# Lithium reverses mechanical allodynia through a Mu opioid-dependent mechanism

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<td>Weinsanto, Ivan; Centre National de la Recherche Scientifique Mouheiche, Jinane; Centre National de la Recherche Scientifique Laux-Biehlmann, Alexis; Centre National de la Recherche Scientifique Aouad, Maya; Centre National de la Recherche Scientifique Maduna, Tando; Centre National de la Recherche Scientifique Petit-Demoulière, Nathalie; Centre National de la Recherche Scientifique Chavant, Virginie; Centre National de la Recherche Scientifique Poisbeau, Pierrick; Centre National de la Recherche Scientifique Darbon, Pascal; Centre National de la Recherche Scientifique Charlet, Alexandre; Centre National de la Recherche Scientifique Giersch, Anne; INSERM Parat, Marie-Odile; University of Queensland, PACE Goumon, Yannick; Centre National de la Recherche Scientifique,</td>
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## Abstract:

**Background:** Lithium is widely used to treat bipolar disorders and displays mood stabilizing properties. In addition, lithium relieves painful cluster headaches and has a strong analgesic effect in neuropathic pain rat models.

**Objectives:** Investigate the analgesic effect of lithium on the cuff model of neuropathic pain.

**Methods:** We used behavioral and pharmacological approaches to study the analgesic effect of a single injection of lithium in wild-type and mu opioid receptor (MOR) null cuffed neuropathic mice. Mass spectrometry and ELISA allowed to measure the levels of endogenous MOR agonist beta-endorphin, as well as monoamines in brain and plasma samples 4 hours after lithium administration.

**Results:** A single injection of lithium chloride (100mg/kg, ip) alleviated mechanical allodynia for 24 hours and this effect was absent in MOR null neuropathic mice. Biochemical analyses highlight a significant increase of beta-endorphin levels by 30% in the brain of lithium-treated mice compared to controls. No variation of beta-endorphin was detected in the blood.

**Conclusions:** Together, our results provide evidence that lithium induces a long-lasting analgesia in neuropathic mice presumably through elevated brain levels of beta-endorphin and the activation of MORs.
Lithium reverses mechanical allodynia through a Mu opioid-dependent mechanism

Abbreviated title: lithium relieves neuropathic pain

Ivan Weinsanto¹, Jinane Mouheiche¹, Alexis Laux-Biehlmann¹, Maya Aouad¹,
Tando Maduna¹, Nathalie Petit-Demoulière¹, Virginie Chavant¹,², Pierrick Poisbeau¹,
Pascal Darbon¹, Alexandre Charlet¹, Anne Giersch³,
Marie-Odile Parat⁴ & Yannick Goumon¹,²* 

¹ CNRS UPR3212, Institut des Neurosciences Cellulaires et Intégratives, Centre National
de la Recherche Scientifique and University of Strasbourg, Strasbourg, France

² Mass spectrometry facilities of the CNRS UPR3212, Institut des Neurosciences
Cellulaires et Intégratives, Centre National de la Recherche Scientifique, Strasbourg,
France

³ INSERM U-1114, Fédération de Médecine Translactionnelle de Strasbourg (FMTS),
Département de Psychiatrie, Hôpitaux Universitaires de Strasbourg, Strasbourg, France

⁴ School of Pharmacy, University of Queensland, PACE, 20 Cornwall Street,
Woolloongabba, Australia

*To whom correspondence should be addressed: Dr. Yannick Goumon, INCI, CNRS
UPR3212 ; 5, rue Blaise Pascal, F-67084 Strasbourg Cedex, France, Tel Phone : (33)-3-88-
45-67-18 ; Fax: (33)-3-88-60-16-64. E-mail: yannick.goumon@inserm.u-strasbg.fr

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Abstract

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Results: A single injection of lithium chloride (100mg/kg, ip) alleviated mechanical allodynia for 24 hours and this effect was absent in MOR null neuropathic mice. Biochemical analyses highlight a significant increase of beta-endorphin levels by 30% in the brain of lithium-treated mice compared to controls. No variation of beta-endorphin was detected in the blood.

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Keywords

Lithium, neuropathy, analgesia, mu opioid receptor, beta-endorphin, monoamines.
Background

Lithium is a metallic ion displaying chaotropic and denaturating properties (Breslow and Guo, 1990). This metal is described to treat bipolar disorders (Chiu and Chuang, 2010), to be able to reduce painful cluster headache (Leone et al., 2010) and to decrease inflammation (Nassar and Azab, 2014). Several research groups reported functional interactions between lithium and the opioid system. In particular, lithium affects morphine-induced analgesia (Johnston and Westbrook, 2004; Dehpour et al., 1994), and reduces morphine tolerance and dependence (Dehpour et al., 1995; Alborzi et al., 2006). Lithium attenuates thermal hyperalgesia and mechanical allodynia in different models of neuropathic pain in rats via a naloxone-sensitive mechanism, suggesting that lithium action is opioid receptor-dependent (Banafshe et al., 2012; Shimizu et al., 2000). In addition, lithium prevents paclitaxel-induced peripheral neuropathy (Mo et al., 2012), increases survival by allowing the use of higher doses of paclitaxel and prevents paclitaxel-induced cardiac abnormalities.

The present study reveals that a single injection of lithium alleviates neuropathic pain symptoms in a mouse model of sciatic nerve chronic constriction and demonstrates for the first time MOR involvement in lithium analgesia. In this context the potential variations of endogenous opioid, as well as monoamine levels have been studied after lithium injection.

Methods

Animals

Experiments were performed with 45 day-old adult male C57BL/6J mice (25±4 g; Charles River, L’Arbresle, France). Mu opioid receptor (MOR) null mice were a
generous gift from Pr. B. Kieffer (IGBMC, Illkirch-Graffenstaden, France). Animals were given free access to food and water, with a 12 h light–dark cycle at a temperature of 22°C±2°C. All procedures were performed in accordance with European directives (2010/63/EU) and were approved by the regional ethics committee and the French Ministry of Agriculture (license No. 00456.02 to Y.G.). The right sciatic nerve was cuffed with a section of polyethylene tubing (cuff group) as previously described (Benbouzid et al., 2008). Briefly, surgeries were done under aseptic conditions and ketamine/xylazine was used for anesthesia (ketamine: 17 mg/mL, i.p., xylazine: 2.5 mg/mL, i.p., 4 mL/kg; Centravet, Taden, France). After a performing a 1.5 cm skin incision of the right hind thigh, a 2 mm long polyethylene tubing was placed on the common branch of the right sciatic nerve (ID = 0.38 mm, ED = 1.09 mm; PE-20, Harvard Apparatus, Les Ulis, France) and sutures were used to close the skin (Benbouzid et al., 2008). Sham-operated mice underwent the same surgical procedure as cuffed animals without implantation of the cuff.

Response to mechanical stimuli

The mechanical threshold for hind paw withdrawal was determined using Von Frey hairs as previously described (Benbouzid et al., 2008). Intraperitoneal injections of a solution of 100mg/kg of lithium chloride (corresponding to 16.4 mg/ml of lithium; Sigma-Aldrich, St. Louis, U.S.A.) diluted in NaCl 0.9% (w/v; saline), naloxone (Sigma-Aldrich) diluted in saline or an equivalent volume of saline were performed at 10 am. Injections of naloxone (0.1 mg/kg, s.c.) were performed 4 h after the injections of lithium (corresponding to the
peak of analgesia observed for neuropathic mice) (Desmeules et al., 1993). Hind paw withdrawal was determined 15 min later.

*Response to thermal stimuli*

Mice were placed during 15 min in clear Plexiglas boxes (7 cm x 9 cm x 7 cm) on a glass surface (Hargreaves et al., 1988). The infrared beam of the radiant heat source (7370 Plantar Test, Ugo Basile, Comerio, Italy) was applied to the plantar surface of each hindpaw. The cut-off to prevent damage to the skin was set at 15 s. The paw withdrawal latency was tested 3 times 4 h after lithium injection and was averaged for each hindpaw.

*Sample preparation*

Plasma was prepared from blood recovered in tubes containing 50 µl of 2% EDTA (w/v) and protease inhibitors (cOmplete Mini EDTA-free, Roche, Basel, Switzerland).

Brain was homogenized in 2 ml of 0.5 µM ascorbic acid containing protease inhibitors and sonicated for 10 s at 90 W and centrifuged (20,000 g, 15 min, 4°C). Supernatant was recovered and protein content was determined using the Protein Assay kit (Bio-Rad, Marnes-la-Coquette, France).

*Monoamines and catecholamines derivatization*

The presence of L-DOPA; dopamine, adrenaline; noradrenaline, serotonin and adenosine was studied. 20 µl of tissue extracts or plasma were derived with the AccQ-Tag Ultra Derivatization kit (Waters, Guyancourt, France). 20 µl of the sample were added to 30 µl
of borate buffer (provided within the kit) and 10 µl of internal standards ([2H3]-L-DOPA, [2H4]-dopamine, [2H6]-adrenaline, [13C6]-noradrenaline, [2H4]-serotonin, [13C5]-adenosine; Sigma Aldrich and Alsachim, Illkirch, France). Derivatization was performed by addition of 10 µl of AccQtag Ultra reagent (10 min, 55°C under agitation). 10 µl of this solution were analyzed using a LC-MS/MS approach.

**Beta-endorphin ELISA**

Beta-endorphin concentrations in the brain and plasma were quantified using a direct ELISA (M0184 ELISA, Clinisciences-Elabscience, Nanterre, France) according to the manufacturer's instruction. Samples (50 µl) were analyzed in duplicate. All samples with a duplicate CV>5% were retested to obtain a CV below or equal to 5%. Detection Range was 15.63-1000 pg/ml and sensitivity was 9.38 pg/ml of beta-endorphin.

**LC-MS/MS instrumentation and analytical conditions**

Analyses were performed with a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, USA) coupled with a triple quadrupole Endura. The system was controlled by Xcalibur v2.0 software. Samples were loaded onto an Accucore RP-MS column (100x2.1 mm, 2 µm, Thermo Electron) heated at 50°C. Buffer A was H2O 99.9%/formic acid 0.1% (v/v), whereas buffer B was ACN 99.9%/formic acid 0.1% (v/v). Gradients used are detailed in Table 1.
Table 1 - LC and MS conditions for the purification and the detection of catecholamines and monoamines and their respective heavy tagged counterparts. Buffer A corresponded to ACN 1% / H₂O 98.9% / formic acid 0.1% (v/v/v), whereas buffer B was ACN 99.9% / formic acid 0.1% (v/v).

Electrospray ionization was achieved in the positive mode with the spray voltage set at 3,750 V. Nitrogen was used as the nebulizer gas and the ionization source was heated to
250°C. Desolvation (nitrogen) sheath gas was set to 45 Arb and Aux gas was set to 15 Arb. Ion transfer tube was heated at 350°C. Q1 and Q2 resolutions were set at 0.7 FWHM, whereas collision gas (CID, argon) was set to 2 mTorr. Identification of the compounds was based on precursor ion, selective fragment ions and retention times. Selection of the monitored transitions and optimization of collision energy and RF Lens parameters were manually determined (see Table 1 for details). Qualification and quantification were performed in MRM mode using Quan Browser software (Thermo Scientific). Limits of detection (LOD) and of quantification for each compound are indicated in Table 2. All amounts of opiates present in samples fit within the standard curve limits, Precision values were <1% for same-day measurements and <5% for inter-day measurements.

<table>
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<th>Compound</th>
<th>LOD (fmol ± SEM)</th>
<th>LOQ (fmol ± SEM)</th>
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<tr>
<td>Adenosine</td>
<td>9,77 ± 1,20</td>
<td>32,53 ± 3,99</td>
</tr>
<tr>
<td>Adrenaline</td>
<td>0,71 ± 0,29</td>
<td>2,36 ± 0,97</td>
</tr>
<tr>
<td>Dopamine</td>
<td>4,64 ± 1,54</td>
<td>15,46 ± 5,14</td>
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<tr>
<td>L-DOPA</td>
<td>43,31 ± 1,04</td>
<td>144,24 ± 3,45</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>3,39 ± 0,74</td>
<td>11,29 ± 2,45</td>
</tr>
<tr>
<td>Serotonin</td>
<td>24,04 ± 0,22</td>
<td>80,05 ± 0,73</td>
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Table 2- Limits of detection (LOD), limits of quantification (LOQ) for all compounds.

LOD was defined as the lowest detectable amount of analyte with a signal-to-noise (S/N) ratio >3. LOQ was defined as the lowest detectable amount of analyte with a signal-to-noise (S/N) ratio >10. Data are presented as the mean ± SEM of 5 measurements.

Statistics

Statistical analysis was performed using GraphPad Prism 6 Software. Results were presented as mean values ± standard error of the mean (SEM). Groups were compared using ANOVA tests with Bonferroni correction.
RESULTS

Effect of lithium on the mechanical nociceptive threshold in the cuff neuropathic pain model

Cuff and Sham mice were tested for mechanical pain threshold using the Von Frey filaments test. The cuff group showed a significant mechanical allodynia compared to the sham group (Fig. 1A). Lithium injection performed six days after surgery did not affect the mechanical pain threshold of the sham group. Conversely, in the neuropathic cuff group, lithium normalized the ipsilateral mechanical pain threshold to sham group values. This analgesic effect of a single injection lasted for about 24 h (Fig. 1A). The contralateral paw mechanical pain threshold was unaffected (Fig. 1B) and no statistical difference before and after lithium injection was observed. Additional experiments were designed to determine the effect of lithium 4 h after administration on thermal hyperalgesia (6 days after the surgery). The Hargreaves test (Fig. 1C) shows a significant relief of thermal hyperalgesia for the ipsilateral paw compared to the pretest condition (before lithium administration; n=6; Mann–Whitney U test; p<0.01). Heat-nociceptive threshold of the contralateral paw was not affected by lithium,
Fig. 1 - Analgesic effect of lithium chloride. Antinociceptive effect of lithium chloride (100 mg/kg, i.p., day 6) vs vehicle (NaCl) on sham and cuffed mice. A, Effect of lithium administration on ipsilateral paw mechanical allodynia. B, contralateral paw. n=6; Two way ANOVA test with a Bonferroni correction. **, p<0.01). C, effect of lithium 4 h after administration on thermal hyperalgesia (6 days after the surgery; Hargreaves test); n=6; Mann–Whitney U test; **, p<0.01). Values are means ± SEM.

Effect of naloxone on lithium-induced analgesia

In order to determine if this analgesic effect involves the endogenous opioid system, naloxone (0.1 mg/kg, s.c.), a non-specific opioid receptor antagonist, was administered 4 h after lithium injections (corresponding to the peak of analgesia observed for neuropathic
mice; Fig.1A). Naloxone alone did not modify the mechanical threshold, but significantly decreased the analgesic effect of lithium compared to pre-test values (Fig. 2A) when testing the ipsilateral paw. The contralateral paw mechanical pain threshold was not affected.

Fig. 2- Mu opioid receptor-dependent analgesic effect of lithium chloride on neuropathic mice. A, Effect of naloxone (0.1 mg/kg, s.c.) on lithium-induced analgesia (100 mg/kg, i.p.). n=6 per group; two way ANOVA test with a Bonferroni correction; $=p<0.01. B$, Effect of lithium chloride on MOR null neuropathic mice; n=6 per group, ANOVA test ; n=6; ns, non significant. Values are means ± SEM. Pretesting group corresponds to mechanical threshold before lithium and/or naloxone injections. 4h group corresponds to mechanical threshold observed 4h after lithium and/or naloxone injections.

Effect of lithium on neuropathic MOR-null mice

To assess whether MORs were necessary to observe lithium analgesia, sham and cuff MOR null mice were treated with a single injection of lithium. Lithium-induced analgesia was never observed in MOR-null mice (Fig. 2B) as illustrated by the stability of the mean
mechanical threshold observed in lithium and vehicle-treated cuffed mice. No effect was observed on the contralateral paw mechanical pain threshold.

**Effect of lithium on the level of endogenous mediators**

While MORs have been long known to promote analgesia, opioid ligands such as beta-endorphin as well as non opioid neurotransmitters may be produced locally or after recruitment of classical pain controls acting in CNS circuits or at the periphery. Plasma and brains of saline and lithium-treated neuropathic animals were analyzed using biochemical approaches aimed at measuring plasma levels of noradrenaline, adenosine, serotonin and beta-endorphin. No differences between saline- and lithium-treated cuff groups were found (Fig. 3). Adrenaline, dopamine and L-Dopa were below detection levels. Values obtained for beta-endorphin, serotonin, noradrenaline and adenosine were in agreement with values published in the literature (Fell et al., 2014; Grouzmann et al., 2003; Ziu et al., 2014; Hu et al., 2016).
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Fig. 3- Effect of lithium chloride on noradrenaline, adenosine, serotonin and beta-endorphin plasma levels of neuropathic mice. Li, Lithium chloride; n=7 or 8. Mann-Whitney test.

In the brain, adrenaline, noradrenaline, dopamine, L-Dopa, adenosine and serotonin levels were not affected by a lithium treatment (Fig. 4) and were in agreement with levels described for healthy animals (Gogos et al., 1998; Delaney et al., 1998; Bengel et al., 1998). In sharp contrast, beta-endorphin levels were significantly increased by 30% (p=0.0047; 54.9±4.1 pg/mg of brain protein) in lithium-treated cuff mice compared to the saline group (42.1±2.1 pg/mg). Similarly, the values of beta-endorphin brain levels (low pg/mg physiological range) are consistent with previous studies (Gong et al., 2014).
**Fig. 4** Effect of lithium chloride on adrenaline, noradrenaline, dopamine, L-Dopa, adenosine, serotonin and beta-endorphin brain levels of cuffed mice. Li, Lithium chloride; n=8, 7 or 6 (please see graphs). Mann-Whitney test.; *, p<0.05; **, p<0.01. Values are means ± SEM.

**DISCUSSION**
In addition to the physiological processing of acute pain by the nociceptive system, inflammatory and neuropathic insults may sometimes result in chronic pain that persists long after recovery from the initial lesions (taxonomy., 1986). In such pathological states, pain no longer plays its physiological warning role. Most therapeutic approaches aimed at alleviating chronic pain symptoms have beneficial effects but suffer from adverse side effects (Dworkin et al., 2010; Ho and Siau, 2009). This is the case for morphine and related opiates that are widely prescribed to chronic pain patients and may lead to the development of tolerance, opioid-induced hyperalgesia and addiction (Roeckel et al., 2016; Williams et al., 2013). Therefore, alternative therapeutic strategies are needed.

Studies examining the role of lithium on pain responses are unfortunately contradictory so far. On one side, lithium seems to induce hyperalgesia and to decrease morphine-induced analgesia (Wiertelak et al., 1994; McNally and Westbrook, 1998; Johnston and Westbrook, 2004). In addition, lithium overdose can, in a few cases, cause peripheral neuropathy or myopathy in patients (Timmer and Sands, 1999). In mice and rats, it has been hypothesized that lithium induces a biphasic effect on morphine-induced analgesia that is dependent on lithium concentration and on the pain test used (de Gandarias et al., 2000; Dehpour et al., 1994; Raffa and Martinez, 1992). On the other hand, several groups did not observe any direct effect of lithium on pain perception using a mouse model (Karakucuk et al., 2006).

Interestingly, lithium was shown to relieve cluster headache, which causes pain episodes of extreme intensity. Lithium also reduced the associated autonomic symptoms in humans (Robbins et al., 2016). Moreover, acute and chronic administration of lithium induce a direct analgesia in neuropathic pain states (Mannisto and Saarnivaara, 1972; Banafshe et
al., 2012; Shimizu et al., 2000; ), and potentiate morphine analgesia in mouse and rat models (Karakucuk et al., 2006; Jensen, 1974). Lithium treatments also reversed thermal hyperalgesia, as well as the mechanical and cold allodynia induced by a partial sciatic nerve ligation in rats in a naloxone-sensitive manner (Banafshe et al., 2012; Shimizu et al., 2000; ). Furthermore, lithium has been shown to prevent paclitaxel-induced peripheral neuropathy in mice (Mo et al., 2012). In humans, a clinical trial performed with lithium medication had a favorable effect on sciatic nerve injury (SNI) neuropathic pain (Yang et al., 2012); moreover, lithium was able to relieve tricyclic antidepressants-refractory fibromyalgia (Tyber, 1990). Our present data confirm the long-lasting analgesic effect of acute lithium administration on a well characterized mouse neuropathic model (Benbouzid et al., 2008). In addition, we show that MORs are necessary for lithium-induced analgesia, as no analgesic effect was observed in MOR-null mice.

Lithium acts on numerous targets that have been recently reviewed (Alda, 2015; Oruch et al., 2014). Different mechanisms of action have been proposed and include a possible substitution of Na\(^+\) by Li\(^+\) impacting homeostasis of electrolyte balance and therefore neuronal firing, or a modulation of the membrane transport of different ions and neurotransmitter precursors (Jope et al., 1978). In rats, lithium aversive effects (place-preference conditioning procedure) were abolished by naloxone, suggesting a beta-endorphin-dependent mechanism (Shippenberg et al., 1988). In addition to being a modulator of NO production in the brain, lithium inhibits Gi and Gs proteins leading to an inhibition of adenylate and guanylate cyclases and of different protein kinases (Schubert et al., 1991). Moreover, lithium affects GSK-3\(\beta\) (glycogen synthase kinase 3 (Klein and Melton, 1996)) acting therefore on both Akt (protein kinase B) and Wingless-related...
integration site (Wnt) signaling (Valvezan and Klein, 2012). This compound is also able to inhibit inositol monophosphatase and inositol polyphosphate-1-phosphatase (Lopez-Coronado et al., 1999; Shaltiel et al., 2009), influencing inositol-dependent regulatory processes. It also reduces CREB phosphorylation and decreases CREB-dependent gene expression (Boer et al., 2008). Lithium’s anti-inflammatory properties lead to a down-regulation of both proinflammatory cytokines and TNF-alpha interleukin and intracellular mechanisms including GSK-3β (Nassar and Azab, 2014). Finally, lithium has also been shown to regulate the biosynthesis of different neurotransmitters and/or associated receptors (e.g., modulation of serotonin and glutamate synthesis and secretion) (Lenox and Wang, 2003; Massot et al., 1999; Scheuch et al., 2010; Jope, 1999; Shippenberg et al., 1988).

Our results indicate that acute lithium treatment has a strong anti-allodynic effect as well as a stimulatory effect (+30%) on the production of brain beta-endorphin, a MOR agonist displaying strong analgesic properties. In good agreement with our data, it has been described that stress-induced analgesia is absent in mice lacking beta-endorphin (Rubinstein et al., 1996) and that ultraviolet light induces both analgesia and addiction through a 35% increase of plasma beta-endorphin levels (Fell et al., 2014). More recently, photobiomodulation therapy performed on the chronic constriction injury mouse model (CCI) correlated the level of pain relief to an increase of beta-endorphin levels (de Andrade et al., 2017). However this effect is still unclear since previous studies reported that in vivo chronic treatment with lithium did not modify beta-endorphin levels in different rat brain structures (Shippenberg and Herz, 1991) whereas in vitro and ex vivo experiments demonstrated that an acute stimulation with lithium increases the release of hypothalamic
beta-endorphin (Burns et al., 1990). As a 35% blood beta-endorphin increase induces a strong elevation of mechanical and thermal thresholds (Fell et al., 2014), the 30% increase in beta-endorphin brain content we observed after lithium injection should likely be sufficient to induce a robust analgesia.

Plasma levels of beta-endorphin are tightly linked to secretions from the pituitary and adrenal gland, whereas brain and cerebrospinal fluid levels are mainly dependent on the arcuate nucleus of the hypothalamus and of the brainstem nucleus tractus solitarius. In addition, beta-endorphin displays a short half-life in rodents’ blood (2 to 10 min) (Houghten et al., 1980; Silman et al., 1977) while in the CNS, degradation of beta-endorphin is described to be extremely long (Burbach et al., 1979; Silman et al., 1977).

Therefore, the beta-endorphin elevation observed in the brain 4 h after a lithium injection is likely due to (i) an up-regulation of beta-endorphin production from the arcuate nucleus and the nucleus tractus solitarius associated with a (ii) longer brain half-life. While the cuff is a model of peripheral neuropathy, CNS beta-endorphin secretion can normalize the ipsilateral paw threshold by acting on supraspinal (e.g. periaqueductal gray, rostral ventromedial medulla) or spinal nociceptors.

Together with other studies showing the analgesic effect of acute lithium treatment on chronic pain, our results suggest that lithium analgesia involves the upregulation of beta-endorphin synthesis in the CNS. This would explain, at least in part, the MOR-dependent nature of the analgesic properties of lithium.
ACKNOWLEDGMENTS

The authors declare that they have no competing interests.

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