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Lithium Induces Expression of HCNP and ChAT in the Septo-Hippocampal Cholinergic System of Rats

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Abstract: This study was conducted to examine if intraperitoneal lithium at high dose, sufficient to induce conditioned taste aversion learning, increases expression of Hippocampal Cholinergic Neurostimulating Peptide (HCNP) and choline acetyltransferase (ChAT) in the brain septo-hippocampal system. Quantitative real-time RT-PCR analysis demonstrated that an intraperitoneal injection of lithium chloride (0.15 M, 12 mL kg⁻¹) acutely increases HCNP mRNA levels in the hippocampus. ChAT immunoreactivities in the medial septal nucleus and the hippocampus, which were determined by immunohistochemistry and western blot analysis, also increased with intraperitoneal lithium. These results suggest that intraperitoneal lithium may acutely activate the septo-hippocampal cholinergic system, via increasing expression of HCNP and ChAT. Additionally, we suggest that activation of the septo-hippocampal cholinergic system with increased HCNP expression may be a part of lithium’s action as unconditioned stimulus in conditioned taste aversion learning.

Key words: Choline acetyltransferase, hippocampal cholinergic neurostimulating peptide, lithium chloride

INTRODUCTION

The septo-hippocampal cholinergic system is important for memory formation and learning and it has been proposed that cholinergic dysfunction is involved in the dementia of Alzheimer’s disease (Francis et al., 1999; Levey, 1996). It was reported that precursor expression of hippocampal cholinergic neurostimulating peptide (HCNP) is decreased in the hippocampus of patient with Alzheimer’s disease (Maki et al., 2002). HCNP is a novel acetylated undecapeptide originally purified from young rat hippocampus, highly expressed during the perinatal period and considered to be crucial for the phenotypic development of the septo-hippocampal cholinergic system (Ojika et al., 1992, 2000; Yuasa et al., 2001). The septo-hippocampal cholinergic system comprises hippocampus and basal forebrain area including medial septal nucleus and the septal cholinergic projections are abundantly innervated to the hippocampal region (Amaral and Kurz, 1985). It has been reported that the septal cholinergic and the hippocampal glutamatergic neurotransmitter pathways exert a reciprocal influence over HCNP precursor mRNA expression (Iwase et al., 2001). In vitro studies have demonstrated that HCNP up-regulates acetylcholine synthesis and production of choline acetyltransferase (ChAT) in the rat septal cholinergic neurons (Ojika et al., 1992, 1994, 2000). It has been reported that a reduction of acetylcholine, due to inactivation of ChAT, was ascertained in animal model of dementia and in patients with Alzheimer’s disease (Burns et al., 1997; Perez et al., 2007).

Lithium has been used clinically for over 30 years and many studies related to its therapeutic effects have been done (Pilcher, 2003; see for review). Lithium is usually administrated systemically in both therapeutic and experimental treatments. For many effects of lithium, however, the action site of lithium is unknown since, as a small ion, lithium rapidly diffuses throughout the brain and body (Stern et al., 1977). In animal studies, intraperitoneal injection of lithium has been widely used as conventional stimulus in conditioned taste aversion (CTA) learning for its toxic effect. Intraperitoneal injection of lithium at high doses increases c-Fos expression, marker for neuronal activation, in several brain regions implicated in CTA learning including the hippocampus (Chen et al., 1999). Hippocampus has been reviewed to be implicated in taste memory (Manrique et al., 2007) and rats with neonatal hippocampal lesion showed impairments in CTA learning (Angst et al., 2007). It has been reported that cholinergic activity may be implicated in the
acquisition (Gonzalez et al., 2000; Naor and Dudai, 1996) and recovery (Miranda and Bermudez-Rattoni, 1998) of CTA learning and that lithium increases acetylcholine synthesis and release in the rat brain (Jope, 1979). In addition, we have previously found that lithium treatment increases HCNP expression in PC12 cells (unpublished observation). Taken together, it is suggested that lithium as unconditioned stimulus in CTA learning may increase cholinergic neurotransmission, perhaps HCNP expression as well, in the septo-hippocampal system. This study was conducted to examine if intraperitoneal lithium at high dose, sufficient to induce CTA formation, acutely increases expression of HCNP mRNA and ChAT in the brain septo-hippocampal system, in order to understand the molecular basis of lithium’s action in CTA learning.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats (200-250 g) were purchased (Orient, Co. Korea) and acclimated to the laboratory condition in a specific-pathogen-free barrier area where the temperature (22±1°C) and humidity (55%) were controlled constantly with a 12/12 h light/dark cycle (lights-on at 07:00 am). Rats had ad libitum access to standard laboratory food (Purina Rodent Chow, Purina Co., Seoul, Korea) and membrane filtered purified water. The experimental protocol was approved by the Committee for the Care and Use of Laboratory Animals at Seoul National University and rats were cared according to the Guideline for Animal Experiments, 2000, edited by the Korean Academy of Medical Sciences, which is consistent with the NIH Guidelines for the Care and Use of Laboratory Animals, revised 1996. The time period of research project was from May 2005 to February 2008.

Drug treatment: Lithium chloride (LiCl, Sigma Co., Mo, USA) was dissolved in distilled water at a concentration of 0.15 M and autoclaved. Rats (300-350 g) received intraperitoneal injection of 0.15 M LiCl or aseptic saline (0.15 M NaCl) at a dose of 12 mL kg⁻¹.

Real time RT-PCR: Rats were rapidly anesthetized in the carbon dioxide chamber at 6 h after the intraperitoneal injection of LiCl or NaCl (n = 7, total 14 rats) and then decapitated once unresponsive. Brains were rapidly removed and tissue samples of the hippocampus and the medial septum were dissected on ice and frozen immediately in LN₂ until used.

The tissues were homogenized in a single detergent lysis buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 1% Triton X-100; protease and phosphatase inhibitor cocktail 0.5%) and then centrifuged at 13,000 g for 20 min at 4°C. The supernatants were transferred into new tubes and the protein contents were determined using a protein assay kit (Biorad DC, Biorad, Inc., Hercules, CA). Protein samples were aliquoted at an 80 µg/20 µL concentration in lysis buffer and stored at -80°C, otherwise used in the same day. The samples were mixed with loading buffer (100 mM Tris, pH 6.8; 200 mM dithiothreitol; 4% SDS; 20% glycerol; 0.2% bromophenol blue) at 1:1 dilution, boiled for 5 min, quickly chilled on ice and then electrophoresed on 12% SDS-polyacrylamide Tris-glycine gels. The proteins were transferred onto nitrocellulose membranes (Hybond-C, Amersham, Bucks, UK) and the
membranes were treated with 5% nonfat dry milk in 1x phosphate buffered saline-Tween (PBST) (1.46 mM NaH$_2$PO$_4$, 8.05 mM Na$_2$HPO$_4$, 144.72 mM NaCl, 5% Tween 20) overnight at 4°C. The membranes were reacted with goat anti-ChAT antibodies (Chemicon, CA) at 1:1000 dilutions for 1 h and then reacted with HRP-conjugated horse anti-goat antibodies (Vector Laboratories Inc., CA) at 1:1000 dilutions for 1 h at room temperature. The bound antibodies were detected with chemiluminescence according to the manufacturer’s instructions (NEN Life Science Products, Inc., MA) and quantified using a digital image analysis system.

**Immunohistochemistry:** Six hours after the intraperitoneal injection of LiCl or NaCl, rats (n = 4, total 8 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), followed by 400 mL of ice-cold 4% paraformaldehyde (Sigma Co., MO) in 0.1 M sodium phosphate buffer (PB). Brains were immediately dissected, blocked, post-fixed for 2 h and transferred into 30% sucrose (Sigma Co., MO) for cryoprotection. Forty micron coronal sections were cut from the hippocampus and the basal forebrain cholinergic complex including the medial septal nucleus by using a freezing, sliding microtome (HM440E, Microm Co., Germany). Free-floating 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 goat anti-ChAT antibodies (1:500 dilution, Chemicon, CA). Sections were washed twice in PBS-BSA and incubated for 1 h with biotinylated anti-goat IgG (1:200 dilution, Vector Laboratories, CA), then bound secondary antibodies were amplified with the ABC kit (Vectastain Elite Kit, Vector Laboratories, CA). Antibody complexes were visualized with 0.05% of diaminobenzidine (Sigma Co., MO) for 5 min. Sections were mounted in an anatomical order onto gelatin-coated slides from 0.05 M PB, air dried, dehydrated through a graded ethanol to xylene and coverslipped. The number of ChAT immunopositive cells was blind-counted using a Nikon YS2 microscope (Nikon Co., Tokyo, Japan). Cell counts from three sections of the medial septal nucleus (closest sections to bregma 0.60 mm) in each rat were averaged per section, respectively and the individual mean counts were averaged across rats within experimental groups. All coordinates were based on Paxinos and Watson (1986, 2005).

**Statistical analysis:** All data were analyzed by one-way or two-way ANOVA and preplanned comparisons with the control performed by post-hoc Fisher’s PLSD and presented by means±SE. For all comparisons, the level of significance was set at p = 0.05.

**RESULTS**

HCNP mRNA expression levels in the hippocampus were determined at 1 or 6 h after an intraperitoneal injection of 0.15 M LiCl or NaCl at a dose of 12 mL kg$^{-1}$ by quantitative real-time RT-PCR analysis. The hippocampal HCNP mRNA level was acutely increased by lithium and the increase became statistically significant (p<0.05, LiCl vs. NaCl) by 6 h after the drug injection (Fig. 1).

ChAT immunoreactivities (-ir) in the hippocampus and the medial septal nucleus were examined 6 h after an intraperitoneal LiCl or NaCl (0.15 M, 12 mL kg$^{-1}$). ChAT-ir appeared to be increased by lithium in both brain regions observed (Fig. 2A). Quantification analysis of ChAT-ir showed that the number of ChAT-positive neurons increased significantly (p<0.05) in the medial septal nucleus of lithium treated rats, compared to the saline injected controls (Fig. 2B). Western blot analysis of ChAT peptide level performed at 6 h after an intraperitoneal LiCl or NaCl (0.15 M, 12 mL kg$^{-1}$) supported the lithium-induced increase of ChAT-ir in both brain regions observed, i.e., in the medial septum (p<0.001 vs. NaCl) and the hippocampus (p<0.05 vs. NaCl) (Fig. 3).

![Fig. 1: HCNP mRNA expression levels in the hippocampus determined by real-time RT-PCR. Rats were sacrificed 1 or 6 h after an intraperitoneal injection with 12 mL kg$^{-1}$ of 0.15 M LiCl or saline (0.15 M NaCl). *p<0.05 vs. NaCl at 6 h. Data are presented by means±SE](image-url)
Fig. 2: ChAT immunohistochemistry in the brain regions at 6 h after an intraperitoneal injection with 12 mL kg⁻¹ of 0.15 M LiCl (b,d) or saline (a,c). A. Representative photos of the hippocampus (a,b) and the medial septal nucleus (c,d). B. Number of ChAT-positive neurons in the median septal nucleus. *p<0.05 vs. NaCl. Scale bars; 200 μm. Data are presented by means±SE.

Fig. 3: Western blot analysis of ChAT immunoreactivities in the hippocampus and the medial septal nucleus at 6 h after an intraperitoneal injection with 12 mL kg⁻¹ of 0.15 M LiCl or saline. *p<0.05, **p<0.001, data are presented by means±SE.

DISCUSSION

In this study, IHCNP mRNA level was significantly increased in the hippocampus at 6 h after an intraperitoneal lithium injection. Lithium, as a small ion, rapidly diffuses throughout the brain and body (Stern et al., 1977). Intraperitoneal injection of lithium at high dose induces a transient expression of c-fos in several brain regions including the hippocampus (Chen et al., 1999). c-fos consists heterodimer with c-Jun, binds to the DNA AP-1 site and HCNP gene contains AP-1 site in its upstream promoter region (Teohoh et al., 1997). It was also reported that lithium increases transcription factor binding to AP-1 site in cultured neurons and rat brain (Ozaki and Chuang, 1997). Together, it is suggested that intraperitoneal lithium may acutely increase HCNP mRNA expression in the hippocampus, likely, in mediation of c-fos expression.

Besides the HCNP mRNA expression, intraperitoneal lithium acutely increased ChAT immunoreactivity not only in the septal nucleus but also in the hippocampus. The present result concurs with previous reports that lithium increases acetylcholine synthesis and release in the rat brain (Jope, 1979) and enhances cholinergic neuronal activity (Jope and Williams, 1994) and suggests that increased ChAT synthesis may contribute to the lithium-induced activation of the septo-hippocampal cholinergic system. In vitro studies have reported that HCNP up-regulates acetylcholine synthesis and ChAT production in the rat septal cholinergic neurons (Ojika et al., 1992, 1994, 2000). These reports support an idea that increased expression of HCNP mRNA in the hippocampus by lithium might have contributed, at least partly, to the increased ChAT production in this study. Interestingly, it was reported that HCNP is released from hippocampal neurons in vitro following N-methyl-D-aspartate (NMDA) receptor stimulation (Ojika et al., 1998). Previous reports have demonstrated that chronic lithium
treatment inhibits NMDA receptor-mediated calcium influx (Nonaka et al., 1998) and proposed that chronic lithium at therapeutic doses suppresses glutamatergic neurotransmission, particularly via NMDA receptors (Bauer et al., 2003; Farber et al., 2002; Krystal et al., 2002; Stewart and Reid, 2002; Zarate et al., 2003). However, acute lithium above the therapeutic doses has been reported to enhance excitatory synaptic transmission in the hippocampus (Rinaldi et al., 1986; Valentin et al., 1997), which is mediated by NMDA receptors (Colino et al., 1998). We conclude that intraperitoneal lithium may increase not only HCNP expression but also its release in the hippocampus, possibly via NMDA receptors and lead to the activation of septo-hippocampal cholinergic system with increased ChAT production. On the other hands, Iwase et al. (2001) have reported that the septal cholinergic and the hippocampal glutamatergic neurotransmitter pathways exert a reciprocal influence over HCNP precursor mRNA expression, suggesting a possible influence of increased cholinergic activity by lithium over the hippocampal HCNP expression. Further studies are required to define the molecular mechanisms underlying lithium-induced HCNP and ChAT expression.

Lithium dose used in this study is sufficiently high to induce CTA formation (Jahng et al., 2004) and activated the septo-hippocampal cholinergic system with increased expression of HCNP in the hippocampus. Cholinergic activation in brain regions has been implicated in the acquisition of CTA learning (Ferreira et al., 2002; Gonzalez et al., 2000; Naor and Dudai, 1996). Also, the hippocampus has been reviewed to be involved in taste memory (Manrique et al., 2007) and CTA learning (Angst et al., 2007). We suggest that the activation of septo-hippocampal cholinergic system with increased HCNP expression may be a part of lithium’s action in CTA learning as unconditioned stimulus.

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