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Different pharmacokinetics of lithium orotate inform why it is more potent, effective, and less toxic than lithium carbonate in a mouse model of mania

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ABSTRACT

Lithium carbonate (LiCO) is a mainstay therapeutic for the prevention of mood-episode recurrences in bipolar disorder (BD). Unfortunately, its narrow therapeutic index is associated with complications that may lead to treatment non-compliance. Intriguingly, lithium orotate (LiOr) is suggested to possess unique uptake characteristics that would allow for reduced dosing and mitigation of toxicity concerns. We hypothesized that due to differences in pharmacokinetics, LiOr is more potent with reduced adverse effects. Dose responses were established for LiOr and LiCO in male and female mice using an amphetamine-induced hyperlocomotion (AIH) model; AIH captures manic elements of BD and is sensitive to a dose-dependent lithium blockade. LiCO induced a partial block of AIH at doses of 15 mg/kg in males and 20 mg/kg in females. In contrast, LiOr elicited a near complete blockade at concentrations of just 1.5 mg/kg in both sexes, indicating improved efficacy and potency. Prior application of organic anion transport inhibitors, or inhibition of orotate uptake into the pentose pathway, completely blocked the effects of LiOr on AIH while sparing LiCO effects, confirming differences in transport and compartmentalization between the two compounds. Next, the relative toxicities of LiOr and LiCO were contrasted after 14 consecutive daily administrations. LiCO, but not LiOr, elicited polydipsia in both sexes, elevated serum creatinine levels in males, and increased serum TSH expression in females. LiOr demonstrates superior efficacy, potency, and tolerability to LiCO in both male and female mice because of select transport-mediated uptake and pentose pathway incorporation.

1. Introduction

Lithium salts have been used for more than half a century to combat the psychiatric manifestations of bipolar disorder and, while antipsychotics and anticonvulsants have gained in popularity, lithium remains a frontline therapeutic option (Culpepper, 2014). Of the presently prescribed lithium formulations, lithium carbonate (Li_2CO_3; LiCO henceforth) is the most administered, and is one of the most effective medications for the prevention of mood-episode recurrences (Machado-Vieira et al., 2009; Malhi et al., 2020; Miura et al., 2014; Severus et al., 2014; Won and Kim, 2017; Zivanovic, 2017) as well as treatment of acute mania/hypomania (McKnight et al., 2019). Unfortunately, LiCO-based therapy displays a narrow therapeutic window with a dose-dependent side effect profile that ranges from mild-to-moderate during short-term use (*e.g.,* polydipsia, polyuria) to potentially severe following chronic prescription (*e.g.,* nephrogenic diabetes insipidus, hypothyroidism). Consequently, treatment non-adherence is a frequently encountered issue with LiCO therapy (Öhlund et al., 2018).

Lithium orotate ($LiC_5H_3N_2O_4$; LiOr henceforth), most notable for its use and advocacy by Hans Nieper in the 1970s (Nieper, 1973), may represent a treatment option that displays lower dosage requirements relative to LiCO with a subsequent reduction in side effect incidence. Nieper proposed that orotic acid was a mineral carrier that could more readily transport inorganic ions – such as lithium, magnesium, or calcium – across biological membranes (Nieper, 1970, 1973). Although evidence for enhanced brain availability was initially found (Kling et al., 1978), research into LiOr was discontinued largely due to studies that demonstrated LiOr to increase impairment of kidney function when used at concentrations equivalent to LiCO (Smith and Schou, 1979). While renal toxicity is a concern, we propose that the purported improved bioavailability enables reduced dosage requirements that will mitigate safety concerns. However, given the relative paucity of data surrounding its efficacy, tolerability, and mechanisms of action, as well as its widespread availability and non-prescription usage, as recently summarized

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(Pacholko and Bekar, 2021), additional research into the pharmacological properties of LiOr are not only warranted, but necessary.

The present study explored the efficacy and potency of LiOr relative to LiCO across a range of concentrations, with the typical therapeutic dose of lithium (adjusted for a murine model) serving as the upper bound. To this end, amphetamine-induced hyperlocomotion (AIH), which has been shown to be attenuated by lithium in a dose-dependent manner (Gould et al., 2007), was used to assess dose requirements of the different lithium compounds.

2. Methods and materials

2.1. Animals

Male and female C57Bl/6NCrl mice (Charles River, Canada) aged 8 weeks were used for all studies. Mice were housed in pairs and kept on a 12-hr light/dark cycle. All experiments were approved by the University of Saskatchewan Animal Research Ethics Board and done according to the Canadian Council on Animal Care.

2.2. Drugs

2.2.1. In vivo studies

LiCO – purchased as a powder from Sigma-Aldrich (ON, CA) – was dissolved in distilled water before adjusting the sodium chloride (NaCl) concentration to 0.9%. LiOr was synthesized by combining lithium hydroxide (Sigma-Aldrich; ON, CA), and orotic acid (Sigma-Aldrich; ON, CA) in a 1:1 M ratio in distilled water; the NaCl concentration was adjusted to 0.9% and pH to 7.4. For all *in vivo* studies, lithium compound weights are reported as elemental lithium (Li^+) for ease of comparison. Dextroamphetamine (*d*A) sulfate tabs (5 mg) were dissolved in saline and administered at 6 mg/kg (0.1 ml/10 g bodyweight) via intraperitoneal injection (IP). The polyethylene glycol-400 (PEG-400; Sigma-Aldrich; ON, CA) solution was prepared by adding PEG-400 to distilled water in a 1:1 ratio (50% final concentration), while naringin (Sigma-Aldrich; ON, CA) and 6-azauracil (Sigma-Aldrich; ON, CA) were dissolved in saline. All drugs were administered in 0.1 ml/10 g bodyweight, with 50% PEG-400 delivered via oral gavage (OG) and 100 mg/kg naringin/30 mg/kg 6-azauracil delivered via IP injection.

2.2.2. Ex vivo studies

LiCO was added to aCSF to yield a 0.6 mM concentration. For 0.6 mM LiOr, the drug was first synthesized in heated distilled water by adding LiOH and orotic acid in a 1:1 M ratio; the individual components of aCSF were then added before bringing the solution to the final volume with distilled water. For all *ex vivo* studies, drugs were added to the artificial cerebrospinal fluid (aCSF) used to perfuse the slices during recording. The 1% PEG-400 solution was prepared by adding 1 ml of PEG-400 to 99 ml of aCSF. For the 50 μM naringin solution, naringin was dissolved in 100 μl of DMSO then added to 99.9 ml of aCSF. A 1.25 μM 6-azauracil solution was prepared by dissolving 6-azauracil in 100 μl of hot 1 M NH_4OH, then adding the resultant mixture to 99.9 ml of aCSF.

2.3. Behavioral tests

2.3.1. Amphetamine-induced hyperlocomotion

Mice were administered *d*-amphetamine (*d*A, 6 mg/kg) or saline intraperitoneally (IP), placed into an open field arena (35 × 35 × 35 cm) for 120 min, and scored for total locomotion offline using Ethovision XT 11 (Noldus, Wageningen, The Netherlands). Drug efficacy was measured as ability of the tested lithium compound – administered IP 30 min prior to *d*A – to diminish AIH. For PEG-400, naringin, and 6-azauracil trials, 50% PEG-400, 100 mg/kg naringin, or 30 mg/kg 6-azauracil solutions were delivered OG (PEG) or IP (naringin/6-azauracil) 30 min prior to lithium injection. Locomotion is reported as percentage of the *d*A response maintained. Minimal Effective Concentration (MEC) is defined as the lowest lithium concentration used to affect AIH attenuation.

2.3.2. Rotarod (locomotor function)

Male mice were injected with saline or LiCO/LiOr 60 min prior to being placed on an I-755 rotarod apparatus (Campden Instruments, Leicster, United Kingdom). The rod was accelerated from 4 rpm to 45 rpm over 2 min. The average time to fall of the last three of four trials for each animal was recorded.

2.3.3. Forced swim test

Male mice were injected with saline or LiCO/LiOr 60 min prior to being placed into a 4 L beaker filled with 3 L of room temperature water. Activity was recorded for 8 min and analyzed for time spent immobile offline using Ethovision XT 11 software (Noldus, Wageningen, The Netherlands).

2.4. Biochemistry

Animals were anaesthetized using urethane (0.2 mg/ml) and xylazine (150 mg/ml) prior to sacrifice (0.1 ml/10 g body weight). Whole blood and brains were subsequently harvested. Blood was collected via cardiac puncture, deposited into a 1.5 ml Eppendorf, and allowed to clot on ice for 24 h at 4 °C prior to centrifugation at 1500 rcf at 4 °C for 15 min using a 5804 R centrifuge (Eppendorf, Framingham, MA, United States). Serum aliquots were held at −80 °C. Mouse brains were rapidly removed, flash frozen in isopentane, and stored at −80 °C. Frozen brains were ground into fine powder, mixed with chilled 0.1M PBS/0.5% tween-20 (5 μg tissue/ml), mechanically homogenized via sonication with three separate 10-s pulses and centrifuged at 20,000 rcf at 4 °C for 15 min (5804 R centrifuge, Eppendorf). Supernatants were additionally ultracentrifuged at 200,000 rcf at 4 °C for 30 min using an Optima XE ultracentrifuge (Beckman Coulter, Brea, CA, United States); final supernatants were stored at −80 °C.

Subsequent spectrophotometric quantification of target serum analytes was performed using a Spectramax M5 spectrophotometer (Molecular Devices, San Jose, CA, United States). See below for relevant methodological details.

2.4.1. Lithium colorimetric assay

Brain and serum Li^+ content was assayed using a commercially available colorimetric assay (Abcam, item no. Ab235613). Brain samples required adjustment of the sample:sodium-masking-agent:assay-buffer ratio to 15 μl: 15 μl: 120 μl from the kit recommended 5 μl: 15 μl: 130 μl for serum.

2.4.2. BUN colorimetric assay

5 μL of serum was diluted 1:9 in 45 μL of distilled water. The diluted samples were assessed for BUN content using a commercially available BUN colorimetric assay (Invitrogen, item no. EIABUN).

2.5. Creatinine ELISA

15 μL of serum was assayed for creatinine content using a commercially available creatinine kinetic colorimetric assay (Cayman chemical, item no. 700460).

2.5.1. TSH ELISA

30 μL of serum was diluted 1:3 in 90 μL of assay diluent (provided with kit). The diluted samples were assessed for TSH content using a commercially available mouse TSH ELISA kit (Elabscience, item no. E-EL-M1153).

2.5.2. AST ELISA

2 μL of serum was diluted 1:99 in 200 μL of assay diluent (provided with kit). The diluted samples were assessed for AST content using a

commercially available mouse AST ELISA kit (Abcam, item no. ab263882).

2.5.3. GSK3β activity assay

5 μL of 2 mM LiOr or LiCO were contrasted for their ability to blunt GSK3β *in vitro* activity using a commercially available GSK3β activity-based kinetic colorimetric assay (BPS Bioscience, item no. 79700). The assay required use of the Kinase-Glo Max Luminescent reagent (Promega, item no. V6071). Concentrations were chosen based on the IC50 for lithium-induced inhibition of GSK3β activity being 2 mM.

2.5.4. Resistivity assay

Resistivity was measured using a patch clamp amplifier (Axopatch 700B; Molecular Devices, San Jose, CA, United States) and pClamp 10 software (Molecular Devices, San Jose, CA, United States). A 10-mV voltage jump in current clamp mode was performed during solution transitions from 20 mM LiCl to 20 mM LiOr. A 20 mM concentration was used for each compound to increase the ease at which alterations to current flow could be detected.

2.5.5. Acute live brain slice electrophysiology

Six-to ten-week-old male C57BL/6 mice were anaesthetized with isoflurane and decapitated. The brain was rapidly removed and submersed in ice-cold aCSF. A Leica VT 1200 vibratome (Leica Biosystems; ON, CA) was used to cut 350 μm thick coronal sections that included the hippocampus. Brains were sliced in chilled aCSF containing the following (in mM): 130 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$-$2H_2O$, 2 $MgSO_4$-$7H_2O$, 0.1 Na-ascorbate, 24 $NaHCO_3$, 10 dextrose, and 1 lactate. The solution had a pH of 7.4 when saturated with 95% O_2 and 5% CO_2. Slices were transferred to a recovery chamber filled with the same aCSF saturated with 95% O_2 and 5% CO_2 for 2 h at room temperature prior to experimentation.

Slices were placed into a 2 ml chamber continually perfused with the same aCSF (saturated with 95% O_2 and 5% CO_2) at a rate of ~4 ml/min at 32 °C. Slices were imaged using a Nikon SMZ1000 microscope (Nikon; ON, CA) for placement of stimulating and recording electrodes. Field excitatory postsynaptic recordings were obtained with a differential amplifier (DP311; Warner Instruments; CT, US) connected to a Digidata 1440A (Molecular Devices; CA, US) using Clampex 10.7 software (Molecular Devices; CA, US). Signals were captured at 2 kHz, high-pass filtered at 1 Hz and low-pass filtered at 300 Hz. Recording electrodes (borosilicate glass filled with 0.9% saline; 4–6 MΩ) were placed within the stratum radiatum of CA1 200–500 μm from the stimulating electrode. Stimulation (30% of max value; only recordings with a max greater than 2.0 mV were used in this study) was applied to the Schaffer collaterals using a concentric bipolar stimulating electrode (TM88CCINA; WPI; FL, US) via a constant current stimulator (Iso-Flex; Microprobes; MD, US) every 20 s for the duration of all baseline and post-long-term potentiation (LTP) experiments. LTP was induced via theta-burst stimulation (TBS) where 8 bursts (at 5 Hz) of 4 pulses (at 100 Hz) were delivered 3 times, 60 s apart and repeated a second time 300 s after the first.

For each experiment, baseline recordings were performed for 15 min in the presence/absence of LiCl or LiOr. For tests involving PEG-400, naringin, or 6-azauracil, the drugs were washed-in via the perfusate for a full 15-min prior to commencement of the baseline recording. Following TBS, post-LTP field potentials were recorded for a full 30 min. Exposure to the relevant drugs (LiOr, LiCl, PEG-400, naringin, 6-azauracil, or a combination of each) was maintained during the TBS and post-LTP phases. The amplitudes of each of the final 10 sweeps of the 30-min post-LTP recording were expressed as a percentage of the amplitudes of the final 10 sweeps of the baseline recording.

2.6. Statistics

Data are expressed as mean ± SEM and compared using one-way or two-way ANOVA with Dunnett's (one-way) or Bonferroni's (two-way) post-hoc tests to assess differences between treatment groups (GraphPad Prism V8.1.2; GraphPad Software, Inc. SD, CA). $p < 0.05$ used as the threshold for significance. SigmaStat 4.0 (Systat Software, Inc. SJ, CA, United States) was used for the construction of dose-response curves.

3. Results

3.1. Amphetamine-induced hyperlocomotion (AIH)

Like humans, mice display hypomanic/manic-like phenotypes when administered dopaminergic stimulants (Mamelak, 1978; Murphy et al., 1971; Peet and Peters, 1995; Wingo and Ghaemi, 2008). Administration of *d*A in rodents produces an elevation in central dopamine levels leading to hyperlocomotor activity that reflects manic aspects of bipolar disorder (Ashok et al., 2017; Sharma et al., 2016). The administration of 6 mg/kg *d*A to mice consistently resulted in two distinct peaks of easily quantified hyperlocomotor activity between minutes 5–35 and 70–120 (Fig. 1). The 70-120-min window was selected to contrast the effects of LiCO and LiOr in these studies because locomotion was particularly robust during this period and potential confounds (IP injection stress, acclimation to novel environment, different pharmacokinetics) for the other peak were of less concern.

3.2. LiOr is more efficacious, potent, and long-lasting than LiCO in the blockade of AIH

To assess the ability of LiCO/LiOr to attenuate AIH, we injected the compounds at various concentrations 30 min prior to *d*A administration. We found that single administration of LiCO or LiOr blunted AIH in a dose-dependent manner from minutes 70–120 post-*d*A, with LiOr demonstrating a substantially reduced minimal effective concentration (MEC) relative to LiCO in both males and females (Fig. 2A). In males, the MEC was 15 mg/kg for LiCO, and 1.5 mg/kg for LiOr (Fig. 2A, top). Interestingly, the MEC of LiCO demonstrated a rightward shift from 15 mg/kg to 20 mg/kg in females while the MEC for LiOr remained at 1.5 mg/kg (Fig. 2A, bottom). Concurrent with these reduced dose requirements, the strength of blockade elicited by LiOr (75.46 ± 16.95% males; 97.45 ± 6.78% females) was greater than that produced by LiCO (67.18 ± 9.64% males; 82.4 ± 3.99% females). In fact, the MEC for LiOr (1.5 mg/kg in both males and females) elicited a more robust block than the maximum dose used for LiCO.

The effects of the MEC for each compound on baseline locomotion and locomotor capacity were assayed using the open field and forced swim/rotarod tests, respectively. These experiments were performed to determine the contributions, if any, of either suppressed exploratory activity (open field) or diminished locomotor function (forced swim/rotarod) to the suppression of AIH elicited by each lithium compound. No changes to baseline activity in the open field and/or impairments to locomotor function in the forced swim and rotarod tests were induced by either LiCO or LiOr in the absence of *d*A in male mice (Fig. 2B), thereby signifying that the suppression of hyperlocomotion was not due to any lithium-induced reductions in baseline locomotor activity and/or ability. Also of note, the improved effects of LiOr relative to LiCO are not attributable to orotic acid alone; sodium orotate had no effect on AIH (Fig. 2B).

Given that LiOr has previously been demonstrated to lead to a progressive increase in central Li^+ levels over the span of 24-h (Kling et al., 1978), we sought to determine whether LiOr could blunt hyperlocomotion when dosed 12, 24 or 36 h prior to *d*A challenge. We observed that 15 mg/kg LiCO failed to elicit a significant effect at any time-point beyond 30 min (Fig. 2C). In contrast, 2.5 mg/kg LiOr was found to block 66%, 56%, and 52% of AIH at the 12-, 24-, and 36-h post *d*A-injection time-points, respectively (Fig. 2C). Thus, LiOr demonstrates improved potency (improved effect at reduced concentrations), efficacy (greater blockade of hyperlocomotion), and duration in the attenuation

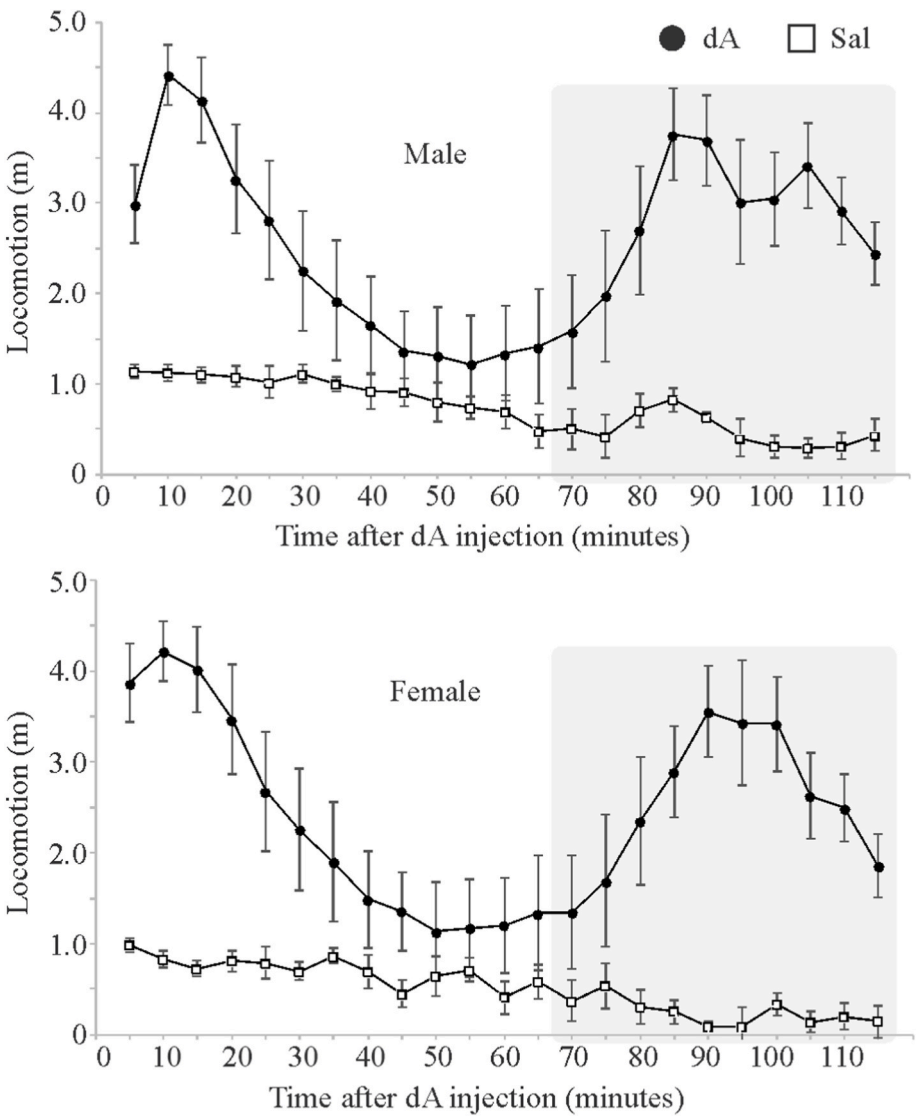

Fig. 1. Effects of *d*-amphetamine on locomotor activity.
The administration of 6.0 mg/kg *d*A consistently resulted in two periods of hyperlocomotion in both males and females. For consistency, the shaded 70–120 min period was used to contrast the efficacy and potency of each compound. Error bars represent mean ± SEM. For the females, n = 5 for *d*A and the saline control; for the males, n = 11 for *d*A and 8 for the saline control. *d*A - d-amphetamine; Sal - 0.9% saline.

of hyperlocomotion relative to LiCO.

3.3. Evidence for an altered biodistribution of LiOr relative to LiCO

One of the ways in which LiOr is proposed to differ from LiCO is in its lack of dissociation within physiological solutions (Marshall, 2015; Nieper, 1973). Low resistivity is indicative of a solution that allows current flow (Tenny and Keenaghan, 2022). Thus, resistivity can be used to assess the degree of a compound's dissociation/ionization (the lower the electrical resistivity, the greater the degree of ionization). When the dissociation of LiOr and LiCO was contrasted in distilled water, we found that the electrical resistivity of a 20 mM LiCO solution was markedly lower than that of a 20 mM LiOr solution, indicating that LiCO undergoes a greater degree of ionization (Fig. 3A). A 20 mM concentration was used for each compound to enable the detection of altered current flow (this is difficult to achieve when using lesser concentrations). These results were confirmed using a more physiologically relevant GSK3β activity assay, where 2 mM LiCO, but not 2 mM LiOr, resulted in an ~50% reduction in GSK3β activity (Fig. 3B). Inhibition of GSK3β is an established outcome of lithium therapy (Grimes C and Jope R, 2001; Muneer, 2017; Williams et al., 2002; Yu and Greenberg, 2016), and is believed to have clinical relevance in BD (Jope, 2011; Jope and Roh, 2006; Muneer, 2017; Yu and Greenberg, 2016). This inhibition is made possible by the similar ionic radii of Li^+ and magnesium, which allows for Li^+ to displace magnesium (a required cofactor) from the catalytic core of the enzyme. Thus, Li^+ must first be liberated from its carrier before it can interact with GSK3β. Consequently, the lack of inhibition elicited by 2 mM LiOr heavily suggests that the compound did not dissociate into its constituent ions at physiological pH, which was not the case for 2 mM LiCO. Of note, the IC50 for lithium-induced inhibition of GSK3β *in vitro* is ~2 mM (Kirshenboim et al., 2004), which means that a 50% attenuation of enzyme activity is expected for a fully dissociated lithium compound.

If LiOr does not readily dissociate into orotic acid and Li^+, then it likely moves throughout the body in a different manner than LiCO (which becomes Li^+ and CO_3^{2-}). Organic anion transporting polypeptides (OATPs) may be involved in transport of LiOr owing to their affinity for large hydrophobic organic anions and abundant localization within both the brain and blood-brain-barrier (BBB) (Roth et al., 2012). To explore the potential role of OATPs in the uptake and subsequent efficacy of LiOr, we probed the abilities of PEG-400 and naringin – specific inhibitors of OATP1A2/Oatp1a1/Oatp1a4 (Bailey et al., 2007; Engel et al., 2012b) – to affect the efficacy of LiOr and/or LiCO in the attenuation of AIH. The application of either 50% PEG-400 (OG) or 100 mg/kg naringin (IP injection) 30 min prior to IP injection of lithium completely prevented the blockade of AIH ordinarily induced by

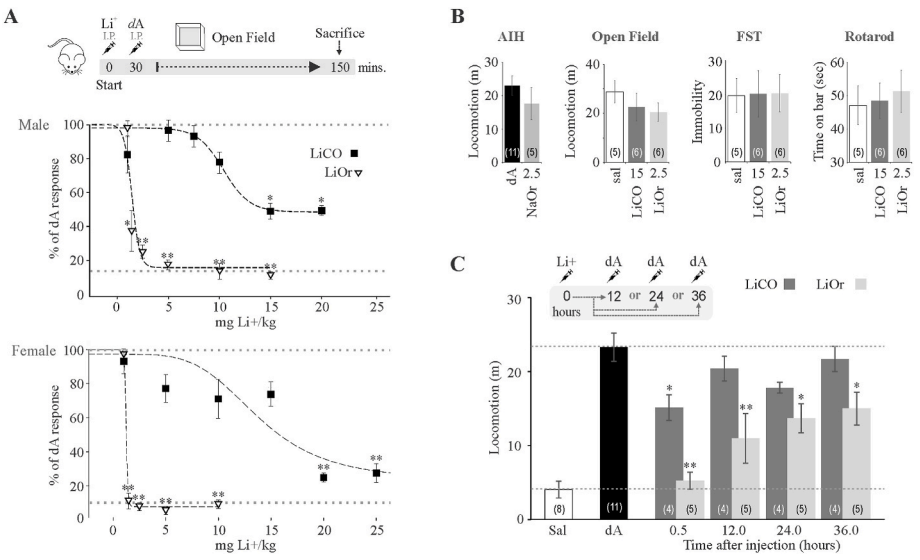

Fig. 2. Comparison of LiOr and LiCO effects on lithium-sensitive amphetamine-induced hyperlocomotion.

A) LiOr and LiCO were administered 30 min before *d*A (6 mg/kg). Locomotor scores were expressed as a percentage of *d*A-induced responses. Dashed lines represent 0% (top; *d*A) and 100% (bottom, near the 13% mark on the y-axis; saline) blockade of hyperlocomotion. **B)** The effects of 2.5 mg/kg LiOr and 15 mg/kg LiCO on baseline activity and motor capacity was assessed using the Open Field Test, Forced Swim Test, and rotarod. The contribution of sodium orotate (2.5 mg/kg) to the effects of LiOr on AIH were also assessed in the Open Field. **(C)** The efficacy of LiOr (2.5 mg/kg) and LiCO (15 mg/kg) against AIH were contrasted 12, 24, or 36 h post-administration. All lithium concentrations are presented as mg of elemental lithium per kg of body weight. Error bars represent the mean ± SEM. All groups were compared to the *d*A control via one-way ANOVA with Dunnett's post-hoc testing. *P < 0.05. **P < 0.01. For the male groups in panel A, n = 11 for dA, 9 for saline, 6 for LiOr 10 and LiOr 15, 7 for LiOr 1, LiOr 1.5, LiOr 2.5, and LiOr 5; for the female groups in panel A, n = 5 for all groups other than LiOr 1.5 and LiCO 15, which had 6. For panels B and C, sample sizes are enclosed within the parentheses situated near the bottom of each histogram. LiOr - lithium orotate; LiCO - lithium carbonate; *d*A - d-amphetamine; Sal - 0.9% saline; NaOr - sodium orotate.

administration of 2.5 mg/kg LiOr, whereas the 20 mg/kg dose of LiCO was unaffected and continued to blunt AIH as expected (Fig. 3C; *interaction* Li^+ x PEG, p = 0.0002; *interaction* Li^+ x naringin, p = 0.0474). The 2.5 mg/kg LiOr and 20 mg/kg LiCO treatments were chosen for these studies because they represent the doses at which a maximal blockade of hyperlocomotion was observed for each respective compound in male mice.

To generalize our findings to additional experimental contexts as well as assess the contributions of the BBB, we repeated the above experiments using an *ex vivo* live brain slice platform that bypasses the BBB. As 0.6 mM LiOr and 0.6 mM lithium chloride (LiCl) strengthen the LTP observed within the CA1 hippocampal subfield after TBS (Fig. 3D, left panel), we assessed whether 15-min pre-treatment of slices with 1% PEG-400 or 50 μM naringin in aCSF would blunt the LTP enhancing effects of either lithium compound. Consistent with the AIH model, pre-application of either PEG-400 or naringin blunted the LTP promoting actions of 0.6 mM LiOr but not 0.6 mM LiCl (Fig. 3D). These results indicate that a) the impact of these drugs on LiOr's efficacy extends to multiple experimental paradigms and that b) their effects are not limited to the BBB and/or peripheral vasculature (*e.g.*, enteric or peritoneal blood vessels). LiCl was used in place of LiCO because they both equally dissociate in solution, it has a near identical pharmacokinetic profile (Morrison et al., 1971), and does not alter the pH of the aCSF solutions.

To exert its therapeutic influence, LiOr must eventually dissociate from its carrier. Hans Nieper proposed that LiOr preferentially targets cell types rich in pentose phosphate pathway (PPP) activity (Nieper, 1973). Given the associations between this pathway and pyrimidine biosynthesis (Stincone et al., 2015), we theorized that the dissociation of LiOr may be mediated by uridine monophosphate synthase (UMPS) during the incorporation of the orotic acid carrier into the biosynthetic pathway. In short, UMPS catalyzes the decarboxylation of orotic acid (Huang and Graves, 2003), and may thus liberate lithium from its carrier by cleaving the carboxyl group to which it is bound. To assess this, the UMPS inhibitor 6-azauracil (Krsiak and Janků, 1969) was employed in both the *in vivo* AIH and *ex vivo* brain slice models in a manner identical to that of PEG-400 and naringin. Pre-application of 6-azauracil in either the AIH model (30 mg/kg IP; 30 min prior to lithium injection) or brain slice model (1.25 μM; 15 min prior to lithium wash-in) completely blunted the effects of 2.5 mg/kg LiOr on hyperlocomotion or 0.6 mM LiOr on LTP, respectively, whereas 20 mg/kg LiCO (AIH) and 0.6 mM LiCl (brain slice) were unaffected (Fig. 3C and D, right).

3.4. In contrast to LiCO, LiOr shows no effect on water intake or kidney and thyroid function

The potential early adverse effects of LiOr and LiCO on kidney and thyroid health – characterized, in part, by aberrant serum TSH, AST, BUN and/or creatinine levels – were contrasted in male and female mice at concentrations of 1x, 2x or 3x the MEC (MEC was 1.5 mg/kg for LiOr and 15 mg/kg for LiCO) once daily for 14 consecutive days. When allometrically scaled, the LiCO concentrations used herein roughly correlate to the therapeutic range employed in humans, *i.e.*, 15–45 mg Li^+/kg in mice translates to ~400–1200 mg of total LiCO in an adult patient (correction factor ratio for human to mouse scaling) (Nair and Jacob, 2016). All mice were sacrificed on the 15th day, 24 h after receiving their final lithium dose.

As polydipsia is a frequent adverse effect of lithium use, we compared the water intake of mice treated with either compound. LiCO, but not LiOr, elicited polydipsia when administered at concentrations greater than or equal to 2x the MEC, with the first signs of excessive water intake observed on day 5 for the 3x dose and day 10 for the 2x dose (Fig. 4A). While the dose-dependent effects of LiCO were similar in each sex, the degree of induced polydipsia was more pronounced in males and demonstrated a progressive increase over time at all concentrations (Fig. 4A, top), whereas water intake plateaued on days 10-through-15 in females treated with the 3x dose (Fig. 4A, bottom). Body weight was unaffected by either treatment (Fig. 4B).

Next, we assessed treatment effects on serum BUN and creatinine, which are waste products used to assess kidney function. Consistent with the lack of effect on polydipsia, LiOr did not alter serum creatinine levels, even when employed at concentrations three-fold greater than its MEC (Table 1). In contrast, we observed that the 3x dose of LiCO significantly elevated creatinine levels above control in the male cohort (Table 1). Serum BUN levels were unaffected (Table 1).

No alterations in serum AST content, which can indicate kidney and/or liver damage when increased, were observed at this early time-point for either LiCO or LiOr (Table 1).

Finally, we assessed the impacts of each lithium treatment on serum

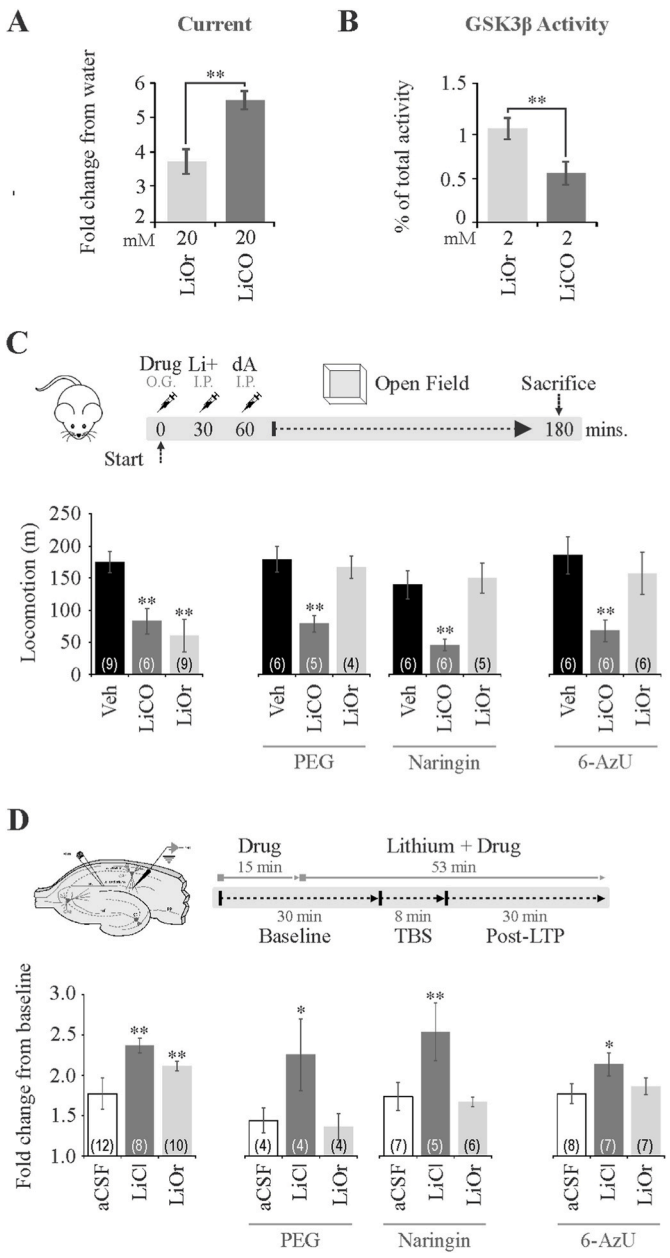

Fig. 3. Comparison of LiOr and LiCO differences in biodistribution.
A) Small voltage steps were applied to either 20 mM LiCO or LiOr dissolved in water to measure the resistivity of each solution. **B)** The effects of 2 mM LiCO and 2 mM LiOr on GSK3β activity was assessed. The IC50 for lithium-induced inhibition of GSK3β *in vitro* is ~2 mM. **C)** PEG-400, naringin, or 6-azauracil were applied 30 min prior to IP injection of LiOr/LiCO in the AIH model. **D)** Live slices were exposed to PEG, naringin or 6-azauracil via the perfusate 15-min prior to wash-in of 0.6 mM LiOr or LiCl. The effects of each inhibitor on the ability for LiOr/LiCl to strengthen hippocampal LTP were assessed. Error bars represent mean ± SEM. For panels **A** and **B**, LiOr and LiCO were contrasted using an unpaired *t*-test. *P < 0.05, **p < 0.01. For panels C and D, all groups were contrasted to the *d*A or aCSF controls, respectively, via two-way ANOVA with Bonferroni's post-hoc testing. Sample sizes are enclosed within parentheses. LiCl – lithium chloride; LiCO - lithium carbonate; LiOr - lithium orotate; *d*A - d-amphetamine; PEG-400 – polyethylene glycol 400; 6-AzU – 6-azauracil; TBS – theta-burst stimulation; LTP – long-term potentiation.

TSH, which serves as a clinical marker of lithium-induced hypothyroidism. LiCO, but not LiOr, elevated TSH expression in females, whereas the male mice were unaffected (Table 1; two-way ANOVA *interaction*, $p < 0.001$).

In summary, LiOr did not elicit any adverse effects on either water intake or serum biomarkers of lithium toxicity, even when dosed at three times its MEC. In contrast, treatment with LiCO elevated serum TSH in females, serum creatinine in males, and polydipsia in both males and females.

3.5. *LiOr retains efficacy at concentrations that are undetectable within the serum*

We observed that serum Li^+ levels were elevated in mice treated with LiCO or LiOr relative to saline alone in both males and females (Fig. 5A; saline not shown due to failure to meet the 0.1 mM detection threshold). Interestingly, the serum Li^+ levels resultant of LiOr administration were only detectable at concentrations 1.67 (males) or 3.33 (females) times greater than the MEC of 1.5 mg/kg, whereas LiCO generated detectable levels when employed at concentrations well below its MEC of 15 mg/kg (Fig. 5A).

As LiOr has previously been found to increase central Li^+ levels relative to LiCO (Kling et al., 1978), brain Li^+ was contrasted in LiCO and LiOr treated mice. We found that both lithium compounds significantly increased brain Li^+ levels relative to control at all tested concentrations, with LiOr-treated mice displaying higher brain Li^+ levels than LiCO at concentrations greater than or equal to 10 mg/kg in both males (Fig. 5B left) and females (Fig. 5B right).

4. Discussion

Despite early evidence of reduced dosage requirements relative to LiCO, the use of LiOr in psychiatric applications has gone largely unexplored over the past 50 years (Kling et al., 1978; Nieper, 1973; Sartori, 1986). Using the AIH model of mania (Fig. 1) – which was selected for its high-throughput and established dose-sensitivity to lithium – we found LiOr to be more potent, efficacious, and long-lasting than LiCO in the blockade of hyperlocomotion (Fig. 2). These differences likely relate to the observations that LiOr did not readily dissociate in solution and utilized alternative transport mechanisms (Fig. 3). Furthermore, we observed an absence of adverse effects on markers of kidney and thyroid health with LiOr (Figs. 4 and 5), supporting the notion that at the lower effective dosages, LiOr is a safer alternative to LiCO.

The lines of evidence supporting the translational potential of the improved potency and efficacy of LiOr relative to LiCO are manifold. First, the actions of lithium against hyperlocomotion are dependent upon amelioration of the amphetamine-induced increase in GSK3β signaling downstream of dopamine receptors (Beaulieu et al., 2008); amphetamine elevates GSK3β activity, in part, through inhibition of the dopamine transporter (DAT), which subsequently results in an enhanced dopaminergic tone. As excessive GSK3β output (Jope, 2011; Jope and Roh, 2006; Muneer, 2017; Yu and Greenberg, 2016), increased expression of dopamine receptors, and reduced availability of DAT have been implicated in BD pathogenesis (Anand et al., 2011; Ashok et al., 2017; Milienne-Petiot et al., 2017; van Enkhuizen et al., 2014), the odds that the improved potency/efficacy of LiOr noted in the AIH model will translate to the human condition appear promising. Second, while no clinical trials for the use of LiOr in BD have been conducted, the disparity in the MEC between LiOr and LiCO pertaining to blockade of AIH is mirrored in studies exploring their efficacy in the cessation of alcohol abuse. LiOr has shown success in reducing alcohol consumption when administered daily for 6 months at a dose of 150 mg/day (~6.4 mg of Li^+) (Sartori, 1986), whereas LiCO is either mildly efficacious (Fawcett et al., 1987) or outright ineffective (Dorus et al., 1989) when employed at substantially greater doses (>600 mg/day; ~112 mg of Li^+). While these studies on alcohol consumption may not translate to BD, they nevertheless provide an additional context within which LiOr elicits an effect at a substantially reduced dose relative to LiCO, which would not be the case if the two compounds were in fact identical regarding their pharmacokinetic and pharmacodynamic properties.

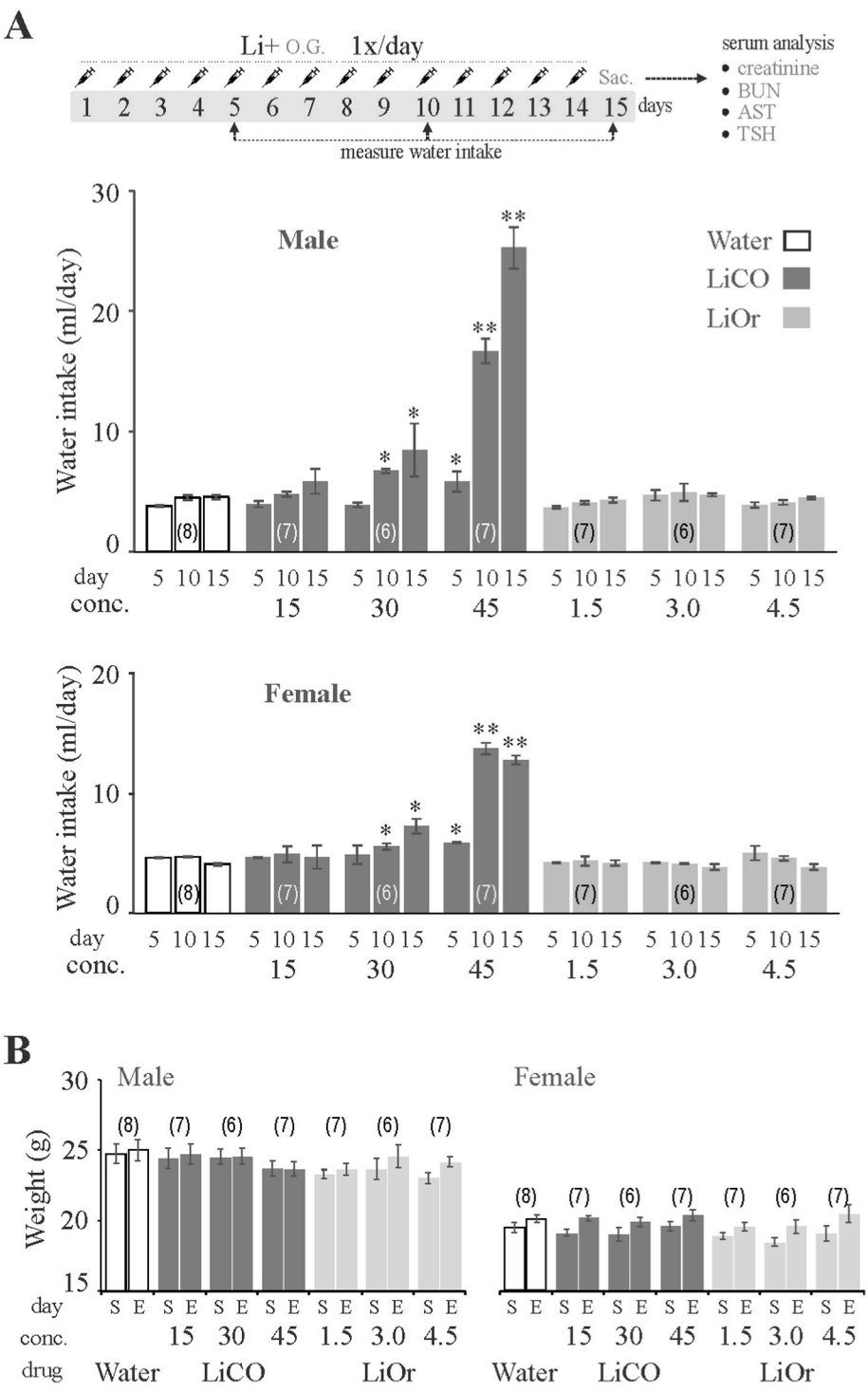

Fig. 4. Comparison of LiCO and LiOr effects on water intake in male and female mice.
A) All compounds were delivered via oral gavage once daily for 15 consecutive days. Water intake was measured every 5 days. **B)** Animal weights were recorded at the outset and conclusion of the protocol for all treatment groups. Error bars represent the mean ± SEM. For the assessment of water intake **(A)**, all groups were compared to the water-sustained control group via one-way ANOVA with Dunnett's post-hoc testing. Weight gain or loss was assessed via one-way ANOVA with Tukey's post-hoc testing (starting weights were compared to end weights for each group). *P < 0.05. **P < 0.01. Sample sizes are enclosed within parentheses. LiOr - lithium orotate; LiCO - lithium carbonate; W - water; S - start weight; E – end weight.

Finally, the MEC for LiCO translates to ~500+ mg of LiCO/day in an 80 kg man or 70 kg woman when scaled from rodent to human, which aligns with the lower end of the effective range employed during lithium therapy and supports the idea that the dose necessary for blockade of AIH roughly correlates with the therapeutic dosages used for the control of mania.

In concert with its reduced dosage requirements, LiOr did not elicit any adverse kidney health-related outcomes (elevated BUN, creatinine, polydipsia, etc.) at doses up to three-fold greater than its MEC. Given the positive association between serum Li^+ levels and toxicity (Malhi, 2015), it is possible that this tolerability is attributable to the fact that the MEC for LiOr does not give rise to detectable levels of Li^+ within the serum. Additionally, the increased duration of effect noted for LiOr (12–36 h post-administration) may result in a smoother serum Li^+ curve over time that minimizes the incidence of "Li^+ spikes"; lithium-induced toxicity is worsened by acute spikes in serum Li^+ levels (Malhi, 2015). In contrast, LiCO elicited elevations in serum creatinine content concurrent with severe polydipsia in male mice, suggesting an impaired ability to concentrate urine in a manner that may reflect vasopressin resistance, which is a frequent complication of lithium use. While the differences between LiOr and LiCO at this early time point are insufficient to definitively state that LiCO will display toxicity during chronic

Table 1
Measures of kidney and thyroid toxicity.

Sex	Drug	Conc. xMEC[a]	Creatinine mg/dl	BUN mg/dl	AST pg/ml	TSH ng/ml
Male	Control	0x	1.64 ± 0.16 (7)	23.17 ± 1.70 (6)	5284 ± 909 (6)	5.59 ± 0.57 (7)
	LiCO	1x	1.88 ± 0.24 (6)	23.77 ± 0.94 (6)	5847 ± 701 (5)	5.34 ± 0.65 (6)
		2x	1.72 ± 0.13 (6)	21.81 ± 0.80 (6)	3970 ± 167 (5)	4.97 ± 0.70 (6)
		3x	**2.43* ± 0.13 (6)**	24.22 ± 1.74 (6)	5722 ± 150 (5)	5.06 ± 0.34 (7)
	LiOr	1x	1.72 ± 0.27 (6)	21.55 ± 0.75 (6)	7845 ± 2650 (3)	5.59 ± 0.40 (5)
		2x	1.66 ± 0.15 (6)	22.31 ± 0.49 (6)	4933 ± 270 (5)	5.58 ± 0.93 (4)
		3x	1.67 ± 0.15 (7)	22.50 ± 0.52 (6)	5850 ± 684 (5)	4.70 ± 0.46 (7)
Female	Control	0x	1.52 ± 0.07 (6)	20.87 ± 1.53 (6)	5190 ± 503 (4)	7.87 ± 1.17 (6)
	LiCO	1x	1.62 ± 0.10 (6)	21.62 ± 1.11 (6)	7007 ± 1427 (5)	10.09 ± 1.10 (6)
		2x	1.71 ± 0.22 (6)	21.02 ± 1.15 (6)	6988 ± 1077 (5)	**18.86* ± 3.79 (6)**
		3x	1.68 ± 0.10 (7)	18.48 ± 0.79 (6)	7119 ± 656 (4)	**13.09* ± 1.87 (7)**
	LiOr	1x	1.86 ± 0.12 (6)	18.53 ± 1.32 (6)	5134 ± 477 (4)	9.82 ± 1.21 (7)
		2x	1.33 ± 0.08 (6)	18.07 ± 1.02 (6)	4384 ± 498 (5)	9.57 ± 2.55 (6)
		3x	1.63 ± 0.14 (7)	19.70 ± 0.83 (6)	5386 ± 758 (4)	8.65 ± 0.93 (6)

*P < 0.05 via one-way ANOVA with Bonferroni post-hoc testing. All groups compared to control.
BUN – blood-urea-nitrogen; TSH – thyroid stimulating hormone; AST – aspartate aminotransferase; LiOr – lithium orotate; LiCO – lithium carbonate.
The numbers enclosed within parentheses represent sample size.
[a] Concentrations for LiOr and LiCO are based on 1x, 2x or 3x of the MEC determined using the AIH model.

treatment while LiOr will not (Gitlin, 2016), the failure of LiOr to elicit water-balance-associated side-effects, which are frequently encountered during the early stages of lithium therapy, suggests that LiOr will demonstrate superior long-term tolerability. This supposition is supported by the absence of any reported cases of serious side effects in over 40 years of LiOr use in North America (Devadason, 2018).

In line with our observations pertaining to kidney health, LiCO, but not LiOr, elicits an elevation in serum TSH at therapeutically relevant concentrations in female mice, which suggests that LiOr may spare thyroid output. Thus, the seemingly improved tolerability of LiOr may be of particular benefit to female BD patients, who are known to be at greater risk for the development of lithium-induced hypothyroidism than their like-aged male counterparts (Henry, 2002).

Opposing our submission of improved tolerability, some have suggested the improved efficacy of LiOr to be attributable to reduced glomerular filtration rates that ultimately culminate in worsened renal health outcomes (Kling et al., 1978; Smith and Schou, 1979). However, our present results are supported by a recent 28-day toxicological evaluation of LiOr at doses up to 400 mg/kg/day in rats (elemental Li^+ ~ 15 mg/kg/day) in which no adverse effects were found (Murbach et al., 2021). Furthermore, the most well-known case of LiOr-induced toxicity seemingly highlights its safety. In 2007, a case report detailing a scenario in which 18 LiOr tablets (3.83 mg Li^+/tablet) were ingested showed that the patient merely displayed nausea, minor tremors, and normal vital signs, with all symptoms resolving after 3 h of observation without intervention (Pauzé D and Brooks D, 2007). The MEC for LiOr in the attenuation of AIH is roughly equivalent to just 2–3 LiOr tablets in a reasonably sized human man (80 kg) or woman (70 kg).

The early proponents of LiOr argued that the improved efficacy of the compound is linked to the utilization of uracil-specific transport systems as well as affinity for tissues highly expressing the pentose phosphate pathway (Nieper, 1970, 1973). The structure of LiOr closely resembles that of 5-fluorouracil, which is a non-charged pyrimidine known to be an exogenous substrate for the ubiquitously expressed equilibrative nucleotide transporters (Wohlhueter et al., 1980). While intriguing, the greatest support for the notion that LiCO and LiOr differ in terms of transport may be our findings that a) LiOr does not readily dissociate into its constituent ions, and that b) PEG-400 and naringin completely prevent the inhibition of AIH and strengthening of LTP induced by LiOr while sparing the effects of LiCO. As PEG-400 and LiOr were administered via different routes in the AIH model (OG and IP, respectively), it is likely that the effects of PEG-400 are chiefly attributable to its inhibition of OATPs. OATP1A2 (Oatp1a1 and Oatp1a4 in mice) appears to be of particular importance, as it is localized within neurons, glial cells, and the endothelium of the BBB (Schäfer et al., 2021), and is a specific target for inhibition by both PEG-400 (Engel et al., 2012a) and naringin (Bailey et al., 2007). This may explain the absence of polydipsia (kidney vasopressin resistance) observed in the LiOr treated mice; LiOr may not be concentrated in the kidneys to the same extent that LiCO is. Thus, while LiCO requires large serum Li^+ concentrations in order to "drive" Li^+ into cells, the putative transport- and dissociation-related properties of the orotic acid carrier may reduce dose requirements by allowing delivery of Li^+ directly to the intracellular target site (bypassing other organ systems?), as was originally proposed by Hans Nieper in the early 1970s (Nieper, 1973). The theory that LiOr targets and dissociates within cells that display high rates of PPP activity (Nieper, 1973) is supported by our observation that inhibition of UMPS robustly attenuates the actions of LiOr, but not LiCO, on AIH and LTP. UMPS, an intracellular enzyme within the *de novo* pyrimidine biosynthesis pathway (for which orotic acid is a substrate), may enable the dissociation of LiOr via cleavage of the carboxyl group to which Li^+ is bound. Additionally, pyrimidine biosynthesis and the PPP are in close association (Stincone et al., 2015), which suggests that a UMPS-mediated dissociation of LiOr would preferentially lead to accumulation of Li^+ in cells with high PPP expression.

As always, this study is not without limitations. First, it would be of great benefit to generalize the differential dose response characteristic of LiOr and LiCO to additional experimental contexts, such as the ouabain-induced model of mania. Second, a lengthened toxicity protocol (*e.g.,* 6 months) would strengthen our findings of the improved tolerability of LiOr relative to LiCO; however, it is worth noting that other toxicological evaluations of LiOr support its safe usage (Murbach et al., 2021).

In closing, the reduced dosage requirements observed for LiOr in the present study appear to dispel the concerns regarding renal toxicity raised in 1979 (Smith and Schou, 1979) as well as ameliorate the dose-dependent, compliance-disrupting side-effects associated with current LiCO therapy. Given the potency, efficacy, apparent tolerability, and wide-spread availability of this over-the-counter nutraceutical, clinical trials for the use of LiOr are warranted.

Author statement

Anthony Pacholko: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft, Writing – Review & Editing. **Lane Bekar:** Conceptualization, Writing – Review & Editing,

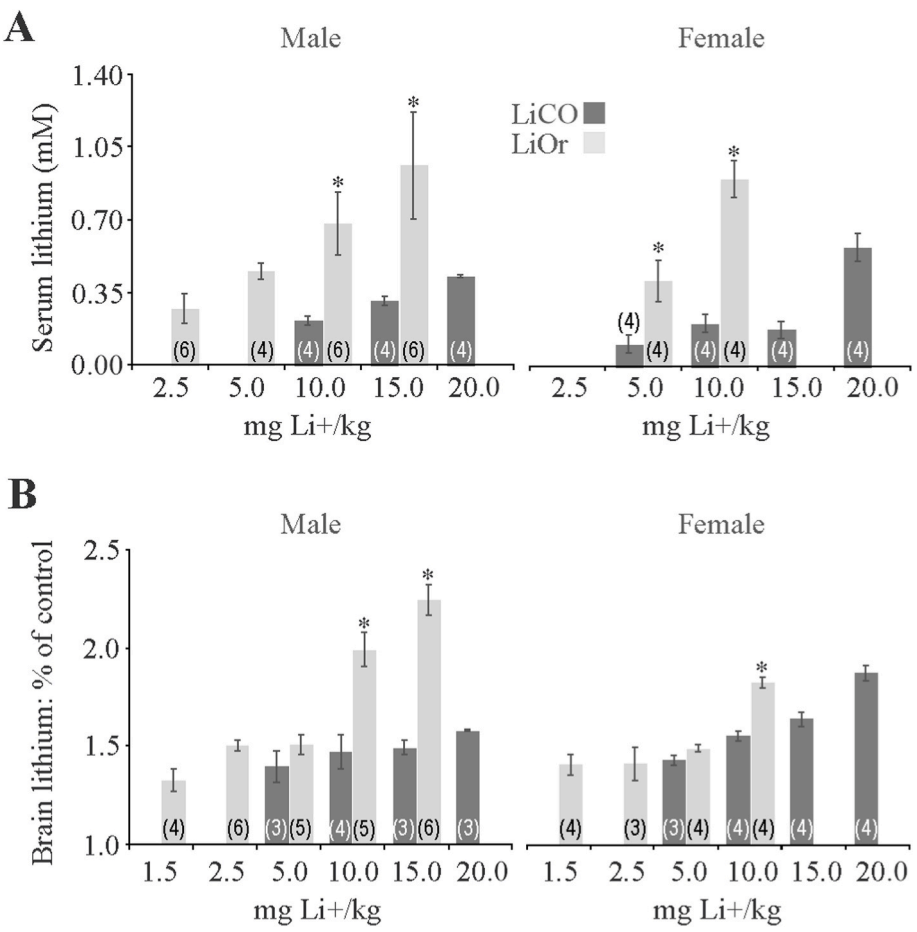

Fig. 5. LiOr yields greater brain and serum lithium levels than LiCO.
A) The levels of lithium within the serum were determined for all treatment groups via colorimetric assay. **B)** Brain lithium levels were assessed for all treatment groups using homogenized and purified tissues. Error bars represent mean ± SEM. Brain lithium content for all groups was contrasted to the saline control via one-way ANOVA with Dunnett's post-hoc (matched concentrations, e.g., LiOr 5 versus LiCO 5, were compared using Tukey's post-hoc test). *P < 0.05, **p < 0.01. The detection limit was 0.1 mM. Sample sizes are enclosed within parentheses. LiOr – lithium orotate; LiCO – lithium carbonate.

Supervision, Funding acquisition.

Declaration of competing interest

Dr. Bekar and Mr. Pacholko report no biomedical financial interests or potential conflicts of interest.

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