cells were detected in the paraventricular nucleus (PVN), the central nucleus of amygdala (CeA), and the nucleus tractus of solitarius (NTS) (Fig. 1). Intracerebroventricular administration of L-NAME (250 $\mu\text{g}/5\mu\text{l}$) 30 min prior to the lithium injection did not significantly change the number of c-Fos expressing neurons induced by the lithium injection in all the brain regions examined (Fig. 2). In one bottle sucrose test paradigm, the strength of the CTA acquisition induced by lithium chloride was not altered by the central L-NAME administration (Fig. 3).

DISCUSSION

In the present study, neither the lithium-induced CTA nor c-Fos expression was influenced by an intracerebroventricular L-NAME at a dose of 250 $\mu\text{g}/5\mu\text{l}$ given 30 min prior to lithium. It has been reported that 250 μg or even lower dosage of intracerebroventricular L-NAME produces its inhibitory effects on the brain nitric oxide system within 5-15 min.¹⁸⁻²⁰ Therefore, the possible assumption which the dose and/or the treatment time of L-NAME might have been insufficient to block the enzyme activity of nitric oxide synthase in this study can be excluded.

Wegener, et al.¹³ reported that the systemic administration of nitric oxide synthase inhibitors, 7-Nitroindazole and methylene blue, failed to influence the aversion produced by lithium. We also found that the systemic inhibition of nitric oxide synthase with L-NAME given 30 min prior to lithium did not influence the acquisition of lithium-induced CTA. However, the systemic L-NAME significantly attenuated the lithium-induced c-Fos in the brain regions.¹⁶ It has been reported that the brain c-Fos expression induced by US correlates with the CTA formation.^{1,3,7} Taken all together, it was suggested that nitric oxide may be involved in the neuronal activation induced by lithium, an unconditioned stimulus (US), but not in the lithium-induced CTA, an associative memory acquired by an interaction

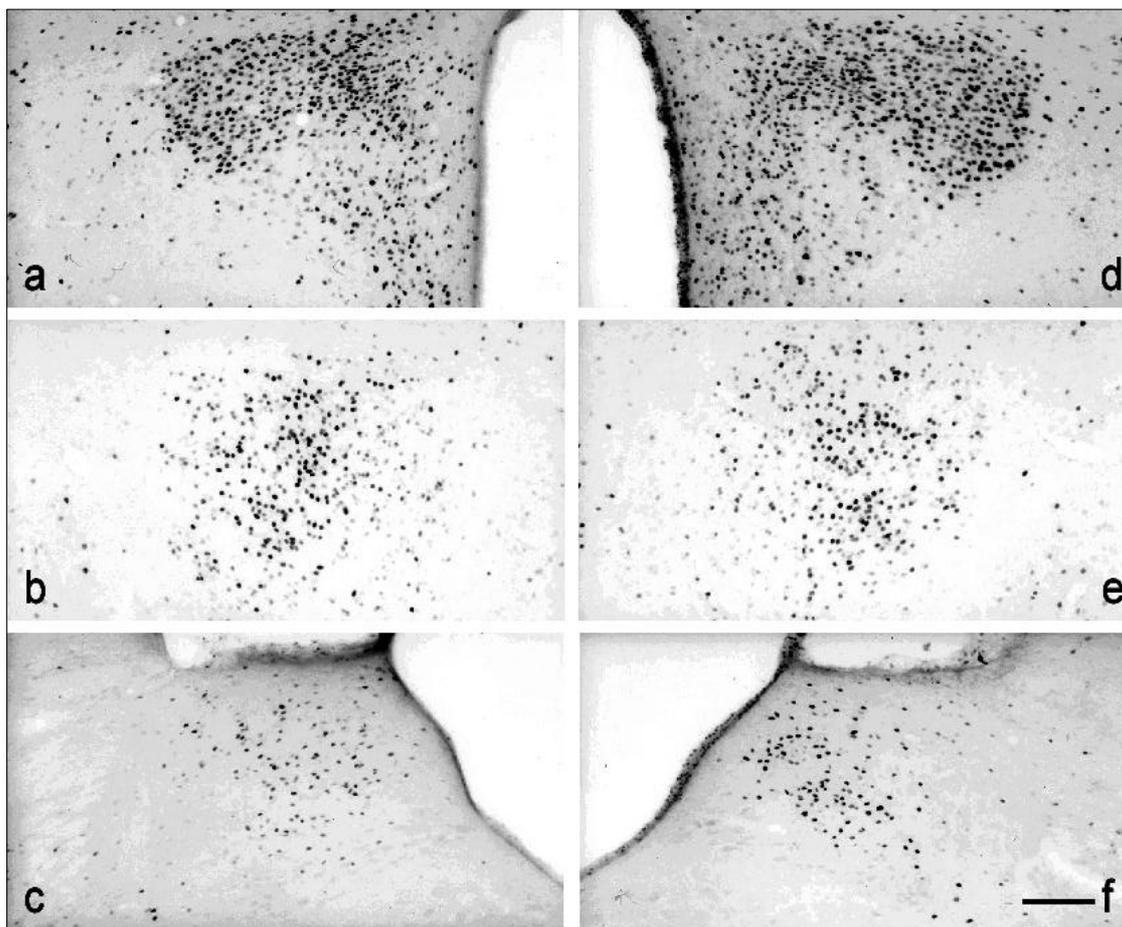


Fig. 1. Microphotographs of c-Fos immunoreactive (-ir) neurons in the hypothalamic paraventricular nucleus (PVN; a, d), the central nucleus of amygdale (CeA; b, e), and the nucleus tractus of solitarius, intermediate (iNTS; c, f) of rats. An intracerebroventricular injection of L-NAME (250 μ g/5 μ l in saline) or physiologic saline (5 μ l) was given 30 min before the intraperitoneal lithium (76 mg/kg, 12 ml/kg of 0.15 M), and the brains were processed for c-Fos immunohistochemistry 1 h after lithium chloride. Large numbers of c-Fos-ir cells were detected in the PVN (a), the CeA (b), and the iNTS (c) of the saline/lithium injected rat. The intracerebroventricular administration of L-NAME 30 min prior to the lithium injection did not appear to change the lithium-induced c-Fos expression in all three regions (PVN, d; CeA, e; iNTS, f). Scale bar: 100 μ m.

between the visceral illness produced by lithium and the novel taste, sucrose. Additionally, the amount of brain c-Fos expression induced by US may not always refer the strength of CTA formation. However, the central inhibition of nitric oxide synthase failed to influence not only the CTA formation but also the brain c-Fos induced by lithium in the present study. Thus, it is concluded that the brain nitric oxide may not be involved in the neuronal activation during the lithium-induced CTA formation, and that the peripheral nitric oxide may, at least partly, mediate the malaise effect of lithium on the

neuronal activation in the formation of aversive memory.

Inconsistent results have been reported about nitric oxide involvement in CTA learning. It has been reported that nitric oxide donors,^{12,13} or the inhibitors of nitric oxide synthase all produces a CTA.^{11,13} These may reveal that a CTA formation is a complicated process which numerous parameters are involved. It seems that nitric oxide may be one of the systemic parameters influencing CTA learning, because all the previous reports about the role of nitric oxide in CTA learning have been made with the systemic administration

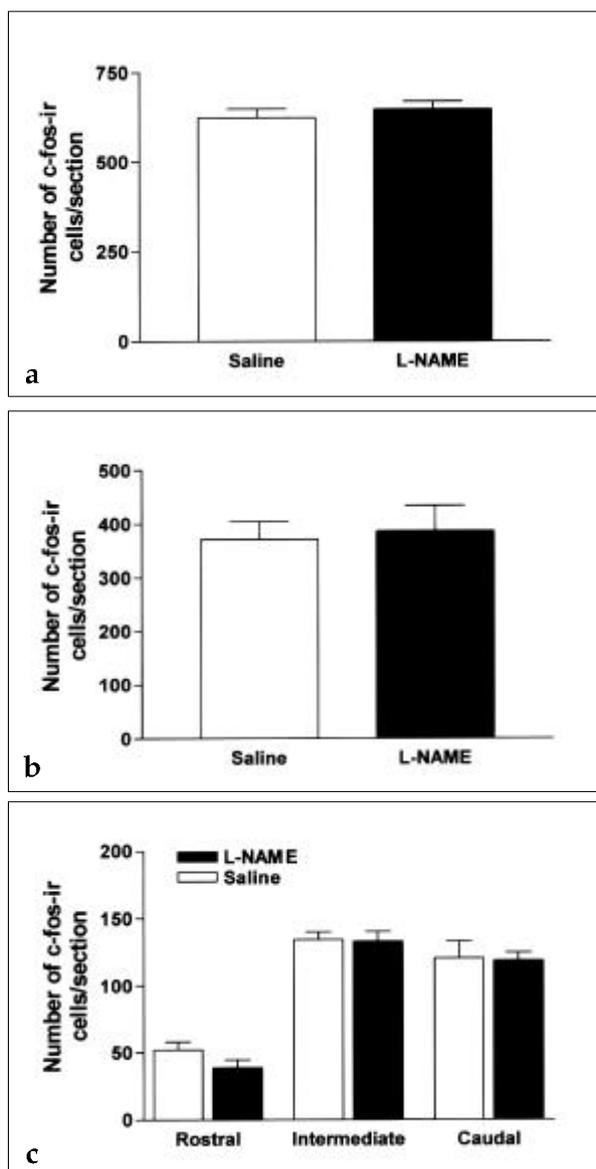


Fig. 2. Number of c-Fos-ir cells in the PVN (a), the CeA (b), or the NTS (c). L-NAME pretreatment did not change the number of c-Fos-ir cells induced by lithium chloride in all brain regions examined ($n=6$). Rostral NTS tends to have a reduced number of c-Fos-ir cells with L-NAME pretreatment, however, a statistical significance was not found. Data was analyzed by unpaired t-test. Bars represent mean \pm s.e.m.

of drugs, and furthermore, in our present study the central modulation of nitric oxide system was found to have no effect on a CTA formation.

In conclusion, this study suggests that the brain nitric oxide may not be involved in the neuronal activation of the relevant brain regions during

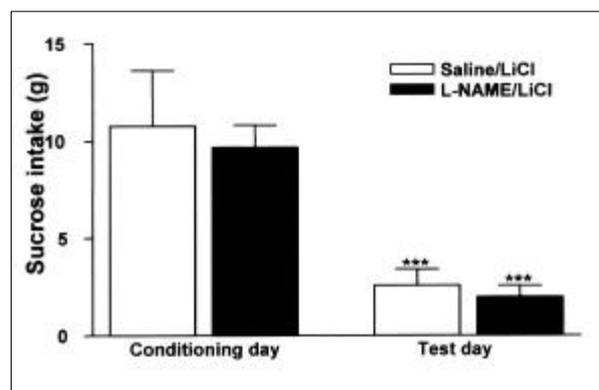


Fig. 3. Effect of the central L-NAME on the acquisition of lithium-induced conditioned taste aversion (CTA). An intracerebroventricular L-NAME ($250 \mu\text{g}/5 \mu\text{l}$ in saline) or physiologic saline ($5 \mu\text{l}$) was given immediately after the sucrose drinking, and then an intraperitoneal lithium injection was followed with a 30 min interval on the conditioning day. The sucrose drinking test was performed 2 days after the conditioning. L-NAME pretreatment did not significantly change the strength of CTA acquisition induced by lithium chloride ($n=7$). Data was analyzed by paired t-test. *** $p < 0.001$; test day vs. conditioning day. Bars represent mean \pm s.e.m.

lithium-induced CTA formation. This is the first report on the effect of the central inhibition of nitric oxide synthase in CTA learning.

ACKNOWLEDGEMENTS

The authors thank Dr. Seoul Lee for reading the manuscript and help with statistical analysis, Dr. Ti Yuen Zhang for technical help with image analysis. This work was supported by the Neurobiology Research Program from the Korea Ministry of Science and Technology (# M1-01-08-00-0021) to JWJ.

REFERENCES

- Haupt TA, Philopena JM, Wessel TC, Joh TH, Smith GP. Increased c-fos expression in nucleus of the solitary tract correlated with conditioned taste aversion to sucrose in rats. *Neurosci Lett* 1994;172:1-5.
- Lamprecht R, Dudai Y. Differential modulation of brain immediate early genes by intraperitoneal LiCl. *Neuroreport* 1995;7:289-93.
- Sakai N, Yamamoto T. Conditioned taste aversion and c-fos expression in the rat brainstem after admini-

- stration of various USs. *Neuroreport* 1997;8:2215-20.
4. Schafe GE, Bernstein IL. Forebrain contribution to the induction of a brainstem correlate of conditioned taste aversion: I. The amygdale. *Brain Res* 1996;741:109-16.
 5. Schafe GE, Seeley RJ, Bernstein IL. Forebrain contribution to the induction of a cellular correlate of conditioned taste aversion in the nucleus of the solitary tract. *J Neurosci* 1995;15:6789-96.
 6. Swank MW, Ellis AE, Cochran BN. c-Fos antisense blocks acquisition and extinction of conditioned taste aversion in mice. *Neuroreport* 1996;7:1866-70.
 7. Yamamoto T, Shimura T, Sako N, Azuma S, Bai WZ, Wakisaka S. C-fos expression in the rat brain after intraperitoneal injection of lithium chloride. *Neuroreport* 1992;3:1049-52.
 8. Dun NJ, Dun SL, Forstermann U. Nitric oxide synthase immunoreactivity in the rat pontine medullary neurons. *Neuroscience* 1994;59:429-45.
 9. Krukoff TL, Khalili P. Stress-induced activation of nitric oxide-producing neurons in the rat brain. *J Comp Neurol* 1997;377:509-19.
 10. Vincent SR, Kimura H. Histochemical mapping of nitric oxide synthase in the rat brain. *Neuroscience* 1992;46:755-84.
 11. Prendergast MA, Buccafusco JJ, Terry Jr. AV. Nitric oxide synthase inhibition impairs spatial navigation learning and induces conditioned taste aversion. *Pharm Biochem Behav* 1997;57:347-52.
 12. Rabin BM. Free radicals and taste aversion learning in the rat: nitric oxide, radiation and dopamine. *Prog Neuro-psychopharmacol Biol Psychiatry* 1996;20:691-707.
 13. Wegener G, Volke V, Bandpey Z, Rosenberg R. Nitric oxide modulates lithium-induced conditioned taste aversion. *Behav Brain Res* 2001;118:195-200.
 14. Bagetta G, Corasaniti MT, Melino G, Paoletti AM, Finazzi-Agro A, Nistico G. Lithium and tacrine increase the expression of nitric oxide synthase mRNA in the hippocampus of rat. *Biochem Biophys Res Commun* 1993;197:1132-9.
 15. Bagetta G, Massoud R, Rodino P, Federici G, Nistico G. Systemic administration of lithium chloride and tacrine increases nitric oxide synthase activity in the hippocampus of rats. *Eur J Pharmacol* 1993;237:61-4.
 16. Jahng JW, Lee JH, Houpt TA. Systemic L-NAME attenuated lithium-induced c-Fos expression in the brain, but no effect on the acquisition of lithium-induced conditioned taste aversion in rats. 24th AChemS Abstr 2002.
 17. Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 2nd ed. New York: Academic Press Inc; 1986.
 18. Chen KK, Chang LS. Involvement of L-arginine/nitric oxide pathway at the paraventricular nucleus of hypothalamus in central neural regulation of penile erection in the rat. *Int J Impot Res* 2002;14:139-45.
 19. De Paula D, Steiner AA, Branco LG. The nitric oxide pathway is an important modulator of stress-induced fever in rats. *Physiol Behav* 2000;70:505-11.
 20. Kadekaro M, Terrell ML, Liu H, Bui V, Summy-Long JY. Indomethacin prevents the L-NAME-induced increase in plasma levels of oxytocin in dehydrated rats. *Brain Res* 2000;877:371-3.