LITHIUM OROTATE AS AN IMPROVED THERAPEUTIC AGENT IN THE TREATMENT OF MANIA: PRECLINICAL EVIDENCE FROM A MURINE MODEL

A Thesis Submitted to
the College of Graduate and Postdoctoral Studies
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
in the Department of Anatomy, Physiology, and Pharmacology
University of Saskatchewan
Saskatoon
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ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Lane Bekar, for his guidance, support, and friendship over the past 6 years. He took a chance on a former high school teacher with no laboratory experience, and for that I will be forever grateful. I wouldn’t be here today if not for his superb mentorship.

I would also like to thank the members of my thesis advisory committee, Dr. Grzegorz Sawicki (chair), Dr. John Howland, Dr. Changiz Taghibiglou, and Dr. Rohit Lodhi (cognate member), for their time, guidance, support, and constructive feedback over the tenure of my graduate schooling. I greatly appreciate the atmosphere of positivity and growth that they established for each of my TAC meetings; I was always made to feel comfortable and respected, and for that I am truly thankful.

Finally, I would like to thank my wife, Ashley Breault, for her tireless support throughout this process. Her relentless encouragement and refusal to allow me to do anything other than pursue my wildest dreams has altered the course of my life beyond anything I once thought to be possible. I also owe a tremendous debt of gratitude to Gordon Pacholko and Danielle Pacholko, my amazing father and sister, as well as my late mother, Diane Pacholko, an incredible woman whose memory I will forever hold close. They have believed in me since day one, even when I didn’t believe in myself. Their guidance, moral lessons, and friendship have proved invaluable in my life, and I couldn’t imagine a better support system.

All the work presented herein was funded by College of Medicine Research Awards (bridge funding), whereas my salary support was provided by an NSERC PGSD3 doctoral scholarship.
CONTRIBUTIONS

Anthony Pacholko was involved in every aspect of the following work. This includes formulating ideas, performing experiments, collecting and analyzing data, interpreting results, and preparing experimental findings for publication (figure creation, manuscript writing, revision, etc.). Apart from one figure (Box 3), all content presented herein was collected, analyzed, and prepared by Anthony Pacholko with no external influences or contributions.

The data in chapters 3-5 was included, in part, within: (Pacholko et al., 2023). AG Pacholko was responsible for performing the experiments, analyzing the data, and preparing the manuscript for publication (including initial submission, revision, and resubmission).
**ABSTRACT**

Lithium carbonate (LiCO) is a mainstay therapeutic for the prevention of mood-episode recurrence in bipolar disorder (BD). Unfortunately, lithium therapy is plagued by high rates of treatment non-compliance due to the unpleasant adverse effects associated with presently prescribed medications. Given that lithium-induced toxicities correlate with serum exposure, a lithium-based therapeutic that displays similar efficacy concurrent with lesser dosing requirements would likely improve treatment regimen adherence. Lithium orotate (LiOr) may meet this criteria, as previous research suggests it to possess unique uptake characteristics that may enable reductions in dosing and subsequent amelioration of toxicity. Thus, we hypothesized that LiOr is more potent than LiCO due to differential transport, allowing mitigation of side effect incidence consequent to lesser dosing requirements. Dose response curves 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 block. LiCO induced a partial attenuation 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, signifying superior potency and efficacy. Prior application of inhibitors of organic anion transporting polypeptides, or inhibition of orotate uptake into the pyrimidine biosynthesis pathway, completely abolished the effects of LiOr on AIH while sparing those of LiCO, thereby confirming the differential transport and compartmentalization of the two compounds. As proposed in our hypothesis, the increased potency of LiOr translated to improved tolerability: after 14 consecutive days of once daily administration, LiCO, but not LiOr, elicited polydipsia in both sexes, elevated serum creatinine expression in males, and increased serum TSH levels in females. In summation, LiOr demonstrates superior efficacy, potency, and tolerability to LiCO in both male and female mice, likely because of select transport-mediated uptake and incorporation into the pyrimidine biosynthesis pathway.
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LIST OF ABBREVIATIONS

BD – bipolar disorder
LiCO – lithium carbonate
LiOr – lithium orotate
Li⁺ – elemental lithium
UMPS – uridine monophosphate synthase
CNS – central nervous system
OATP – organic anion polypeptide
ENT – equilibrative nucleoside transporter
AIH – amphetamine-induced hyperlocomotion
MEC – minimal effective concentration
TSH – thyroid stimulating hormone
LTP – long-term potentiation
PEG-400 – polyethylene glycol-400
GSK3β – Glycogen synthase kinase-3β
IMPase – inositol monophosphatase
D2R – type-2 dopamine receptor
DAT – dopamine transporter
NKA – Na⁺,K⁺-ATPase
BDNF – brain-derived neurotrophic factor
CREB – cyclin-AMP response element binding protein
PI – phosphatidylinositol cycle
BBB – blood-brain barrier
PIP2 – phosphatidylinositol-4, 5-bisphosphate
PIP3 – phosphatidylinositol 3,4,5-triphosphate
PI3K – PI3 kinase
IP3 – inositol 1,4,5-triphosphate
PKC – protein kinase C
TNFα – tumor necrosis factor-α
IL-6 – interleukin-6
NHE – sodium-proton exchanger
ISBD – International Society of Bipolar Disorders
OUA – ouabain
GluR6 – glutamate receptor 6
NaCl – sodium chloride
dA – dextroamphetamine
OG – oral gavage
IP – intraperitoneal
aCSF – artificial cerebrospinal fluid
BUN – blood-urea-nitrogen
AST – aspartate aminotransferase
TBS – theta-burst stimulation
GFR – glomerular filtration rate
LTD – long-term depression
NDI – nephrogenic diabetes insipidus
AQP2 – aquaporin 2
CHAPTER 1: INTRODUCTION

Bipolar disorder (BD) encompasses a host of related conditions of incompletely understood origin characterized by cycling manic and depressive states (1). Lithium salts, made popular in the early 1950s through late 1960s by John Cade (2) and Samuel Gershon (3), have been used for more than half a century to combat the psychiatric manifestations of BD. While antipsychotics and anticonvulsants have gained in popularity, lithium remains a frontline therapeutic option (1) for its efficacy in limiting suicidality and preventing mood episode recurrence (4-9). Of the presently prescribed formulations, lithium carbonate (Li$_2$CO$_3$; LiCO henceforth) and lithium citrate are the most frequently administered. Unfortunately, these compounds have narrow therapeutic windows with dose-dependent side effect profiles that range from mild-to-moderate during short-term use (e.g., polydipsia, polyuria) to potentially severe following chronic prescription (e.g., nephrogenic diabetes insipidus, hypothyroidism), ultimately culminating in high rates of treatment non-adherence (10).

Lithium orotate (LiC$_5$H$_3$N$_2$O$_4$; LiOr henceforth) may represent an alternative which, relative to LiCO, displays lesser dosing requirements and a concomitant reduction in side effect incidence. One of the earliest supporters of mineral orotates, the controversial Hans Nieper, offered that orotic acid conjugates more readily transport inorganic ions across biological membranes than do their carbonate-based counterparts (11, 12). If true, then the superior transport-related properties of LiOr could be leveraged to reduce the concentrations of elemental lithium (Li$^+$) employed during therapy, which would in turn improve patient compliance by limiting the appearance of adverse effects; lithium-induced toxicity correlates with the ion’s concentration within the plasma (13). In support, a 1978 study performed by Kling and associates found that 24-hour brain lithium levels are ~3-fold greater in rats when administered as an orotate as opposed to as a carbonate (14). With that said, the question of exactly how, and if, LiCO and LiOr differ remains unanswered.

Intriguingly, the differential transport characteristics hypothesized for LiOr and LiCO may be attributable to a set of complementary factors. First, proponents of LiOr argue that mineral orotates do not dissociate at physiological pH, and thus present in sera as electrically neutral compounds. Such complexes could potentially cross biological membranes with greater efficiency than the Li$^+$ ions resultant of LiCO (12, 15), which dissociates readily in solution and thus has its ingress into the brain limited by the regulatory mechanisms that govern sodium flux (16-19). Second, LiOr has
been theorized to preferentially target and dissociate within cells that display high rates of pentose-phosphate pathway activity, such as neurons and glia (12). Given that lithium is bound to the carboxyl group of orotic acid, a site-specific dissociation of LiOr could potentially be triggered by uridine monophosphate synthase (UMPS); UMPS is a constituent enzyme within the pyrimidine biosynthesis pathway that serves, in part, to decarboxylate orotic acid during its conversion to uridine monophosphate. As de novo pyrimidine synthesis and the pentose phosphate pathway are in close association (20), a UMPS-mediated dissociation would explain why LiOr demonstrates a putative bias toward accumulation within the central nervous system (CNS) (14). Lastly, the non-dissociated nature of LiOr likely enables routes of transport unavailable to LiCO, such as organic anion transporting polypeptides (OATPs), which mediate the passage of a plethora of exogenous compounds (21), or equilibrative nucleoside transporters (ENTs), which facilitate the translocation of several non-charged pyrimidines structurally related to LiOr (e.g., 5-fluorouracil (22)).

Despite early promise (14), research into LiOr was discontinued due to observations of reduced kidney function in rats when administered at concentrations equivalent to LiCO (23). While renal toxicity is a concern, we propose that the purported improved bioavailability of LiOr enables reduced dosing concomitant with mitigation of safety concerns. 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 (24), additional research into the pharmacological properties of LiOr is not only warranted, but necessary.

With the above in mind, the goals of this thesis were three-fold: 1) contrast the efficacy of LiOr and LiCO in a mania-mimetic rodent model; 2) assess the tolerability of LiOr relative to LiCO; and 3) elucidate the dissociation- and transport-related properties of LiOr that underly its putative improved therapeutic functionality.

**Dissertation at a glance**

In objective 1 we explored the efficacy and potency of LiOr relative to LiCO across a range of concentrations in both male and female wild-type (C57Bl/6) mice, with the typical therapeutic dose of lithium (adjusted for a murine model) serving as the upper bound. To this end, amphetamine-induced hyperlocomotion (AIH), a high-throughput behavioural model of mania that displays excellent dose-sensitivity to lithium (25), was used to contrast LiOr/LiCO dosage requirements. The strength of blockade elicited by the minimal effective concentration (minimal
the effective concentration) of LiOr (75.46 ± 16.95% males; 97.45 ± 6.78% females) was substantially greater than that produced by the minimal effective concentration of LiCO (67.18 ± 9.64% males; 82.4 ± 3.99% females), despite the required dose of LiOr being 10-to-13.3-fold less. This improved suppression of hyperlocomotion by LiOr was not due to any reductions in baseline locomotor activity or capacity, as evidenced by its lack of effect on exploratory activity in the open field or locomotor function during the rotarod test. Of note, the differential dosing requirements of LiCO and LiOr were maintained against two separate concentrations of amphetamine, indicating that our results are not exclusive to a particular dosage.

In objective 2, the relative toxicities of LiOr and LiCO were contrasted over their effective ranges, which were defined as 1x, 2x, or 3x the minimal effective concentration of each respective compound. When adjusted for allometric scaling from mouse to man, these concentrations roughly correlate with the low, intermediate, and high doses of LiCO used in human therapy. After 14 days of once daily LiOr/LiCO administration, serum markers of kidney and thyroidal health were assessed post-mortem. While LiOr had no adverse effects on any measure, LiCO induced polydipsia in both sexes concurrent with elevations in serum creatinine in males (3x minimal effective concentration) and thyroid stimulating hormone (TSH) in females (2x and 3x minimal effective concentration).

For objective 3, both AIH (in vivo) and long-term potentiation (LTP) of synapses within the CA1 hippocampal sub-field (ex vivo) were used as output measures by which the impacts of pharmacological inhibition of transporters potentially linked to the movement of LiOr, such as OTAPs or ENTs, were assayed. As lithium enhances LTP and blunt AIH (25), an inhibitor-induced loss of either effect would implicate the targeted transporter in the movement of LiOr. We found that polyethylene glycol-400 (PEG-400) and naringin, which are non-selective and selective inhibitors of OATP1A2 (26, 27), respectively, disrupted the actions of LiOr, but not LiCO, on both AIH and hippocampal LTP, suggesting that OATP1A2 is involved in the transport of LiOr across membranes.

Given that lithium must eventually separate from its carrier to affect cellular functions, we also explored if LiOr dissociates under physiologically relevant conditions, and if not, whether UMPS mediates its dissociation via the uptake of orotic acid into the de novo pyrimidine biosynthesis pathway (for which it is a substrate). To contrast the dissociation states of LiOr and LiCO in
solution, an electrical current was applied and the resistivity to current flow was recorded. Solutions containing LiOr demonstrated substantially greater resistivity than those containing LiCO, suggesting less ionization. These results were recapitulated under more physiologically relevant conditions using a GSK3β activity assay. 2 mM LiOr failed to affect GSK3β whereas 2 mM LiCO blocked 50% of its activity, indicating that only LiCO was fully dissociated; the IC50 for lithium-induced inhibition of GSK3β in vitro is ~2 mM (28). If the presence of UMPS, which was absent from the above experimental paradigms, is responsible for the eventual dissociation of LiOr, then its inhibition should prevent LiOr from affecting either LTP or AIH. Consistent with this prediction, application of the UMPS inhibitor 6-azauracil (29) attenuated the effects of LiOr on both AIH and LTP while sparing the actions of LiCO.

In summary, LiOr was found to be both more potent (lesser dosage requirements) and efficacious (more robust effects) than LiCO in the attenuation of AIH, which translated to an improved safety profile during a 14-day toxicological probe. This improved functionality appeared to be due to utilization of OATPs for transport and UMPS for intracellular dissociation. If the translational potential of these results proves sound, then the reduced dosage requirements of LiOr may ameliorate the dose-dependent, compliance-disrupting side-effects frequently encountered during LiCO therapy.

1.1 Bipolar disorder: Etiology and pathogenesis

1.1.1 Prevalence, symptomatology, and heritability

BD pertains to a complex cluster of severe, lifelong conditions linked with extreme suicidality: roughly 30-50% of sufferers will attempt suicide at least once (30-32). Unsurprisingly, these disorders substantially affect psychosocial functioning and are associated with a 10–20-year reduction in anticipated lifespan (33).

In general, the illness is characterized by depressive/major depressive and manic/hypomanic mood cycles, where the severity and duration of each varies according to the specific class of BD in question, e.g., BD I, BD II, or cyclothymia (Fig. 1.1A) (1). Depressive states are characterized by feelings of persistent sadness, anhedonia, sleep disturbance, and fatigue, whereas mania and hypomania are noted for euphoria, loss of inhibition, impaired decision making, and/or reduced need for sleep (Fig. 1.1B) (34). BD I is defined by the presence of at least one manic episode, and while patients may experience additional hypomanic or depressive symptoms, they are not a part
of its diagnostic criteria. In contrast, BD II involves the presence of syndromal hypomanic and major depressive mood states sans incidence of mania. Cyclothymia is a milder form of BD that displays frequent and rapidly cycling episodes of hypomania and mild depression (1, 33).

The etiology of BD has a strong genetic component: independent genome-wide association studies have identified a series of BD-associated loci which contain genes encoding proteins involved in ion conductance, synaptic plasticity, neurotransmission, and signal conductance (35, 36). The argument for the genetic basis of BD is further supported by twin studies which reveal a heritability of ~60-80% with a concordance of 45% in monozygotic twins and 6% in dizygotic twins (37). Disorders that fall under the BD umbrella also share genetic risk alleles with other mental health conditions in a class-dependent manner, e.g., BD I is highly genetically correlated with schizophrenia whereas BD II is genetically linked to major depressive disorder (35).

Taken together, it is probable that genetics play a larger role than environmental factors in illness development and progression. Consequently, BD is a lifelong condition that requires persistent medlicative therapy, which thereby necessitates the identification of treatments which maintain their efficacy over the long term while limiting side effect incidence.

1.1.2 Processes implicated in bipolar disorder pathogenesis

Although the underpinnings of BD have not been fully elucidated, theories abound regarding its etiopathogenesis. Factors such as an abnormal circadian clock (38); aberrant glycogen synthase kinase-3β (GSK3β) (39-42) and inositol monophosphatase (IMPase) activity (42); catecholamine dysfunction (43, 44) and serotonin dysregulation (45, 46); neuroinflammation (47-49); and lack of neurotrophic factor support (49, 50) are implicated in the etiology and pathological progression of BD.
Thus, it is unsurprising that the illness lacks a unifying pathophysiological hypothesis (51). The more prominent of these hypotheses are discussed in the sections below.

1.1.2.1 Dopamine dysregulation

The role of dopamine in BD has been discussed since the early 1970s (52). Early theoretical frameworks focused on relationships between mania and elevated dopamine signaling, citing parallels between the behavioural effects of dopaminergic stimulants and the antimanic actions of antidopaminergic drugs (53-56). The idea was that if hyperdopaminergia underlies manic expression, as evidenced by the attenuation of manic episodes by agents which diminish dopaminergic neurotransmission, then hypodopaminergia might manifest as the depressive phase (43, 57). While the hypothesis has been fueled by recent discoveries of elevated type-2 dopamine receptor (D2R) availability in mania (58), altered dopamine transporter (DAT) activity in both phases (58-61), and the presence of single nucleotide polymorphisms in the DAT gene promoter of at risk individuals (62, 63), a pivotal question remains: how does the cycling between mania and depression occur?

Modern incarnations of the dopamine dysregulation hypothesis (Fig. 1.2) propose that failure of

![Diagram of dopamine dysregulation hypothesis](image)

the intrinsic homeostatic mechanisms which regulate dopaminergic function precipitate cyclical changes in dopaminergic neurotransmission (43, 64, 65). In brief, hyperdopaminergia in the manic
phase elicits downregulation of D2R sensitivity and increased DAT function, resulting in transition to a hypodopaminergic condition. This state, which is believed to be depressive, is reversed by compensatory upregulation of D2R expression concurrent with a reduction in DAT availability, thereby restoring the manic phase (43). Intriguingly, proinflammatory cytokines, which are elevated in mania, increase DAT activity, suggesting a link between neuroinflammation and the transition from hyperdopaminergia to hypodopaminergia (elevated DAT will reduce overall dopaminergic tone) (66).

Perhaps the strongest evidence for the hypothesis is pharmacological in nature. In patients being treated for Parkinson’s disease, the combination of levodopa and dopaminergic agonists elicits a cyclical mood dysregulation reminiscent of BD: elevated mood, increased activity, and sexual disinhibition during treatment are followed by profound depression upon dosage reduction (67). Similar patterns have been described for amphetamines, which initially evoke symptoms of mania followed by depressive characteristics during withdrawal (68). Unsurprisingly, individuals with BD are far more sensitive to the effects of amphetamine and levodopa than the general population, as evidenced by the high risk of manic or hypomanic induction following ingestion of either class of compound (69, 70).

In closing, failure of the homeostatic mechanisms which regulate dopaminergic activity may underly the cyclical nature of BD by allowing successive alternations between hyperdopaminergic states associated with mania and hypodopaminergic states associated with depression. Evidence for this proposal is provided by the differential expression of D2R availability, D2R sensitivity, and DAT functionality in mania versus depression, as well as the BD-mimetic effects of psychostimulant treatment and subsequent withdrawal.

1.1.2.2 Disrupted ion balance

Numerous genes related to ion balance are implicated in BD etiopathogenesis. One such gene is ATP1A3, which encodes the Na\(^+\),K\(^+\)-ATPase (NKA) α3 sodium pump (71). NKA is central to the sodium-potassium ATPase hypothesis of BD (Fig. 1.3) which proposes, in part, that a mild insufficiency in pump activity precipitates an increase in intracellular sodium concentrations, which in turn drives activity of the sodium-calcium exchanger. The resultant influx of calcium into the cell induces a state of cellular hyperexcitability believed to be linked to the emergence of manic phenotypes. If the suppression of NKA function is quite robust, the excessive concentrations of
sodium and calcium within the cell may lead to a reduction in cellular activity (perhaps courtesy of a depolarization block). Proponents of the hypothesis argue that this state of hypoexcitability manifests as depression.

In support of the sodium-potassium ATPase theory, genetic analyses performed on post-mortem tissues gathered from BD patients reveal lesser expression of the α3 isoform of NKA in the prefrontal cortex (72). Transgenic animal models provide additional evidence: heterozygous Myshkin (Myk/+) mice carry a missense mutation in NKA α3, leading to inactivity of the isoform and a 36-42% reduction in total NKA activity in the brain (73). Similar to manic humans who engage with novel objects more frequently, show hyperambulation, and demonstrate increased preference for reward relative to the control population, Myk/+ mice display increased novel object exploration, ambulation, and sucrose preference in the novel-object, open field, and sucrose-preference tests, respectively (71). Myk/+ mice also show increased sensitivity to amphetamine, much like human patients (74); and altered circadian rhythms (71), which is a common trait marker of BD (38).

In summary, aberrant ion balance alters cellular excitability. Proponents of the sodium-potassium ATPase hypothesis assert that hyperexcitable states precipitate manic symptomatology, while hypoexcitability elicits induction of depressive phenotypes.

1.1.2.3 Heightened GSK3β signaling

GSK3β is implicated in BD pathology (Fig. 1.4) (39-42): heightened GSK3β expression is observed in human BD patients (75, 76); mood stabilizers return GSK3β activity profiles to a normal state (77); and overexpression of GSK3β in rodents elicits manic endophenotypes (78).
whereas ablation recapitulates the ameliorative benefits of lithium (79, 80). GSK3β is a serine/threonine kinase known to phosphorylate over 100 substrates and is itself regulated by numerous upstream elements through excitatory and inhibitory phosphorylation. This promiscuity and multimodal regulation necessitate that any dysfunction within its interactome will lead to a host of deleterious effects, including impaired neurogenesis (81), reduced neurotrophic factor support (82, 83), and disrupted sleep (84-86).

Hyperactivity of GSK3β precipitates marked reductions in neurogenesis (81), a process required for the maintenance of appropriate hippocampal mass and functionality. Unsurprisingly, deficits in hippocampal volume and function are observed post-mortem in BD patients (87-89). These observations may be attributable, in part, to the disruption of neurogenesis that occurs as a consequence of GSK3β-induced degradation of β-catenin, which is a key element of the canonical wingless pathway known to support growth, differentiation, and survival (90). Additionally, aberrant alterations to regional grey matter structure and function may be a consequence of reduced brain derived neurotrophic factor (BDNF) expression (50, 91). Deficiency of BDNF – a neurotrophic factor that contributes to cell survival (92, 93) – is associated with a plethora of adverse mental health conditions (94-98). GSK3β reduces the transcription of the BDNF gene through negative regulation of its transcriptional promoter, cAMP response element binding protein (CREB) (82, 83), which ultimately leads to suppression of neurotrophic support. Alternatively, excessive GSK3β signaling might drive adverse volumetric changes through promotion of inflammatory responses (99, 100), as evidenced by the protection against inflammation-induced neuronal toxicity that follows pharmacological disruption of GSK3β (100). GSK3β overactivity may also be linked to the sleep disturbances and circadian abnormalities
commonly observed in BD (38, 101). Sleep is intimately linked to the circadian clock, which sets a “rhythm” through cyclic transcription, translation, and degradation of clock proteins. In short, the circadian clock is initiated by the binding of the transcription factors CLOCK and BMAL1 to E-box sequences in the promoter regions of important clock-controlled genes, such as those which encode the transcriptional repressors period and cryptochrome 1. These repressors bind to CLOCK/BMAL1 and inhibit transcription of their targets, themselves included. The process is completed by the coordinated degradation of period and the cryptochromes, thus reactivating transcription of the E-box genes and resetting the circadian cycle (102). GSK3β promotes the translocation of period to the nucleus (86) (and thereby promotes its repressive functionality) and regulates the degradation of CLOCK, BMAL1, and cryptochrome 2 through phosphorylation (84, 85). In mice, chronic activation of GSK3β impairs the rhythmicity of BMAL1 (103), suggesting that enzymatic hyperactivity is associated with sleep disturbances.

Overall, GSK3β hyperactivity appears central to both the pathogenesis of BD and its resultant symptomatology. The significance of this will become apparent when the similarities between BD and the model of mania employed in our studies are discussed in chapters 3 and 6.

1.1.2.4 Altered activity within the phosphoinositide cycle

The phosphatidylinositol cycle (PI cycle) is involved in a diverse array of processes ranging from cell growth to neuronal transmission (104). As the uptake of inositol from the blood into the CNS is limited by the blood-brain barrier (BBB) (42, 105, 106), brain inositol is primarily derived from \textit{de novo} synthesis and recycling of various phosphatidylinositol molecules.

The first step of the PI cycle is the conversion of inositol to phosphatidylinositol by phosphatidylinositol synthase. Next, phosphate groups are sequentially added to the molecule until phosphatidylinositol-4, 5-bisphosphate (PIP₂) is synthesized. PIP₂ is then either converted to phosphatidylinositol 3,4,5-trisphosphate (PIP₃) by PI3 kinase (PI3K) (107) or hydrolyzed into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol by phospholipase C. To regenerate substrates, IP₃ is restored to inositol through the removal of phosphate groups by inositol polyphosphate 5-phosphatase, inositol polyphosphate 1-phosphatase, and inositol monophosphatase, in that order (108). Thus, dysregulation within the PI cycle can influence a plethora of cellular activities downstream of diacylglycerol, IP₃, PIP₂, and PIP₃, such as synaptic plasticity (109, 110), insulin responsivity (111), and neurotransmission (42).
Altered levels of brain inositol, PIP₂, and protein kinase C (PKC) activity (112) are observed in the brains of BD patients post-mortem (113-118). Intriguingly, brain inositol levels appear to vary with illness phase: magnetic resonance spectroscopy studies reveal that frontocortical myo-inositol signals are heightened when manic (119) and reduced when depressive (120). These findings suggest state-dependent aberrations within the phosphatidylinositol second messenger system that adversely affect neurotransmission (42), synaptogenesis (109), and even cell survival (121).

While the functionalities of the PI cycle are manifold, regulation of intracellular calcium flux may be of particular import within the context of BD (Fig. 1.5). Since perturbation of intracellular calcium dynamics influences neurotransmission, autophagy, and apoptotic sensitivity (122), it is possible that heightened PI signaling could promote calcium-induced neuronal hyperexcitability followed by subsequent excitotoxicity and cellular damage. In contrast, suppressed PI turnover could precipitate depressive symptoms through dampened neurotransmission (120, 123, 124); phosphoinositides and their derivatives, notably PIP₂, IP₃, and diacylglycerol, play important roles in mediating pre-synaptic release and post-synaptic responses during glutamatergic neurotransmission (125, 126).

Although the above propositions are intriguing, the exact contributions of aberrant PI cycle activity to BD pathogenesis and symptomology are indeterminate at this time (127).

Fig 1.5. PI signaling in BD. Brain inositol levels are altered in BD, with increased expression implicated in mania and reduced expression in linked to depression. Increased inositol levels set the stage for excessive PI signaling, which heightens PKC activity and intracellular calcium concentrations downstream of DAG and IP₃, respectively. In turn, PKC and increased intracellular calcium levels promote glutamatergic neurotransmission, which is believed to be altered in BD. In contrast, reduced inositol expression results in decreased PI signaling, which may contribute to depressive phenotypes through induction of cellular hyperexcitability and suppressed neurotransmission. Ca⁺⁺ - calcium; GPCR - G-protein coupled receptor; PLC - phospholipase C; PIP₂ - phosphatidylinositol 4,5-bisphosphate; IP₃ - inositol trisphosphate; IP₃R - inositol trisphosphate receptor; DAG - diacylglycerol; PKC - protein kinase C; PI - phosphoinositol; PI Synthase - phosphatidylinositol synthase; PI4K - phosphatidylinositol 4-kinase; PI4P5K - phosphatidylinositol 4-phosphate 5-kinase.
1.1.2.5 Neuroinflammation

Cytokines are signaling molecules involved in either the potentiation or resolution of inflammation, among numerous other functions, and are classified as either proinflammatory, anti-inflammatory, or mixed. While cytokines play an essential role in regulating neuroplasticity under physiologic conditions (128, 129), deleterious sequelae emerge during excessive and persistent bouts of neuroinflammation (48, 99, 100, 130, 131).

Several lines of evidence implicate inflammatory processes in the pathophysiology of BD: autoimmune comorbidities are frequently reported in association with BD (132-134); the expression of proinflammatory cytokines is often elevated in symptomatic patients (132, 135); both bipolar parents and their at-risk children display a genetic profile characteristic of elevated immune-inflammatory signaling (136); and mood episodes are predicted by increased proinflammatory output (137, 138). Additionally, immune responses following infection are linked to increased severity of mood episodes (139) and an elevated risk of BD emergence (134, 140, 141) in human patients; and in murine models, elevated immune-inflammatory activation elicits behavioural indices indicative of a depressive state (131) while knock-out of the proinflammatory cytokines tumor necrosis factor-α (TNFα) (142) and interleukin-6 (IL-6) (143) attenuates phenotypic expression. Taken together, the involvement of neuroinflammation in BD pathogenesis seems clear, but how do peripheral inflammatory events trigger neuroinflammatory processes?

Systemic inflammatory signals propagate to the CNS through several pathways, including bypass of the BBB through the circumventricular organs; transport along afferent vagal fibers; active transport across the BBB; BBB translocation of activated immune cells; and release of cytokines from the BBB endothelium (47). Once centrally expressed, proinflammatory cytokines activate microglial cells, leading to intensification of inflammatory responses through release of reactive oxygen species, reactive nitrogen species, chemokines, and cytokines (144). This undesirable chemical milieu precipitates alterations in astrocytic function, promoting 1) increased activation of indoleamine 2,3-dioxygenase, which favours the metabolism of tryptophan into neurotoxic kynurenine metabolites rather the serotonin (145), and 2) diminution of BDNF and glial cell line-derived neurotrophic factor secretion concurrent with increased cytokine and glutamate extrusion. Astroglia-derived glutamate signals primarily through extra-synaptic N-methyl-D-aspartate receptors, inducing suppression of neuronal BDNF synthesis and stimulation of proapoptotic
cascades (146). Of note, BD chronicity, increased mood episode frequency, and decreased treatment response are associated with imbalanced expression of BDNF relative to inflammatory agents and mediators of oxidative stress (147).

1.1.2.6 Connecting the dots: convergence of mechanisms on neuroprogression?

Investigations into the brain structure of BD patients reveal volumetric reductions in the hippocampus (148, 149), amygdala (150, 151), striatum (152), and prefrontal cortex (153), with the severity of grey matter loss correlating with illness chronicity (154). This ongoing loss of grey matter, termed neuroprogression, is believed to correlate with worsening symptomatology, increased emergence of psychiatric comorbidities, reductions in inter-episode duration, and insensitivity to medicative treatment, lithium included (154-156).

Unlike conventional neurodegenerative disorders, which are marked by profound neuronal loss, glial cell atrophy appears to be the predominant cellular pathology in BD (47). Biochemical, histopathological, and neuroimaging analyses link BD pathogenesis to structural and functional anomalies in glial cells (157, 158), and post-mortem investigations reveal reductions in glial cell number and density within the prefrontal cortex (159) and hippocampus (158). Intriguingly, the mood-stabilizers lithium and valproate appear to mitigate glial loss (160), although only a paucity of data is available at present. Given the critical role of glial cells in supporting overall brain health and cognition (161-163), pathological alterations to astrocytes, oligodendrocytes, and microglia likely contribute to regional grey matter loss.

The volumetric reductions in neuroprogression may be linked to a progressive decline in BDNF content (164) concurrent with elevated immune-inflammatory activation (165) and reduced antioxidant capacity (166, 167). Given the influence of GSK3β and PI signalling over these processes, it is possible that GSK3β hyperactivity and PI-cycle dysfunction converge to drive neuroprogression through promotion of oxidative stress and neuroinflammation (99, 100), suppression of neurotrophic factor support (82, 83), and induction of pro-apoptotic signaling cascades (168). Additionally, neuroinflammation – induced either by GSK3β hyperactivity (100), genetic susceptibility (136), or systemic inflammatory events propagating into the CNS (47) – can itself precipitate reductions in neurotrophic factor expression (146), formation of neurotoxic metabolites (145), and induction of apoptotic cascades (146, 169). Therefore, neuroprogression could represent a point of convergence for the various factors and processes implicated in the
pathogenesis of BD.

1.2 Current treatment options

1.2.1 Antipsychotics

The core function of the antipsychotics is to reduce dopaminergic tone (Fig. 1.1C), either through antagonism of D2R, or combined antagonism of D2Rs and type 2A serotonin receptors (53). Along with the antagonistic effects of lithium downstream of D2R (170), the clinical success of antipsychotics gave rise to the dopamine dysregulation hypothesis of BD (43). Despite knowing their mechanism of action, it is not yet definitely known why these medications are effective in BD; however, normalization of dopaminergic activity, particular within the context of the dopamine dysregulation hypothesis, is a leading theory (43). For a better understanding of the hypothesis and how it relates to antipsychotic medications, please refer to 1.1.2.1 and 1.8.3.

1.2.2 Anticonvulsants

Of the numerous anti-epileptic medications, only valproic acid, carbamazepine, and lamotrigine have demonstrated clinical efficacy (Fig. 1.1C). In general, anticonvulsants are believed to derive their efficacy via suppression of neuronal excitability (171) through blockade of voltage-gated sodium channels (172), modulation of gamma-aminobutyric acid signaling (172), and suppression of intracellular calcium responses(173, 174).

1.2.3 Lithium

Lithium salts have been used for more than 50 years to address the psychiatric manifestations of BD (Fig. 1.1C). While their use has declined in recent years (175, 176), largely in favor of antipsychotics (177, 178), this reduction in employment may be ill-advised, particularly within the context of maintenance therapy (177-179). A meta-analysis performed by Miura et al (2014) found that of 17 treatment options assessed in 33 randomized controlled trials, only lithium and quetiapine prevented manic- and depressive-episode relapse or recurrence when used in monotherapy (180). Of note, trials involving lithium utilized a non-enriched study design, which was not the case for quetiapine (181). In other words, lithium proved to be similarly efficacious to quetiapine despite the latter benefiting from a favourable experimental set-up.

Overall, while antipsychotics represent an effective alternative, lithium maintains a central role in the prevention of mood episode recurrence, either alone or in combination with other medications.
1.3 The role of lithium therapy

1.3.1 A brief overview

Brought to popularity by John Cade in 1949 (79) and Samuel Gershon in 1960 (3), lithium salts now have a well-established role in the control of mania and prevention of mood-episode recurrence. These compounds are believed to address BD symptomatology through a plethora of mechanisms, many of which can be linked back to the antagonism of GSK3β, IPPase, and IMPase (42). Due to their similar ionic radii, lithium and magnesium (a required cofactor) compete for binding at the catalytic core of each protein (182-184), resulting in an attenuation of overall enzymatic activity. As previously discussed, GSK3β hyperactivity and dysregulated phosphoinositide turnover may underpin much of the deleterious sequelae of BD (42).

Additionally, a growing body of evidence links neuroinflammation and neurodegeneration to the pathogenesis of numerous affective disorders, BD included (51, 147, 185). Given that lithium demonstrates anti-inflammatory (186-190) and neuroprotective qualities (4, 191, 192), likely through regulation of phosphoinositide expression and GSK3β signaling, it is possible that the attenuation of neuroinflammation (186-190), promotion of neurotrophic signaling (193), and support of antiapoptotic pathways (194) are therapeutically relevant consequences of lithium therapy.

1.3.2 Lithium pharmacokinetics

Most lithium salts (with the notable exceptions discussed below) are highly bioavailable, with roughly 80-100% of the ingested dose readily absorbed in the upper intestinal tract (195). Interestingly, Li⁺ levels in the blood are almost directly proportional to the amount ingested (196), indicating a relative lack of regulation that is in stark contrast to most other biologically important cations (197). This lack of regulation is attributable to the absence of dedicated transport mechanisms. Instead of utilizing transporters or channels specific for Li⁺, it competes for access to transport pathways specific to ions of similar ionic radii. Perhaps the most significant and physiologically relevant pathway for Li⁺ influx is through competition with sodium for passage through both voltage-gated and non-voltage-gated sodium channels (17, 18, 195, 198). While lithium can also interact with select potassium and calcium channels (199, 200), transport is not achieved, and net alterations to calcium/potassium ion flux are only observed at non-physiological concentrations (197). As for cellular efflux, intracellular Li⁺ largely competes with protons for
access to the sodium-proton exchanger, NHE (201).

Post absorption, the metabolism and excretion properties of Li⁺ are unremarkable. With rare exception, Li⁺ is excreted through the kidneys unchanged (not metabolized), with an elimination half-life of ~16-30 hours (195).

In summation, currently employed lithium therapeutics dissociate shortly after ingestion and are rapidly absorbed into the circulation, which means that the entirety of each salt’s effects outlined in 1.3.3 (below) are evoked by Li⁺ rather than the salt formulation itself (the relevance of this will become apparent in chapter 5).

1.3.3 Potential mechanisms of action

1.3.3.1 Inhibition of GSK3β

Aberrant GSK3β activity is linked to several pathological processes implicated in BD, including disruption of the circadian clock (38, 79, 101, 202); impaired hippocampal function and reduced volume (87-89); and decreased expression of neurotrophic factors (50, 83, 91). Lithium salts are known inhibitors of GSK3β (182), and this antagonism is believed to be central to their therapeutic influence. In support, positive outcomes of lithium therapy are predicted by GSK3β gene polymorphisms (203, 204), insufficient inhibitory phosphorylation of GSK3 isoforms elevates sensitivity to mood disturbances (75), and tissues collected post-mortem display evidence of increased signaling (75). In animal models, GSK3β haploinsufficiency recapitulates behaviours associated with chronic lithium treatment (79, 80), whereas overexpression elicits manic-like hyperactivity (78) and reverses lithium’s behavioural effects (205). On the human front, tissues collected from BD patients display reduced inhibitory phosphorylation of GSK3β, indicating elevated enzymatic activity (75). Taken together, it seems likely that normalization of GSK3β signaling is central to lithium’s therapeutic efficacy, which thus raises the question: what are the phenotypic manifestations of GSK3β inhibition, and how do they contribute to mitigation of the underlying pathophysiology of BD?

As previously discussed, sleep disturbances and circadian abnormalities are common in BD (101), and may be linked to GSK3β hyperactivity. While the evidence is inconclusive at present, lithium is believed to normalize sleeping patterns in BD (101, 206), perhaps through modulation of cellular circadian rhythms via GSK3β inhibition (206, 207). If true, then rescue of sleep could represent a
core therapeutic effect of lithium that occurs consequent to its actions on GSK3β signaling.

While the impacts of lithium on sleep are intriguing, its most important therapeutic effects are likely due to its capacity to promote cellular health and survival through induction of neuroprotective pathways. First, chronic lithium treatment rescues expression of BDNF (193), which is often reduced in BD (50, 91). This is likely achieved through prevention of the GSK3β-induced inhibition of CREB (208) and the consequent suppression of BDNF mRNA transcription that follows. Second, lithium-induced inhibition of GSK3β blunts the phosphorylation and activation of Bax, thereby disrupting its pro-apoptotic properties and preventing stimulation of intrinsic cell death pathways (194). Third, lithium robustly supports signaling within the wingless pathway (209-211), which in turn counteracts the deleterious impacts of GSK3β on β-catenin expression (90). Overall, the actions of lithium support cell survival, cell growth, and synaptogenesis (212, 213), which would be expected to diminish the regional atrophy characteristic of neuroprogression in BD.

Although less is known regarding the role of GSK3α in BD, evidence of elevated enzymatic activity within the periphery has been identified in manic patients, and these aberrations are normalized following chronic lithium therapy (76). Given that lithium inhibits GSK3α (214) and that GSK3α haploinsufficiency recapitulates the behavioural effects of lithium treatment in mice (102), it is possible that antagonism of GSK3α hyperactivity is a therapeutic mechanism of lithium in the treatment of human mania.

In closing, the lithium-induced inhibition of GSK3β (and perhaps GSK3α) may rescue circadian function in BD by normalizing the expression of CLOCK, BMAL1, cryptochrome 2, and period; as well as delay neuroprogression (and thus preserve hippocampal function and volume) by increasing neurotrophic signaling and suppressing the induction of pro-apoptotic cascades.

1.3.3.2 Inhibition of IMPase

Lithium inhibits IMPase activity (215, 216) through displacement of the cofactor magnesium (215), which in turn leads to inositol depletion (217-219) concomitant with prevention of PIP2 resynthesis and IP3 regeneration (183). Given that dysfunction within the PI-cycle is implicated in BD pathology (113-115, 117, 220), and that polymorphisms in genes encoding phospholipase C are associated with responsivity to lithium (221), it is possible that lithium derives some of its therapeutic efficacy from IMPase inhibition and subsequent normalization of PI-cycle signaling;
this idea is supported by the restoration of normal myo-inositol and phosphomonoester concentrations in BD patients on long-term lithium therapy (220). Despite the evidence supporting the inositol depletion hypothesis of lithium’s therapeutic efficacy, observations of the downstream consequences of inositol depletion are uncertain and at times contradictory (222).

With the above in mind, we propose that inositol depletion (114, 217, 223, 224), along with modulation of calcium flux through N-methyl-D-aspartate receptors (225), serves to limit neuronal excitability (125, 226). This modulation of neurotransmission could conceivably limit manic symptomatology while mitigating excitotoxicity and neuroprogression; neuronal hyperexcitability is a potential lithium-sensitive endophenotype in BD (227, 228).

1.3.3.3 Attenuation of neuroinflammation
As previously discussed, elevated immune-inflammatory signalling is seen in both BD patients and their symptomatic or asymptomatic but at-risk children (136). The expression of the proinflammatory cytokines, such as interleukin-4, IL-6, interleukin-1β or TNFα, are often normalized in previously medication-naïve patients following chronic lithium therapy (186-188).

The impacts of lithium on inflammatory cascades (189, 190) appear to involve downregulation of cyclooxygenase-2 and consequent reductions in prostaglandin E2 expression (229), prevention of nuclear factor-κβ transcriptional activities (230), reduced production of reactive oxygen species in response to inflammatory insults (230), and GSK3β antagonism (100). Prostaglandin E2 and nuclear factor-κβ induce inflammation through induction of T helper cell 17 proliferation and transcription-mediated upregulation of cytokine and chemokine expression, respectively, whereas GSK3β supports nuclear factor-κβ transcriptional activity (231, 232). Therefore, it is possible that aspects of lithium’s therapeutic effects are elicited through anti-inflammatory mechanisms.

1.3.3.4 Preservation of grey matter volume
BD has been theorized as a progressive condition where volume reductions in key regions worsen over time (233). Intriguingly, the length of the delay between symptom onset and introduction of lithium is inversely correlated with successful treatment outcomes (234, 235). Given that structural changes are not consistently observed at the time of initial diagnosis and instead become more evident with chronicity (236, 237), it is possible that the neuroprotective effects of lithium (191, 238) are key to its therapeutic efficacy. In brief, lithium-induced diminution of BD-associated grey matter loss (239) may halt neuroprogression, thereby sparing cognitive function and ameliorating
present and future symptom severity. Conversely, a significant delay in the onset of treatment may allow degenerative processes to continue to a point where the neuroprotective influence of lithium ceases to be beneficial (i.e., the damage has been done), hence the association between poor therapeutic outcomes and delayed treatment onset (234, 235). In support, regional changes in grey matter volume are diminished in lithium-treated patients compared with their untreated counterparts (240, 241), and longitudinal studies reveal an association between increased prefrontal grey matter volume during lithium therapy and positive treatment outcomes (242).

As outlined in 1.1.2.6, GSK3β hyperactivity, neuroinflammation, oxidative stress, and
excitotoxicity appear to drive neuroprogression, which suggests that lithium’s various influences downstream of GSK3β, IMPase, and IPPase inhibition may converge on neuroprotection; e.g., resolution of neuroinflammation (189, 190), rescue of BDNF expression (193), and preservation of β-catenin pools (β-catenin is anti-apoptotic) (Fig. 1.6) (210). In line with this proposition, lithium also prevents cellular degeneration through antioxidant mechanisms. In rodent models and in vitro cellular assays, lithium reverses and repairs the oxidative stress resultant of excitotoxicity (243) and neurotoxin exposure (244), respectively; and in human patients, excellent lithium responders and their kin appear to experience a strengthening of their antioxidant defenses through upregulation of glutathione (245). Given that oxidative stress can inflict cellular damage and precipitate induction of cell death pathways, amelioration of oxidative stressors through upregulation of antioxidant factors may limit cell loss and subsequent reductions in grey matter volume.

1.4 The rise of lithium carbonate

The utilization lithium in psychiatry can be traced back to the mid 1800s. While some credit the early interest in lithium to Alfred Baring Garrod, who used the element in the treatment of gout (246), it was William Hammond in 1871 who became the first physician to prescribe lithium for mania, specifically lithium bromide (247). In the late 1890s, LiCO was used in the treatment of 35 patients with melancholic depression in Denmark (248); however, this early Danish work was seemingly forgotten, and the use of lithium in psychiatry would not experience a revival until 1949 courtesy of psychiatrist John Cade (248).

At the Bundoora Repatriation Hospital, Cade theorized that mania was a consequence of excess uric acid within the body. Citing the success of Garrod in using lithium to manage gout (248), Cade treated 10 manic patients with either lithium citrate or LiCO. He noted that many of the patients responded quite well to lithium, with some even meeting the criteria necessary for discharge from the hospital (2).

Unfortunately, the timing of Cade’s success was inopportune, for within that same year lithium chloride would fail as a replacement for sodium chloride in the management of hypertension (249). The excessive use of lithium chloride-based table salt substitutes, such as Milosal and Foodsal, led to widespread reports of lithium poisoning. Following several deaths, the Food and Drug Administration moved to ban the use of lithium salt substitutes.
Despite concerns, trials for the use of LiCO in mania continued in Denmark and Australia (250, 251). Interest in North America began to build in the 1960s following the advent of the Coleman flame photometer (248, 252), which allowed for accurate tracking of Li\(^+\) levels within the blood; and the arrival of Samuel Gershon, who spearheaded the first North American publication on the use of lithium in mania (3). By the late 1960s, numerous investigations into the psychiatric potential of LiCO had been conducted (253-256) with compelling success, leading the FDA to approve lithium (namely LiCO) for use in the treatment of BD in the United States (248).

From these humble beginnings, lithium has gone on to become the gold standard for terminating manic episodes and maintaining euthymia (175); however, an important questions remains unanswered: why did LiCO rise to prominence over the bevy of other salt formulations? While speculative in nature, the answer may reside within the history of lithium therapy. First, the seminal work of John Cade (2) and Samuel Gershon (3) influenced much of the 1960s research that paved the way for the acceptance of lithium as a therapeutic option in BD. Second, while LiCO was gathering attention for its therapeutic efficacy, alternative lithium salts, such as lithium chloride, were more famously associated with cases of lithium poisoning. Third, early research focused chiefly on the general utility of lithium as a therapeutic, and less so on the differences between the various salt formulations. Taken together, it is possible that a confluence of factors pushed LiCO to prominence, such as original safety concerns surrounding alternative compounds (LiCl); LiCO being the first and subsequently most studied formulation for psychiatric applications; and LiCO being the first lithium salt commercially produced for prescription (increased availability).

While undoubtedly efficacious, LiCO comes with an unpleasant side effect profile and long-term toxicity concerns that limit treatment compliance (257). In the sections to follow, the issues with current practices in lithium therapy and the rationale for explorations into the use of LiOr as an alternative to LiCO will be discussed.

1.5 The lithium carbonate problem

1.5.1 What do typical treatment regimens look like?

Most therapeutic guidelines recommend divided daily doses of lithium over bolus administration, with the rationale being to maintain steady plasma Li\(^+\) levels while limiting acute spikes in concentration (258-260). When initiating treatment, lithium levels should be assessed at a steady state (~5 days), and titrated until plasma Li\(^+\) levels between 0.5-1.2 mmol/L (258) are achieved.
over two consecutive tests (261). Given the increased likelihood of adverse effect emergence observed at the higher ends of lithium’s therapeutic range (e.g., 1.2 mM), plasma concentrations of 0.5-0.8 mmol/L should be targeted during treatment initiation, with the dose titrated upwards following mood episode recurrence (262). For long-term maintenance therapy, levels between 0.4 and 0.8 mmol/L are recommended (258-260); however, titration is required according to illness polarity and symptomatic profile (263), with depression-prone patients and mania-prone patients requiring plasma Li$^+$ concentrations of 0.4-0.8 mmol/L and 0.6-1.0 mmol/L for prophylaxis, respectively (264).

1.5.2 Blood Li$^+$ levels must be closely monitored throughout lithium therapy

Several essential factors are to be considered when deciding whether a patient is a suitable candidate for lithium therapy. Per the International Society of Bipolar Disorders (ISBD), baseline assessments of waist circumference, body mass index, haematological parameters, fasting glucose and lipid profiles, TSH expression, and serum calcium levels should be attained prior to the prescription of lithium therapeutics (261).

In addition to baseline assessments, several parameters require ongoing evaluation. As lithium is excreted by the kidneys, it is recommended that adequate renal function first be established, followed by regular evaluation of urinary output, serum creatinine, and serum urea throughout the course of treatment (261). Insufficient renal output can lead to excessive lithium serum concentrations and subsequent toxicity (265). Thyroid and parathyroid function should also be closely monitored. Lithium exerts a deleterious influence over TSH, thyroxine, and triiodothyronine expression (266), particularly in women (267), thereby necessitating annual or biannual performance of a thyroid toxicity panel (261). As for the parathyroid, serum calcium should be tested bi-annually to probe parathyroidal output (261). While relatively uncommon, lithium increases the risk of hyperparathyroidism following chronic treatment (e.g., >10 years) (268). Finally, lithium use is associated with weight gain (269), particularly in patients who are already overweight (270). As per the ISBD, the patient’s weight, waist circumference, and body mass index should be measured every 6 to 12 months (261).

1.5.3 Lithium carbonate dosing protocols lead to frequent incidence of adverse effects and overall poor treatment compliance
LiCO dissociates readily in solution and requires administration of doses large enough to force Li\(^+\) across the BBB and into cells via sodium transporters (16-18). While uncommon, acute lithium toxicity can occur when blood Li\(^+\) levels reach or exceed the upper bounds of the drug’s therapeutic range. Li\(^+\) levels between 1.2 and 1.5 mEq/L are associated with obtundation and locomotor impairments, while concentrations exceeding 2 mEq/L are linked to tonic-clonic seizure, stupor, and coma. Levels exceeding 3 mM are often fatal (5, 271).

Unfortunately, the dosages employed in long-term therapy also precipitate adverse reactions (272, 273) in up to 90% of patients (274), even when serum Li\(^+\) are maintained within the therapeutic range of 0.5-1.2 mM (259). The unpleasant nature of these lithium-induced side effects contribute to high rates of treatment regimen non-adherence (257).

1.5.4 Iatrogenic conditions are often encountered during chronic therapy

The side effects of short-term LiCO use typically involve some combination of polydipsia, polyuria, nausea, diarrhea, or tremor. More severe side effects, such as cognitive impairment, hypothyroidism, and hyperparathyroidism, are observed during the intermediate stages of treatment (273). End-stage renal failure is a possible but largely unlikely outcome of long-term therapy (>10-15 years) (275).

The issues associated with LiCO use are attributable to reduced thyroid hormone synthesis and

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**Fig. 1.7. Schematic of common iatrogenic organ toxicities encountered during lithium therapy.**

A) Lithium precipitates hypothyroidism by inhibiting thyroidal iodine uptake, iodotyrosine coupling, and thyroid hormone secretion. B) Lithium enters the epithelium of the collecting ducts through sodium channels and subsequently impairs urinary concentrative capacity via induction of vasopressin resistance (insufficient vasopressin-mediated insertion of AQP2 into the luminal membrane). This often triggers onset of nephrogenic diabetes insipidus in the short-to-intermediate term, with outright renal failure occasionally developing after ~10 years of treatment. C) Lithium alters the set point of calcium-sensing receptors, leading to elevated parathyroid hormone release (hyperparathyroidism). TSH - thyroid stimulating hormone; TRH - thyrotropin-releasing hormone; PTH - parathormone; T3 - triiodothyronine; T4 - thyroxine; ENaC - epithelial sodium channel; AQP2 - aquaporin-2; IMPase - inositol monophosphatase; CSR - calcium-sensitive receptor; GPCR - G-protein coupled receptor; PLC - phospholipase C; PI - phosphatidylinositol; PIP2 - phosphatidylinositol1,4,5-bisphosphate; IP3 - inositol trisphosphate; DAG - diacylglycerol; PKC - protein kinase C.
release, which manifests as increased serum TSH (276, 277); increased parathyroid hormone signaling and subsequent heart and CNS disturbances (278, 279); and disrupted interactions between antidiuretic hormone and its receptors on the collecting ducts of the nephron, leading to polyuria and secondary polydipsia (Fig. 1.7). Given enough time, these actions may precipitate hypothyroidism, which can occur within months of starting therapy, or renal insufficiency, which is a late-stage outcome (276).

1.5.4.1 Hypothyroidism
Among the plethora of mechanisms proposed for LiCO-induced hypothyroidism (Fig. 1.7A), the inhibition of thyroid hormone synthesis and release coupled with reductions in glandular iodine trapping are believed to be the most influential (274, 280).

The prevalence of hypothyroidism ranges from 19% for the overt condition to 23% for subclinical manifestations (281), with women displaying markedly higher incidence rates than their male counterparts (267). Iatrogenic hypothyroidism is typically managed through thyroid hormone replacement (281).

While LiCO-induced hypothyroidism will often resolve following discontinuation of lithium therapy, the symptoms encountered during treatment are often quite unpleasant and may contribute to non-compliance. These symptoms are identical to those observed in primary cases of the disorder: lethargy, depression, weight gain, and mental slowing (274).

1.5.4.2 Hyperparathyroidism
LiCO independently elevates calcium reabsorption in the kidneys and stimulates release of parathyroid hormone from the parathyroid gland. LiCO is proposed to affect parathyroid hormone release by blunting the activity of protein kinase C downstream of inositol depletion (Fig. 1.7B); PKC inhibits hormone release following the interaction of calcium with membrane-bound calcium-sensitive G-protein coupled receptors which trigger the phospholipase C/IP3 cascade (282).

The chief symptoms of LiCO-induced hyperparathyroidism and secondary hypercalcemia are weakness, fatigue, and renal insufficiency (274, 283). The treatment of iatrogenic cases is similar to that of the primary condition: more frequent monitoring is performed following detection of mild elevations in asymptomatic patients, whereas calcimimetic therapy may be employed when
high levels are observed (284).

1.5.4.3 Vasopressin resistance and renal insufficiency

Excessive urination and thirst are observed in up to 70% of patients prescribed LiCO on a long-term basis (285). The emergence of polydipsia is presumed to be secondary to deficits in urinary concentrating ability. These deficits are believed to be precipitated by lithium-induced vasopressin resistance (Fig. 1.7C), which results in an inability for antidiuretic hormone to trigger the insertion of aquaporin-2 water channels into the luminal surface of the epithelial cells that line collecting ducts of the kidney (265). LiCO-induced alterations to renal function may be progressive, with some finding that severity of impairment correlates with treatment duration (265, 286, 287).

While the renal effects of LiCO are usually reversible, a subset of patients will develop renal insufficiency in the form of interstitial nephritis (288), perhaps as a result of cumulative strain and subsequent morphological aberrations; renal biopsies performed by Hansen et al. on tissues collected from 14 BD patients with severe pathologies revealed an inverse correlation between urinary concentrating capacity and the severity of interstitial nephritis (289). Due to these concerns, lower plasma Li⁺ targets are recommended for long-term maintenance therapy (288).

1.6 Lithium orotate as a superior therapeutic option

Given the issues associated with the current practice of lithium therapy, it is worth considering whether alternative lithium formulations might demonstrate some pharmacokinetic or pharmacodynamic parameters that enable a reduction in the amount of Li⁺ administered, which in turn word limit side effect incidence and subsequently improve treatment regimen adherence. Although controversial, LiOr may represent such an alternative (12, 14).

1.6.1 The controversial history of lithium orotate

In the early 1970s, Hans Nieper claimed to successfully employ LiOr in the treatment of mania (12), citing improvements to efficacy and side effect incidence as a result of increased membrane penetration (11, 12). Shortly thereafter, contrasting studies in 1978 and 1979 yielded two important findings. First, the arguments of Nieper were seemingly supported by the work of Kling et al., who found that rats injected with either 1, 2 or 4 mmol Li⁺/kg of body weight displayed dramatically greater concentrations of Li⁺ in both the brain and blood at each dose when delivered as an orotate instead of as a carbonate (14). Additionally, while LiCO (2 mmol Li⁺/kg) maintained a steady
concentration of 0.5 mmol Li⁺/kg of brain tissue, LiOr elicited a progressive increase from 0.5 to 1.3 mmol over the course of 24 hours (14).

Unfortunately, a follow up study performed in 1979 by Smith and associates raised concerns about the adverse influences of LiOr on renal function when employed at concentrations of 2 mmol Li⁺/kg of body weight. These findings prompted the researchers to opine that the elevations in central and systemic Li⁺ previously reported for LiOr were due to reductions in the renal excretion of Li⁺ consequent to GFR impairment (23).

Following the conclusions of Smith et al., research into the efficacy and tolerability of LiOr was effectively curtailed, leading to the paucity of data concerning LiOr present in today’s literature; the separate works of Kling and Smith in the late 1970s are the only basic scientific studies that have been performed in the past 50 years. The same is true on the clinical front where, aside from the early work of Hans Nieper (12), the only human data for LiOr presently available is found within a single study concerning the treatment of alcoholism. Intriguingly, LiOr has shown success in promoting the cessation of excessive alcohol consumption when administered at 150 mg/day over a 6-month period; out of the 42 patients involved in the study, 23 remained without relapse for up to 10 years (15). In contrast, LiCO seems to range from being mildly efficacious at therapeutic doses (290) to outright ineffective in aiding the avoidance of relapse (291), which has led to the general conclusion that it is not an effective treatment for alcoholism (292). Unfortunately, no additional studies regarding the use of LiOr in the management of alcoholism have been conducted beyond the work of Satori et al. in 1986 (15). Nevertheless, the ability for LiOr to reduce the incidence of relapse in alcoholism while LiCO fails to do so at markedly higher doses lends credence to the theory that LiOr is in fact a more potent formulation.

Despite early reservations, LiOr is once again gathering interest (192, 293, 294) for its putative ability to yield higher serum and brain Li⁺ concentrations than equivalent doses of LiCO (14). A recent editorial penned by Peter Devadason (2018) highlights the growing interest in LiOr as a medication delivered in trace doses, noting the reported benefits of LiOr administration and the general lack of safety concerns associated with low-dose supplementation (294).

While the literature surrounding LiOr is highly interesting (limited though it may be), a few key questions remain unaddressed: First, is LiOr a more potent formulation than other lithium salts, as suggested by Nieper (12) and Kling (14), and if so, will this translate to an improved safety profile
courtesy of reduced serum Li⁺ exposure (13)? Second, what properties of LiOr might account for its proposed differences relative to conventional lithium therapeutics?

1.6.2 Can lithium orotate enter the CNS more readily than lithium carbonate?

1.6.2.1 The environment of the CNS is tightly regulated

The term BBB pertains to the unique properties possessed by the microvasculature of the CNS and its supporting cells that allows for tight regulation over the transport of ions and molecules between the blood and neural tissue (295). The endothelial cells that comprise the BBB lack fenestra and are held together by tight junctions, which greatly limits transcellular and paracellular transport, respectively. Additionally, the highly polarized nature of the BBB endothelium allows for localized expression of efflux and influx transporters, enabling regulation over the entry and egress of substances into the brain (295). The basal lamina, pericytes, and astrocyte end-feet, which contain numerous transporters of their own, envelope the CNS vasculature to complete the BBB (296). Overall, the BBB provides stringent control over the extracellular milieu of the CNS, allowing for precise maintenance of the ionic concentration gradients required for proper neuronal function (295).

1.6.2.2 Due to regulation of sodium transport, the CNS uptake of lithium carbonate is limited

As the Li⁺ borne of dissociated LiCO utilizes sodium transporters for passage, it is subject to the regulation of CNS sodium dynamics exerted by the BBB (17-19). Consequently, the limited degree of passive transcellular or paracellular electrolyte transport across the BBB slows the entry of Li⁺ into the CNS (297), and results in a mean ratio of serum Li⁺ to cerebrospinal fluid Li⁺ of ~2:1 (298). Where such regulatory systems are absent, Li⁺ tends to accumulate to a greater degree; for example, bone, muscle, kidneys, and the thyroid gland display higher levels of Li⁺ per unit of tissue than the brain parenchyma (299, 300). This phenomenon may be attributable to the differential sodium permeability of central versus peripheral capillary walls. In brief, the endothelium of the BBB demonstrates markedly reduced sodium flux relative to the highly fenestrated capillaries of the systemic circulation (19). Resultantly, the doses of LiCO necessary to reach therapeutic concentrations of Li⁺ within the brain lead to excessive accumulation within off-target organs, many of which are associated with the more problematic lithium toxicities (e.g., thyroid and kidneys) (297, 299, 301).

1.6.2.3 Does lithium orotate display enhanced passage across biological membranes?
Hans Nieper originally proposed that orotic acid was a mineral carrier that could more readily transport inorganic ions – such as lithium, magnesium, or calcium – across biological membranes (11, 12). The potential dissimilarities between LiOr and LiCO pertaining to transport may be attributable to their differing dissociative properties. Unlike LiCO, LiOr may not readily dissociate at physiological pH, thus allowing it to exist in sera as a neutral complex that can utilize alternative transport pathways. As a charge-neutral pyrimidine, LiOr may make use of ENTs resident within cell membranes in a similar manner to non-ionized fluorouracil, which is itself a charge neutral pyrimidine and substrate for ENTs (22). Alternatively, OATPs may mediate the translocation of LiOr owing to their affinity for large hydrophilic organic anions and abundant localization within both the brain and BBB (Fig. 1.8) (302).

1.6.2.3.1 Organic anion transporting polypeptides

If LiOr does not dissociate, as claimed by its proponents, then it must utilize transport proteins resident within the endothelium of the BBB. OATPs of the solute carrier superfamily are pharmacologically important uptake transporters for their role in mediating the passage of numerous exogenous compounds across the BBB (21). Owing to their affinity for large, hydrophilic anionic and/or neutral molecules (302), OATPs present an intriguing target for the transport of LiOr (Fig. 1.8); LiOr is large, neutral, and hydrophilic.

Of the 11 human OATPs, OATP1A2 and OATP2B1 are of particular import with regard to the CNS entry of neurotherapeutics. Both transporters are richly expressed in the BBB (303, 304), with OATP1A2 showing additional localization in the neurons (304) and glia (305) of the brain parenchyma.

1.6.2.3.2 Equilibrative nucleoside transporters
ENTs are integral membrane proteins that mediate the transport of nucleosides and, occasionally, nucleobases (306). Of the known ENTs, human ENT1 and human ENT2 are the best characterized (307). Unlike the concentrative nucleoside transporters, ENT1 and ENT2 are capable of transporting select nitrogenous bases, uracil included (307, 308). As LiOr is structurally related to uracil, it is possible that it might utilize ENTs for transport in a similar manner to 5-fluorouracil (Fig. 1.8) (22, 309), which like LiOr is a non-ionized, charge-neutral pyrimidine.

1.6.2.4 Is pyrimidine biosynthesis involved in the dissociation of lithium orotate?

1.6.2.4.1 The role of orotic acid in the de novo biosynthesis of pyrimidines

The primary biological role of orotic acid is to serve as an integral substrate in the de novo pyrimidine biosynthesis pathway (310, 311). In brief, orotate phosphoribosyltransferase – a component of the bifunctional UMPS protein complex – catalyzes the transfer of ribose-5-phosphate-1-pyrophosphate to orotate to form orotidine 5’-monophosphate. Next, orotidine 5’-monophosphate is decarboxylated by orotidine 5’-phosphate decarboxylase to form uridine monophosphate, which is then subsequently processed into associated pyrimidine nucleotides and other bioactive metabolites, such as β-alanine and carnosine (310, 312).

1.6.2.4.2 The putative target specificity of lithium orotate may involve pyrimidine biosynthesis

Given that Li⁺ must eventually separate from its carrier to exert its influence, it is probable that some interaction or enzymatic process triggers the dissociation of LiOr. Hans Nieper originally suggested that LiOr preferentially targets cells exhibiting high rates of pentose phosphate pathway activity (12). As this pathway is in close association with de novo pyrimidine biosynthesis, it is possible that UMPS, an enzyme responsible for the conversion of orotic acid to uridine monophosphate (313), liberates Li⁺ from its carrier during the decarboxylation of the side group to which it is bound, thereby yielding Li⁺, CO₂, and uridine monophosphate as products (Fig. 1.9). If true, then a UMPS-mediated dissociation of LiOr could explain how the compound comes to dissociate and why it accumulates to a greater degree within the CNS (14); increased pentose phosphate pathway has been suggested as a
means to maintain redox homeostasis in highly metabolically active tissues (313), such as neurons, glia, and the BBB endothelium (12).

1.6.2.5 The significance of improved membrane transport and targeted dissociation
As previously discussed, much of BD symptomatology may arise from aberrant GSK3β and PI-cycle signaling. Given that GSK3β, IMPase, and IPPase are intracellularly situated, it is possible that a lithium formulation which more readily crosses biological membranes before dissociating within the intracellular compartment could result in greater control of BD symptoms. Animal studies suggest that LiOr yields higher concentrations of Li⁺ within the brain than LiCO, and that the central expression of Li⁺ progressively builds over 24 hours (14). If inhibition of GSK3β and normalization of the PI cycle are truly responsible for the drug’s therapeutic influence, then these elevations in brain Li⁺ may enable the use of LiOr at doses substantially lower than those currently prescribed for LiCO.

1.6.3 Potential concerns regarding the use of lithium orotate
1.6.3.1 Impaired renal function
As previously discussed, interest in LiOr was greatly curtailed in the late 1970s after Smith et al. (1979) noted that LiOr reduced glomerular filtration rates – though the mechanisms underlying these effects are unknown and may be dose-dependent – and advised against its consideration as a treatment option (23); however, equivalent concentrations of LiOr and LiCO were employed, thereby defeating the purpose of using LiOr in place of LiCO. To accurately assess the tolerability of LiOr, explorations into its efficacy and safety must consider whether the heightened CNS Li⁺ expression it elicits can be leveraged to reduce dosing relative to LiCO.

1.6.3.2 Promotion of cancerous cell growth
Orotic acid accelerates hepatocarcinogenesis in rats acutely exposed to known carcinogens (314, 315). While concerning, there are a couple of factors that must be taken into consideration. First, the stimulation of cancerous cell growth is only observed in initiated rats, i.e., those destined to develop hepatocarcinoma. Second, stimulation of carcinogenesis only occurs when orotic acid consumption exceeds 100 mg/kg (314, 316), which is a substantially greater amount of orotic acid than would ever be ingested by patients (e.g., <1000 mg, or ~10-15 mg/kg). Nevertheless, the use of orotic acid mineral conjugates, be they for magnesium, copper, iron, or lithium, are potentially inadvisable in individuals considered to be at risk for oncogenesis.
1.7 Our research question

Despite its efficacy (4-9), lithium therapy is plagued by high rates of treatment non-compliance (10). Lithium concentrations within the body are poorly regulated (197), which when coupled with the high doses of LiCO required for symptom management, leads to substantial off-target effects on organs such as the thyroid, parathyroid, and kidney. Given that lithium-induced toxicity correlates with serum Li⁺ content (13), the goal of lithium therapy is to ameliorate symptomatology while limiting systemic Li⁺ exposure. With this objective in mind, the rationale behind the work presented herein is to assess whether the proposed superior central accumulation of LiOr (14) can be leveraged to reduce dosing while maintaining efficacy, which would theoretically allow for control over symptom emergence alongside diminution of the adverse effects linked to excessive Li⁺ concentrations within the blood.

Before such research can be performed, a suitable model of BD must be selected. The ideal model will mirror elements of the human condition, be highly granular, and display excellent dose-sensitivity to lithium therapeutics. See 1.8 for the rationale behind the selection of the AIH model.

1.8 Selecting an appropriate animal model for contrasting lithium therapeutics

1.8.1 How are animal models validated?

1.8.1.1 Face validity

Face validity pertains to the resemblance between what is observed in an animal model relative to the targeted human phenotype (317). In general, face validity relies on the substantiation of ethological factors and biomarkers. The validation of ethological factors depends on the similarity between modelled behaviours and those considered pathologically relevant in man, e.g., reduced sucrose preference in models of chronic stress and anhedonia in human depression (318), whereas the reliability of biomarkers rests on the similitude of biological outcomes, such as stress-induced elevations in corticosterone expression in rodents versus cortisol in humans.

1.8.1.2 Predictive validity

Predictive validity encompasses both induction validity and remission validity (317). Induction validity pertains to the relationship between presumed etiological factors and outcomes, be they behavioural or biochemical, whereas remission validity concerns the congruity of treatment effects between organisms (319). These definitions are not to be conflated with the impact of triggering
factors or treatments on the mechanisms producing the effect; rather, predictive validity concerns only the resemblance between how etiological factors and treatments affect behavioural outputs or biomarker expression in animal models relative to human patients. For example, if increasing the dose of a drug induces a linear change in corticosterone expression in an animal, then this should also be found in clinical settings (e.g., altered cortisol levels) if the model displays suitable remission validity. In contrast, mechanistic validity is assessed through direct exploration and comparison of the physiological changes induced by an experimental manipulation.

1.8.1.3 Construct validity
Construct validity pertains to the theoretical rationale of a model and is primarily dictated by the similarity of the processes which lead to phenotype induction between species (317, 320). For instance, chronic stress protocols may display face, predictive, and mechanistic validity for an array of depressive phenotypes, but will not exhibit construct validity for persistent forms of depression that are unrelated to sustained stress exposure; the triggering factor in the animal model differs from that of the human condition. In contrast, a transgenic model of Huntington’s disease with genotypic abnormalities and associated phenotypic expressions which mirror the human disease state can be said to possess a high degree of construct validity.

1.8.1.4 Mechanistic validity
Mechanistic validity refers to the equivalence of mechanisms underpinning the disease state in the animal model to those presumed to be involved in the symptomatology of the human condition (317). It is important to differentiate mechanistic validity from face validity, as the similarity between mechanisms is independent from the similarity between the effects of those mechanisms. To illustrate, even if chronic stress similarly impairs hippocampal neurogenesis in both animal models and depressed human patients, the resultant symptoms and expression of biomarkers may not necessarily be identical. In short, even if a similar mechanistic process is involved in both the model and human condition, the phenotypic expression (e.g., behavioural output) may differ, which means that face validity and mechanistic validity must be considered separately.

1.8.1.5 Homological validity
The homological validity of an animal model depends upon the nature of what is being explored and the suitability of the species and species strain for such explorations (319). For example, mice are considered an appropriate choice for assessing the impact of mood stabilizers on amphetamine-
induced hyperlocomotion (a model of mania), but only when the correct strains are employed. C57Bl/6 mice respond to both amphetamine and lithium in the expected manner (hyperlocomotion and blockade of hyperlocomotion, respectively), whereas CD-1 mice display amphetamine-induced locomotor responses that are refractory to lithium treatment (25).

1.8.2 Models of mania

One of the most troublesome issues encountered during selection of a suitable model of BD is that diagnostic criteria is based, in-part, on phenomenology (321). The problem with this approach is that BD encompasses numerous illnesses which may or may not share behavioural and biochemical features. As the underpinnings of these conditions have yet to be fully elucidated, animal models aim not to recapitulate the full indeterminate disease state, but to model select behavioural phenotypes, such as hyperactivity, or endophenotypes, such as dopaminergic dysregulation (43, 322, 323) or aberrant NKA activity.

Another difficulty with attempting to model mania is that many of the core diagnostic criteria, such as delusions of grandeur, euphoric mood states, and racing thought patterns (324), can not be captured in rodent species. Fortunately, increased motor behaviours are emerging as a core diagnostic marker for mania (325), and can be readily induced in animal models (25, 326-329) through mechanisms believed to be involved in the induction of manic symptoms in human BD patients (e.g., GSK3β hyperactivity and DAT inhibition) (60, 61, 63, 74, 170, 330-334).

Outlined in the sections below are a selection of the most frequently employed models of mania that display hyperactivity and/or hyperlocomotion as a core phenotype.

1.8.2.1 Ouabain-induced hyperlocomotion

The administration of ouabain (OUA), an inhibitor of Na+/K+-ATPase, has been shown to increase locomotor activity in rats (335). The OUA model is base on the “Na+/K+-ATPase” hypothesis of BD, which postulates that decreased activity of the enzyme is involved in the etiopathogenesis of the disorder (336). Unfortunately, the effects of lithium on Na+/K+-ATPase are controversial (337, 338), and while the behavioural phenotypes induced by OUA are reversed by lithium, dose-sensitivity has not been established. In addition, the behavioural effects of OUA are quite brief (335) relative to AIH (~10 minutes versus 120 minutes), which may limit granularity when exploring the efficacy of potential therapeutics.
1.8.2.2 Sleep-wake cycle disruption

Sleep-wake cycle aberrations are primary symptoms of patients suffering from BD (38, 339, 340), and are used as a diagnostic criterion. Manipulation of circadian rhythms has been used to induce manic-like hyperactivity in a manner that is sensitive to blockade via application of lithium or dopamine receptor antagonists (341-343). While intriguing, these models are low-throughput and lack granularity, which makes them unsuitable for dose-response analyses.

1.8.2.3 GluR6 knockdown

Knockout of the glutamate receptor 6 (GluR6) has been shown to elicit manic-like behaviours, such as increased responsiveness to amphetamine, hyperactivity, and aggression. These behaviours are curtailed by chronic lithium administration. However, while the model seemingly displays features of the manic state, the associations between GluR6 perturbation and mania are unknown. Also, the molecular mechanisms underlying the behavioural effects of lithium in these mice remain to be elucidated (344, 345).

1.8.2.4 Amphetamine-induced hyperlocomotion

Amphetamine salts elicit hyperlocomotor responses downstream of increased dopaminergic tone (25, 346, 347). This is achieved through three primary mechanisms: 1) increased cytosolic dopamine concentrations through inhibition of vesicular monoamine transporter 2; 2) reversal of DAT functionality (efflux instead of reuptake) through trace amine-associated receptor 1 stimulation; 3) diminished dopamine reuptake via direct DAT inhibition (348-350).

Due to the dopaminergic effects of amphetamine, the AIH model displays some degree of mechanistic, face, and predictive validity (25, 345, 351). Altered DAT availability (59, 60) and increased D2R density (58) are observed in BD patients and, according to the dopamine dysregulation hypothesis, these alterations set the stage for the emergence of hyperdopaminergia and the subsequent manifestation of manic symptomatology (65). As the striatum – which is particularly enriched with DATs (352) – has been proposed as the principal site of action for amphetamines (353), amphetamine-induced inhibition of DAT may mimic the altered striatal DAT availability implicated in BD pathogenesis (60-63). Importantly, lithium blunts hyperlocomotion in a dose-dependent manner in rodents (25) through modulation of GSK3β signaling downstream of D2R activation (354, 355), and antagonizes the mania-mimetic effects of amphetamine in human patients (55, 56).
1.8.3 The AIH model is ideal for contrasting the efficacy of lithium therapeutics

Of the various models of mania currently available, AIH is the ideal choice for comparing the efficacy of different lithium formulations for a variety of reasons (Fig. 1.10). First, the model is affordable, rapidly inducible, highly granular, and sensitive to lithium in a dose-dependant manner (25), which makes it particularly well suited to the creation of dose-response curves. Second, the AIH model is well-supported in the literature (25, 68, 351, 356), and its hyperlocomotor phenotype is an established and accepted rodent behavioural correlate of mania (351, 357, 358). Lastly, amphetamine generates hyperlocomotion through mechanisms linked to the emergence of manic symptomatology in man (e.g., reduced DAT function and increased GSK3β signaling); lithium blunts these amphetamine-induced phenotypes in rodents by inhibiting GSK3β activity (205, 347, 354, 355), an action which is also believed to be central to lithium’s therapeutic efficacy in human therapy (50, 193, 203, 204).

Fig 1.10. The AIH model is ideal for contrasting the efficacy of lithium-based therapeutics. Schematic representation of the factors which make the AIH model well-suited for contrasting the potency of LiCO and LiOr. In brief, the AIH model demonstrates face, mechanistic, and predictive validity; responds to lithium in a dose-sensitive manner; generates a robust, easily quantified output measure; and is high-throughput. GSK3β - glycogen synthase kinase 3β; IP- intraperitoneal. Created using BioRender.
Central objectives and hypotheses

Lithium therapy is burdened by patient non-compliance resultant of the unpleasant side effect profile associated with LiCO’s narrow therapeutic range. LiOr has been found to yield significantly greater concentrations of Li⁺ within the brain (14), leading proponents to argue that it will display both elevated potency and efficacy relative to other lithium compounds (11, 12). Given the putative unique transport-related properties of LiOr (12), I hypothesize that…

The transport- and dissociation-related properties of the orotic acid carrier improve the efficacy and expand the therapeutic window of LiOr in the control of manic symptomatology.

The project objectives are to 1) contrast the efficacy of LiOr and approved lithium formulations in the AIH model of mania, 2) assess the toxicity profile of LiOr, and 3) elucidate the transport-related characteristics of LiOr that allow for enhanced efficacy (Fig. 1.1).

Objective 1: The AIH model was used to contrast the potency of LiOr relative to LiCO. AIH, which is partially mania-mimetic, is sensitive to a dose-dependent lithium blockade (25). If LiOr is more efficacious than LiCO, then a lesser dose will be required for the attenuation of hyperlocomotion.

Objective 2: The relative toxicities of LiOr and LiCO were contrasted via once daily application for 14 consecutive days. The previously determined effective ranges for each compound were employed. Markers of kidney (serum creatinine, and blood-urea-nitrogen; BUN) and thyroidal (serum TSH) health were measured post-mortem, 24 hours after final drug administration. In addition, the medial concentrations, which roughly correlate with the most oft administered dosages in man, were assessed for their impacts on memory.

Objective 3: If LiOr is dependent upon NTs/OATPs for transport and UMPS for dissociation, then their inhibition should attenuate LiOr’s effects on hyperlocomotion and LTP while sparing those of LiCO/LiCl. Inhibitors of OATPs (PEG-400, naringin) and UMPS (6-azauracil) were employed in both in vivo (AIH) and ex vivo (hippocampal LTP) models to determine their impacts on the efficacy of LiOr/LiCO/LiCl. Also, the dissociation-related characteristics of LiOr and LiCO were explored first via determination of the electrical resistivity of each compound in solution, then by assessment of their abilities to blunt GSK3β activity in vitro (more physiologically relevant). If LiOr does not freely dissociate, then it should display increased resistance to current flow compared to LiCO, as well as a lack of effect on GSK3β during the activity assay.
Fig. 1.11. Thesis background, research objectives, and experimental rationale. The narrow therapeutic window of LiCO leads to excessive side effect incidence and deleterious renal/thyroidal outcomes during long-term therapy. In turn, these adverse effects contribute to high rates of treatment regimen non-adherence. Unlike LiCO, which dissociates readily in solution, LiOr is believed to exist in sera as a non-dissociated compound. If true, then LiOr would need to utilize different transport pathways than LiCO. As LiOr has been observed to elevate central Li⁺ levels to a greater extent than LiCO, it is possible that differential transport characteristics are responsible. Thus, we propose that differential dissociative and transport-related properties improve the CNS penetration of LiOr relative to LiCO, leading to a reduction in dosing requirements that translates to superior tolerability during treatment; lithium-induced toxicities correlate with serum Li⁺ content. In objective 1, the potency and efficacy of LiOr and LiCO were contrasted in the lithium-sensitive A1H model of mania. In objective 2, the relative impacts of each compound on measures of thyroidal and kidney health were contrasted after 14 days of treatment. In objective 3, the dissociative characteristics of LiOr/LiCO were assessed by determining their resistivity in solution (higher resistivity = less ionization) alongside their capacity to inhibit GSK 3β (LiOr/LiCO must dissociate to interact with the enzyme). Next, the transport-related properties of LiOr and LiCO were contrasted by probing the impact of OATP inhibition on the ability of each to affect lithium-sensitive A1H and hippocampal LTP. Finally, as lithium compounds must eventually dissociate to affect cellular functions, the role of UMPS in mediating the decarboxylation and subsequent dissociation of LiOr was assessed. LiOr - lithium orotate; LiCO - lithium carbonate; A1H - amphetamine-induced hyperlocomotion; LTP - long-term potentiation; OATP - organic anion transporting polypeptide; UMPS - uridine monophosphate synthase.
CHAPTER 2: GENERAL METHODS

2.1 Animals
Male and female C57Bl/6NCrl mice (Charles River, Canada) aged 8 weeks were used for all studies, electrophysiology excepted. 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 molar 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 (dA) 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 molar 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 NH₄OH, then adding the resultant mixture to 99.9
2.3 Behaviour

2.3.1 Amphetamine-induced hyperlocomotion

Mice were administered \(dA\) (6 mg/kg) or saline intraperitoneally (IP), placed into an open field arena (35 x 35 x 35 cm) for 120 minutes, then scored for total locomotion offline using Ethovision XT 11 (Noldus, Wageningen, The Netherlands). Drug efficacy was measured as the ability of the tested lithium compound – administered IP 30 minutes prior to \(dA\) – 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 minutes prior to lithium injection. Locomotion is reported as percentage of the \(dA\) response maintained. The minimal effective concentration is defined as the lowest lithium concentration used to affect AIH.

2.3.2 Rotarod

Male mice were injected with saline or LiCO/LiOr 60 minutes 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 minutes. 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 minutes prior to being placed into a 4 L beaker filled with 3 L of 25°C water. Activity was recorded for 8 minutes and analyzed for time spent immobile offline using Ethovision XT 11 software (Noldus, Wageningen, The Netherlands).

2.3.4 Barnes maze

The Barnes maze apparatus consisted of a white escape box (placed beneath the escape hole of the stationary platform), white 100 cm (diameter) rotating platform with twenty 5.5 cm (diameter) escape holes evenly distributed about the perimeter, and a white 100 cm (diameter) stationary base with one escape location. The rotating platform was placed on top of the stationary base (itself 12 inches above the ground) (fig. 2).

*Habituation:* Mice were habituated to the maze on the first day of testing via placement in the center of the testing field (within a translucent container) for 10 seconds, after which the mice were
gently guided toward the escape location. Upon entry, the mice were habituated to the escape box for two minutes. The escape location was rotated every fourth mouse, and the assigned holes were held constant throughout the acquisition protocol.

Acquisition: Mice were placed in the center of the maze under a translucent container. After ten seconds, the container was lifted, and an aversive buzzer was triggered. Animals were allowed 2 minutes to locate the escape hole under duress of the buzzer, aversive overhead lighting, and the scent of a predator (soiled rat bedding). If successful, the buzzer was silenced, and the mice were held in the escape box for two minutes prior to return to their home cages. If unsuccessful, the mice were guided to the escape location, followed by cessation of the buzzer and two-minute reinforcement in the escape box. This procedure was repeated once daily for seven consecutive days. Each mouse was scored for latency to escape (entry of the nose into the escape), total number of errors (exploration of incorrect holes), and search strategy utilization (spatial versus not-spatial).

Probe: Long-term recall of the escape location was assessed 48 hours after the final acquisition trial. Animals were allowed 2 minutes to explore the platform with the escape location closed. Animals were scored for latency to escape, number of errors, and duration within the escape area.

For each acquisition and probe trial, behaviour was analyzed offline using Ethovision XT 11 software (Noldus, Wageningen, The Netherlands).

2.3.5 Modified Y-maze
Mice were placed into the maze with one arm blocked off and allowed to explore the two open arms for five minutes (training phase). 30 minutes later, mice were placed back into the maze for a period of 5 min with all three arms now open (test phase). The preference for the novel arm was assessed as a measure of short-term spatial memory.

2.4 Biochemistry
2.4.1 Organ harvesting, blood extraction, and serum isolation
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 hours at 4°C prior to centrifugation at 1500 rcf at 4°C for 15 minutes 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-second pulses and centrifuged at 20,000 rcf at 4°C for 15 minutes (5804 R centrifuge, Eppendorf). Supernatants were additionally ultracentrifuged at 200,000 rcf at 4°C for 30 minutes 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).

2.4.2 Lithium colorimetric assay
Brain/serum Li⁺ content was assessed 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 : 15µl : 120 µl from the kit-recommended 5 µl : 15µl : 130 µl (used for serum).

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

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

2.4.5 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).

2.4.6 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).
2.4.7 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). The IC50 for lithium-induced inhibition of GSK3β activity is 2 mM.

2.4.8 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.

2.4.9 PAMPA assay

200 µL of 2.5 mM LiOr or LiCO were contrasted for their ability to pass through a seeded lipid bilayer using a commercially available paired-artificial membrane permeability assay (Abnova, item no. KA6223). The donor and acceptor plates, prepared as per kit instructions, were incubated overnight at 37 °C. After incubation, the Li^+ content of both the donor and collection wells was determined using a commercially available colorimetric kit (Abcam, item no. Ab235613).

2.5 Histology and IHC

2.5.1 Haematoxylin and eosin staining

Mouse kidneys were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific) for 48 hours prior to long-term storage in PBS (0.01M, pH 7.4). Fixed tissues were sectioned on a vibratome (60 µm, Leica VT1200) and stained with hematoxylin and eosin for assessment of pathological morphology.

2.5.2 AQP2 anti-body IHC

Mouse kidneys were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific) for 48 hours prior to long-term storage in PBS (0.01M, pH 7.4). Next, fixed tissues were sectioned on a vibratome (60 µm, Leica VT1200) for use in the IHC assay, which was performed as follows: on day one, slices are placed into a 12-well plate (mesh inserts) loaded with PBS (0.01 M PBS; pH
In order, slices were 1) washed (10 min/wash) 3x in PBST (PBS with 0.3% triton x-100); 2) incubated in 0.3% peroxide (45 min); 3) washed (5 min/wash) 3x in PBST; 4) blocked (2 hours) with goat serum (0.05% in PBST); 5) incubated in 1° antibody (Abcam, item no. Ab199975) overnight at 4-8 °C. On day two, slices were 6) washed (5 min/wash) 3x in PBST; 7) incubated in Visucyte-HRP 2° antibody (80 min) (R&D Systems, item no. VC003025); 8) washed (5 min/wash) 3x in PBST; 9) incubated in FastDAB for 120 seconds (Sigma, item no. D4293); 10) mounted on frosted slides; 11) dehydrated via an alcohol series; then 12) fixed and covered with resin.

2.6 Live brain slices

2.6.1 Acute live brain slice preparation

Six- to ten-week-old male C57BL/6 mice were anesthetized with isoflurane and decapitated. The brain was rapidly removed and submerged 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. Chilled aCSF contained the following (in mM): 130 NaCl, 3 KCl, 1.25 NaH₂PO₄, 2 CaCl₂-2H₂O, 2 MgSO₄-7H₂O, 0.1 Na-ascorbate, 24 NaHCO₃, 10 dextrose, and 1 lactate; solution pH was 7.4 when saturated with 95% O₂/5% CO₂. Slices were transferred to an aCSF filled recovery chamber saturated with 95% O₂/5% CO₂ for 2 hours at room temperature prior to use.

2.6.2 Electrophysiology

Slices were placed into a 2 ml chamber continually perfused with the same aCSF (saturated with 95% O₂ and 5% CO₂) 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 seconds for the duration of all baseline and post- 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 seconds apart and repeated a second time 300 seconds after the first.

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

### 2.7 Statistics

Data are expressed as mean ± SEM and compared using one-way or two-way analysis of variance (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. Error bars are expressed as mean ± SEM unless otherwise specified.

### 2.8 Summary of protocols

Behavioural and biochemical experiments are summarized below (Fig. 2.1); standard biochemical assays, serum isolation protocols, and tissue homogenization methods were excluded.
**Chapter 3 (Objective 1)**

**AIIH (single injection)**

![Diagram](image)

Assess the degree to which each compound blunts dA-induced hyperlocomotion.

**AIIH (interval dosing)**

![Diagram](image)

Measure how long LIOr and LiCO are able to attenuate AIIH.

Determine whether the improved potency/efficacy of LIOr is attributable to unanticipated effects on exploratory behaviors (open field) or locomotor capacities (forced swim and rotarod).

**Chapter 4 (Objective 2)**

**Determination of LIOr/LiCO concentrations**

<table>
<thead>
<tr>
<th>HED (mg)</th>
<th>LiCO</th>
<th>LiOr</th>
</tr>
</thead>
<tbody>
<tr>
<td>520</td>
<td>1040</td>
<td>1500</td>
</tr>
<tr>
<td>253</td>
<td>506</td>
<td>760</td>
</tr>
</tbody>
</table>

**AED (mg Li/kg)**

<table>
<thead>
<tr>
<th>Barnes maze</th>
<th>Y-maze</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
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<td>3.0</td>
</tr>
<tr>
<td>4.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

**Toxicity induction**

![Diagram](image)

Assess the impacts of LIOr and LiCO on long-term spatial memory acquisition (training phase) and recall (probe phase).

Assess the impacts of LIOr and LiCO on short-term spatial memory recall.

**Chapter 5 (Objective 3)**

**AIIH (OATP/UMPS inhibition)**

![Diagram](image)

Assess whether PEG-400, naringin, or 6-azauracil abolish the effects of LiCO or LIOr on AIIH.

**Resistivity experiment**

![Diagram](image)

Contrast the resistivity of each solution. High resistivity = less ionization.

**GSK3β Activity experiment**

![Diagram](image)

1. Add LiCO to solution containing GSK3β, cofactors, and physiological media.
2. Assay GSK3β activity (inhibition confirms that LiCO or LIOr dissociated in solution).

**Preparation of live brain slices**

![Diagram](image)

LTP experiments

![Diagram](image)

Assess whether PEG-400, naringin, or 6-azauracil abolish the effects of LiCO or LIOr on hippocampal LTP.

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**Figure 2.1. Schematic of the general experimental protocols employed in each results chapter. AIIH - amphetamine-induced hyperlocomotion; dA - dextroamphetamine; LiOr - lithium orotate; LiCO - lithium carbonate; IP - intraperitoneal; OG - oral gavage; HED - human equivalent dose; AED - animal equivalent dose; BM - Barnes maze; OATP - organic anion transporting polypeptide; UMP - uridine monophosphate synthase; aCSF - artificial cerebrospinal fluid; LTP - long-term potentiation; BUN - blood urea nitrogen; AST - aspartate aminotransferase; TSH - thyroid stimulating hormone; IHC - immunohistochemistry; H&E - hematoxylin and eosin; AQP2 - aquaporin 2; SC - Schaffer collateral; TBS - theta burst stimulation.**
CHAPTER 3: CONTRASTING THE EFFICACIES OF LITHIUM OROTATE AND LITHIUM CARBONATE IN THE ATTENUATION OF AIH

3.1 Rationale

Although evidence for enhanced brain availability relative to LiCO was initially found (14), research into LiOr was effectively curtailed due to fears of worsened renal impairment when used at concentrations equivalent to LiCO (23). Although these concerns are valid, comparisons involving use of the two compounds at equivalent concentrations fail to consider previous reports of LiOr’s superior central expression (14). In brief, it is possible that the purported improved bioavailability of LiOr enables a reduction in dosing that would limit blood Li$^+$ levels while maintaining suitable expression within the brain. Considering that the toxicities associated with lithium therapy correlate with serum Li$^+$ concentrations (13), limiting exposure while retaining efficacy would greatly improve tolerability.

In this chapter, the efficacy and potency of LiOr and LiCO against AIH (25) were explored across a range of concentrations in both male and female wild-type mice, with the typical therapeutic dose of LiCO (adjusted for a murine model) serving as the upper bound. Owing to its mechanistic (60, 65, 205, 326, 329, 346, 349), face (68, 70, 325, 356, 358-360), and predictive validity (25, 55, 205, 354, 361), the AIH model is well suited to the task of comparing lithium therapeutics. In short, amphetamine elicits hyperlocomotion (25) downstream of increased GSK3β activity (347) consequent to an enhanced dopaminergic tone (346) (Fig. 3.1). Lithium antagonizes hyperlocomotion in a dose-dependent manner (25) through inhibition of GSK3β signaling (205, 354, 355). Given the proposed central role of GSK3β in mediating lithium’s therapeutic efficacy (4, 77, 182, 354, 362), it is possible that observations of improved potency

![Figure 3.1: Amphetamine induces hyperlocomotion by increasing dopaminergic tone.](image-url)
in the murine AIH model will translate to the human condition.

3.2 Objectives and hypotheses

Given previous reports of greater levels of Li⁺ within the brain following administration as an orotate as opposed to as a carbonate (14), I hypothesize that...

*LiOr displays reduced dosage requirements relative to LiCO in the attenuation of lithium sensitive AIH.*

The objectives of this chapter were to contrast the efficacy and potency of LiOr relative to LiCO in a lithium-sensitive, mania-like *in vivo* model. To this end, the AIH model was selected because it is partially mania-mimetic and exhibits a dose-dependent loss of phenotype following pre-application of lithium (25) (see 1.8.3 for further details).

3.3 Experimental groups and method modifications

3.3.1 Experimental groups

Male and female C57Bl/6 mice aged 3-4 weeks were used in all AIH studies other than the interval dosing trials. Male mice were used in the open field, forced swim, and rotarod experiments. For the AIH trials, mice were assigned into groups to receive either saline, lithium chloride (1, 5, 10, 15, 20, or 25 mg Li⁺/kg), LiCO (1, 5, 10, 15, 20, or 25 mg Li⁺/kg), or LiOr (1, 1.5, 2.5, 5, 10, or 15 mg Li⁺/kg). In the interval dosing trials, mice were treated with one of saline, LiCO at 15 mg Li⁺/kg, or LiOr at 2.5 mg/kg. For tests of baseline exploration (open field) and locomotor capacity (forced swim/ rotarod), mice received either saline, 15 mg Li⁺/kg LiCO, or 2.5 mg Li⁺/kg LiOr.

3.3.2 Statistics

All treatment groups were compared to the relevant control using one-way ANOVA coupled with Dunnett’s post-hoc testing. For serum Li⁺ quantification, matched LiCO and LiOr concentrations were contrasted using Tukey’s analysis. An alpha value of 0.05 was the threshold for significance.

3.4 Results

3.4.1 Amphetamine-induced hyperlocomotion

When administered stimulants, mice display a mania-mimetic hyperactive phenotype (25, 351). In brief, the administration of *dA* to rodents heightens dopaminergic tone by increasing synaptic concentrations of dopamine (346, 363), leading to induction of a GSK3β-dependent
A hyperlocomotor response that is believed to reflect select aspects of mania (Fig. 3.1) (43, 351).

In our model, the application of 6 mg/kg dA sulfate to wild-type mice results in two distinct periods of hyperlocomotor activity between minutes 0-35 and 70-120, with no differences between males and females (Fig. 3.2). As the influence of both dA (hyperlocomotion) and lithium (blockade of hyperlocomotion) are most pronounced from minutes 70-120, we opted to contrast the efficacies of LiCO and LiOr within this window. Importantly, the drug potency-related data gathered during this period was recapitulated at a lesser dose of dA (3 mg/kg), supporting its generalizability within the overall AIH model (i.e., the observed results are not exclusive to one dosage of dA) (Box 1A).

Figure 3.2. Effects of dA on locomotor activity. The administration of $6.0 \text{ mg/kg} \, \text{dA}$ consistently resulted in two periods of hyperlocomotion in both males and females. For consistency, the shaded 70-120 minute period was used to contrast the efficacy and potency of each compound. Error bars represent mean ± SEM. For the females, $n = 5$ for dA and the saline control; for the males, $n = 11$ for dA and 8 for the saline control. dA = d-amphetamine; Sal = 0.9% saline.

Box 1. LiCO and LiCl are functionally identical; and the improved potency of LiOr is maintained against 3 mg/kg dA-induced hyperlocomotion. A) The maximal effective concentrations of LiOr/LiCO were injected 30 minutes prior to administration of 3 mg/kg dA in male mice. Locomotion was scored over the initial 90 minutes post-injection. The differential dosing requirements of LiOr and LiCO were confirmed, with LiOr eliciting a more robust attenuation of AIH despite being dosed at 1/10th the concentration. While the exact differences in required dose were not elucidated, less LiOr is required for the blockade of AIH induced by either 3 mg/kg or 6 mg/kg of dA. B) LiOr and LiCl were administered 30 minutes before dA (6 mg/kg), and locomotion was scored between minutes 70-120 post-dA injection. The dose response curves for LiCO are identical to those of lithium chloride, signifying that while most lithium salt formulations are functionally identical, LiOr is unique. Additionally, both LiCO and lithium chloride are only efficacious at concentrations of 15-20 mg/kg or more, which when adjusted via allometric scaling from mouse-to-man, roughly correlates with the lower dosage range employed in human patients. Mean ± SEM. All groups compared to the dA control via one-way ANOVA with Dunnett's post-hoc. *P < 0.05. **P < 0.01. LiOr = lithium orotate; LiCO = lithium carbonate; LiCl = lithium chloride; dA = d-amphetamine.
In contrast, minutes 35-70 are characterized by motor stereotypies that a) are difficult to quantify using software and b) are not rodent behavioural correlates for mania (Box 2A). As for the hyperlocomotor response between minutes 0-35, it is potentially confounded by injection stress, acclimation to the novel environment, and the delayed effects of our lithium compounds (Box 2B).

Box 2. Effects of LiOr/LiCO on amphetamine-induced motor stereotypies (minutes 35-70) and early locomotor behaviors (minutes 0-35). LiOr/LiCO were injected 30 minutes prior to dA (6 mg/kg). A) Minutes 35-70 after dA injection are characterized by motor stereotypies. Stereotypic behaviors are defined as those which result in repetitive activity sans locomotion, e.g., repeated licking or gnawing. Instances of locomotion or body elongation were filtered out, and the degree of stereotypy was presented as the ratio of head movement to immobility. Head activity is defined as movement sans ambulation, while immobility is defined as the absence of movement. LiOr partially blunts the expression of stereotypic behaviors at 5 mg/kg in males and 1.5 mg/kg in females. In contrast, LiCO has no effect on male mice, and only elicits an attenuation in females at doses exceeding 20 mg/kg. B) Locomotor activity was observed from minutes 0-35 after the injection of amphetamine. Within this period, LiOr attenuates hyperlocomotion at concentrations greater than or equal to 10 mg/kg in males and 5 mg/kg in females, whereas LiCO is ineffective. While intriguing, the data collected within this time window is potentially confounded by residual stress from the IP injections, the initial period of acclimation to the testing apparatus, and the delayed effects of lithium. Lithium concentrations are presented as mg of elemental lithium per kg of body weight. Mean ± SEM. Groups compared to dA control via one-way ANOVA with Dunnett’s post-hoc testing. *P < 0.05. **P < 0.01. Sample sizes enclosed within parentheses. LiOr - lithium orotate; LiCO - lithium carbonate; dA - d-amphetamine; Sal - 0.9% saline.
3.4.2 Lithium orotate is more efficacious and potent in the blockade of AIH

To assess the ability of LiCO/LiOr to attenuate AIH, the compounds were injected IP at various concentrations 30 minutes prior to dA administration. We found that both compounds attenuate AIH in a dose-dependent manner from minutes 70-120 post-dA (Fig. 3.3A), with LiOr showing a dramatically reduced minimal effective concentration relative to LiCO in both the male and female cohorts (Fig. 3.3B). In males, the minimal effective concentration is 15 mg/kg for LiCO and 1.5 mg/kg for LiOr (Fig. 3.3A, top). While the dose-response curves are similar in female mice, the minimal effective concentration of LiCO did demonstrate a rightward shift from 15 mg/kg to 20

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Figure 3.3. Comparison of LiOr and LiCO effects on amphetamine-induced hyperlocomotion. A) LiOr and LiCO were administered 30 minutes before dA (6 mg/kg). For the dose-response curves, locomotor scores were expressed as a percentage of dA-induced responses. Dashed lines represent 0% (top; dA) and 100% (bottom; saline) blockade of hyperlocomotion. Total locomotion was scored between minutes 70-120 after the injection of amphetamine. B) The MEC of LiCO and LiOr is shown for attenuation of AIH is displayed. C) The effects of 2.5 mg/kg LiOr and 15 mg/kg LiCO on baseline activity and motor capacity were assessed using the open field, forced swim, and rotarod tests. The contribution of sodium orotate (2.5 mg/kg) to the effects of LiOr on AIH were assessed. D) The efficacy of LiOr (2.5 mg/kg) and LiCO (15 mg/kg) against AIH were contrasted 12, 24, or 36 hours post-administration. Lithium concentrations are presented as mg of elemental lithium per kg of body weight. Mean ± SEM. All groups were compared to the dA control via one-way ANOVA with Dunnett’s post-hoc testing. *P < 0.05, **P < 0.01. Sample sizes enclosed within parentheses. LiOr - lithium orotate; LiCO - lithium carbonate; dA - amphetamine; Sal - 0.9% saline; NaOr - sodium orotate; MEC - minimal effective concentration.
mg/kg, whereas the minimal effective concentration of LiOr was unchanged (Fig. 3.3A, bottom). Concurrent with its increased potency, the minimal effective concentration of LiOr elicits a stronger attenuation of hyperlocomotion (75.46 ± 16.95% males; 97.45 ± 6.78% females) than the minimal effective concentration of LiCO (67.18 ± 9.64% males; 82.4 ± 3.99% females). Further, the minimal effective concentration for LiOr produces a more robust blockade than even the maximum effective dose of LiCO, regardless of sex (Fig. 3.3A).

Of note, the dose response curves for LiCO are nearly indistinguishable from those of lithium chloride, signifying that while most lithium salt formulations are functionally identical, LiOr is unique (Box 1B).

3.4.3 The superior efficacy of lithium orotate is not attributable to alterations in baseline locomotor function or exploratory behaviour

The effects of the minimal effective concentration 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 are induced by either LiCO or LiOr in the absence of dA in male mice (Fig. 3.3C), thereby signifying that the suppression of hyperlocomotion is 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 has no discernable effect on hyperlocomotion (Fig 3.3C).

3.4.4 The effects of Lithium orotate on hyperlocomotion are more persistent

Given previous reports concerning the ability of LiOr to progressively increase central Li$^+$ levels over the span of 24-hours (14), we sought to determine whether LiOr could blunt hyperlocomotion when dosed 12, 24 or 36 hours prior to dA challenge. We observed that 15 mg/kg LiCO fails to elicit a significant effect at any time-point beyond 30 minutes. In contrast, 2.5 mg/kg LiOr blocks 66%, 56%, and 52% of AIH at the 12-, 24-, and 36-hour post dA-injection time-points, respectively (Fig. 3.3D). Thus, in addition to LiOr demonstrating improved potency (improved effect at reduced
concentrations) and efficacy (greater blockade of hyperlocomotion), it also extends the duration of the attenuation of hyperlocomotion relative to LiCO.

**3.4.5 Lithium orotate retains efficacy even when associated Li⁺ concentrations are undetectable within the serum**

![Graphs showing serum Li⁺ concentrations and brain Li⁺ levels for male and female rats across different Li⁺ doses. The graphs indicate a significant attenuation of hyperlocomotion with LiOr at lower Li⁺ concentrations compared to LiCO.](image)

**Figure 3.4. LiOr yields greater brain and serum lithium levels than LiCO.** A) The levels of Li⁺ within the serum were determined for all treatment groups via colorimetric assay. LiCO at 5 mg/kg (male only), LiOr at 1 and 1.5 mg/kg (male and female), and LiOr at 2.5 mg/kg (female only) yielded Li⁺ levels that fell below the detection limit. B) Brain Li⁺ levels were assessed for all treatment groups using homogenized and purified tissues. C) The serum Li⁺ levels associated with LiCO or LiOr doses that are % of the minimal effective concentration for each drug are displayed (top). Also, the minimal effective concentrations of LiCO and LiOr are listed alongside the serum Li⁺ levels they produce (bottom). 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. For serum lithium, matched concentrations e.g., LiOr 5 versus LiCO 5 • were compared with Tukey’s post-hoc test (matched pairs can be identified by the black bars situated above the histograms). *p<0.05, **p<0.01. Sample sizes enclosed in parentheses. MEC = minimal effective concentration; LiOr = lithium orotate; LiCO = lithium carbonate; BD = below detection limit; N/A = not-applicable (no samples). The detection limit was 0.1 mM (hence the absence of saline controls). Because serum Li⁺ values were <0.1 mM, statistical testing relative to control could not be performed.
We observed that serum Li\(^+\) levels are elevated in mice treated with LiCO or LiOr relative to saline alone, regardless of sex (Fig. 3.4A; saline not shown due to failure to meet the 0.1 mM threshold). As LiOr has previously been shown to increase central Li\(^+\) levels relative to LiCO (14), brain Li\(^+\) concentrations were contrasted in LiCO and LiOr treated mice. We found that both lithium compounds significantly increase brain Li\(^+\) levels relative to control at all concentrations greater than 1 mg/kg, with LiOr-treated mice displaying higher brain Li\(^+\) levels than LiCO at doses greater than or equal to 10 mg/kg in both males (Fig. 3.4B, left) and females (Fig. 3.4B, right). While significance was not reached, a trend toward increase was observed at 5 mg/kg for LiOr.

Intriguingly, the serum Li\(^+\) levels resultant of LiOr administration are only detectable when doses 1.67 times greater than the minimal effective concentration of 1.5 mg/kg are used (for both sexes), whereas LiCO generates detectable levels when employed at concentrations well below the compound’s minimal effective concentration of 15 mg/kg in males and 20 mg/kg in females (Fig. 3.4C). Given the associations between serum Li\(^+\) content and lithium-induced toxicities (13), this may have implications regarding the tolerability of LiOr (explored in chapter 4).

### 3.5 Discussion

#### 3.5.1 Summary of findings

- Concerning the blockade of hyperlocomotion in the AIH model of mania, LiOr is both more potent and efficacious than LiCO; these differences are not attributable to alterations in baseline locomotor capacity or explorative behaviour
- The effects of LiOr on AIH are not due to any unanticipated influences of the orotic acid carrier post-dissociation
- LiOr demonstrates efficacy even when Li\(^+\) levels are undetectable within sera

#### 3.5.2 The validity of the AIH model supports the translational potential of lithium orotate’s improved potency

These are the first studies to contrast the dose-response characteristics of LiOr and LiCO within the context of BD. Previously, work by Kling et al. demonstrated that greater concentrations of Li\(^+\) within the brain are observed when administered as an orotate as opposed to as a carbonate; this increased central expression persisted for 24 hours, even as serum Li\(^+\) content progressively declined (14). Given that the therapeutic efficacy of lithium is believed to be attributable to
interactions with intracellular enzymes situated within the constituent cells of the brain parenchyma (183, 215, 216), we hypothesized that the potential enhanced CNS penetration of LiOr would increase its potency relative to LiCO in a mania-mimetic animal model. In line with our predictions, we observed that the requisite dose for antagonism of hyperlocomotion was markedly lower in mice treated with LiOr, and that the strength of blockade elicited was demonstrably greater (Fig. 3.3), indicating that the potency and efficacy of LiOr are superior to LiCO within our chosen experimental paradigm. With that said, the strength and translational potential of these findings are dependent on the validity of the AIH model.

In support, the translational potential of our observations is buttressed by 1) the similitude between amphetamine’s mechanism of action and the dopamine-related processes implicated in BD pathogenesis; 2) the generalizability of the differential dosing requirements of LiOr and LiCO; and 3) the similarities between the effective concentrations of LiCO used in the AIH model and those employed in human lithium therapy (after allometric adjustment).

First, the face validity and mechanistic validity of the AIH model are supported by the shared dopamine-dependent and GSK3β-dependent pathways potentially involved in both hyperlocomotion in mice and manic symptomatology in man (Fig. 3.5). In rodents, amphetamine promotes GSK3β signaling downstream of D2R activation (346, 347, 354) via an enhancement of dopaminergic tone consequent to inhibition of DAT-mediated dopamine reuptake (Fig. 3.5A) (346, 351, 363, 364). In turn, this elevation in GSK3β activity precipitates hyperlocomotion (347, 354, 357), which is thought to be a rodent behavioural correlate of mania (Fig. 3.5B) (25, 351, 356, 358, 360, 365). Similarly, excessive GSK3β output (39, 41, 42, 75, 366), increased expression of D2Rs (58), and reduced availability of DAT (59, 60, 63, 367) are observed in manic patients; and these alterations to dopaminergic activity are believed to occur chiefly within the striatum (60, 367), which is amphetamine’s principal site of action (352, 353). In addition, the mania-mimetic effects of dopaminergic stimulants are not exclusive to rodent models: amphetamine precipitates the onset of manic-like symptoms in BD patients (68, 368, 369), and levodopa administration and withdrawal in individuals being treated for Parkinson’s disease elicits cyclical mood dysregulations reminiscent of BD (67).

Regarding the predictive validity of the model, the minimal effective concentration for LiCO (and LiCl) translates to ~500 mg of LiCO/day (total, not elemental) in an average sized adult when
allometrically scaled, which aligns with the lower end of the doses recommended for maintenance
therapy (370, 371). This supports the proposal that the concentrations required for blockade of
hyperlocomotion roughly correlate with therapeutically relevant dosages used in the prevention
of mood episode recurrence (Fig. 3.5C). Although no assessments concerning the use of LiOr in BD
have been performed since the early work of Hans Nieper (12), the disparity in requisite dosing
between LiOr and LiCO in the AIH model reported herein is supported within the literature. First,
Gould et al. found that 100 mg/kg of LiCl administered to mice (~500 mg in human patients) was
sufficient to blunt hyperlocomotor activity in the AIH model, whereas 50 mg/kg was not (25) (50-
100 mg of total LiCl/kg is ~8-16 mg of Li+/kg). These findings are in line with the minimal
effective concentration for LiCO (15 mg Li+/kg) observed in our trials, and further supports the
dosing inequities between LiCO and LiOr demonstrated in this manuscript; recalls that LiOr
curtails hyperlocomotion at just 1.5 mg Li+/kg. Second, in studies exploring their efficacy in the
cessation of alcohol abuse, LiCO was found to be either mildly efficacious (290) or outright
ineffective (291) when employed at doses >600 mg/day (~112 mg of Li\(^+\)), whereas LiOr successfully prevented relapse when used at 150 mg/day (~6.4 mg of Li\(^+\)) over 6 months (15). Finally, the differential dose response curves of LiCO and LiOr observed in our AIH studies were recapitulated in separate \textit{ex vivo} live slice experiments that contrasted the effects of each compound on hippocampal LTP and LTD (Box. 3). While these observations regarding LTP, LTD, and relapse prophylaxis are unrelated to BD, they nevertheless provide additional contexts wherein LiOr elicits an effect at a substantially lesser dose than LiCO, which would not be the case if the pharmacokinetic and pharmacodynamic properties of the two compounds were in fact identical.

**Box 3. Impacts of LiOr and LiCl on hippocampal plasticity within the CA1 field.** LiOr and LiCl were washed over slices via addition to the aCSF perfusate for 15 minutes prior to induction of LTP via TBS (A) or LTD via LFS (B). Lithium compounds remained in the perfusate for the entire experiment. The amplitudes of the final 10 sweeps of the 30-minute post-LTP/LTD recording were expressed as a percentage of the final 10 sweeps of the baseline. LFS involved stimulation at 1 Hz for 10 minutes. TBS involved 8 bursts (at 5 Hz) of 4 pulses (at 100 Hz) delivered 3 times, 60 seconds apart (repeated a second time 300 seconds later). Stimulation was applied to the Schaffer collaterals (30% max amplitude), and fEPSPs were recorded from the CA1 at synapses between the dendrites of the pyramidal cells and the axonal terminals of the Schaffer collaterals. Mean ± SEM. All groups compared to the appropriate aCSF control via one-way ANOVA with Dunnett’s post-hoc. *P < 0.05. **P < 0.01. Sample sizes enclosed within parentheses. LiOr - lithium orotate; LiCl - lithium chloride; aCSF - artificial cerebrospinal fluid; TBS - theta burst stimulation; LFS - low-frequency stimulation; fEPSP - field excitatory post-synaptic potential; LTP - long-term potentiation; LTD - long-term depression. Work performed by Hannah Walk as part of a separate project (data unpublished).

In summary, the odds that the improved potency of LiOr in the AIH model will translate to the human condition appear promising: lithium antagonizes hyperlocomotion through mechanisms believed to be central to its therapeutic efficacy in man (e.g., GSK3\(\beta\) inhibition; Fig. 3.6) (347, 354, 355); amphetamine elicits behavioural correlates of mania through induction of pathways implicated in BD pathophysiology (e.g., reduced DAT function and increased GSK3\(\beta\) signaling) (3.5A); the doses of LiCO required for blockade of hyperlocomotion are similar to those used in
lithium therapy; and the differential dosing requirements of LiOr and LiCO are observed across numerous contexts (Fig. 3.5C; Box 3) (15).

3.5.3 The superior potency of lithium orotate is due to an enhanced blockade of hyperlocomotion rather than off-target effects on drug absorption or excretion

There are many possible explanations for the observed discrepancy in dosage requirements between LiCO and LiOr, including: elevated serum expression of LiOr as a consequence of orotic acid-mediated disruption of glomerular filtration, as was proposed by Smith et al. in 1978 (23); altered dA uptake due to interactions with orotic acid in the peritoneal cavity and/or blood; and differential transport- and dissociation-related characteristics which enable the increased cellular uptake of LiOr relative to LiCO. In support of the latter option, several lines of evidence counter the proposition that the differential effects of LiOr are due to unanticipated influences on the absorption or excretion of either Li⁺ or dA.

If LiOr altered the concentration of dA through modulation of absorption or excretion, we would expect either the incidence of motor stereotypies to dramatically increase (if dA excretion is diminished/absorption is increased) or for the hyperlocomotor response to mirror those observed at lesser doses of dA (if dA excretion is increased/absorption is blocked) (Fig. 3.7) (346), neither of which was observed (Fig. 3.3A). Also, the diminution of hyperlocomotion elicited by LiOr...
against a lesser concentration of \(dA\) (Box 2) proves that LiOr neither promotes the absorption of \(dA\) nor delays its excretion, for if it did, either the resultant hyperlocomotor response would have been greater or motor stereotypies would have emerged between minutes 35-70 post-administration (346).

Regarding the effects of orotic acid on \(Li^+\) absorption/excretion, the fact that just 1.5 mg \(Li^+/kg\) was required for the attenuation of hyperlocomotion when delivered as LiOr makes it highly unlikely that impairments in renal \(Li^+\) clearance are responsible for the compound’s improved potency. Even if orotic acid reduced the rate at which \(Li^+\) was excreted, a 1.5 mg \(Li^+/kg\) concentration would not be able to generate serum \(Li^+\) levels sufficient to affect the hyperlocomotor phenotype (25). In support, serum \(Li^+\) expression was less than 0.1 mM following application of the 1.5 mg/kg dose of LiOr in our AIH trials (Fig. 3.4C), proving that reduced filtration of \(Li^+\) could not have been responsible for the differential effects of LiOr and LiCO. Furthermore, sodium orotate alone does not influence hyperlocomotion (Fig. 3.3B), indicating that orotic acid does not significantly alter GFR at the concentrations employed in our AIH trials.

The idea that the effects of LiOr on AIH are due to impacts on GFR are further opposed by our observation of similar differences in the dose-response curves of LiOr and LiCO in \textit{ex vivo} live slice assessments of each compound’s influence on hippocampal LTP and LTD (Box 3). While these external findings don’t necessarily rule out the potential impacts of LiOr on renal excretion and \(dA\) uptake, it is reassuring that the differential efficacies of LiOr and LiCO are recapitulated
in a model that a) does not involve $dA$, and b) is free from the confounds associated with drug absorption and excretion.

In closing, it appears likely that the improved potency of LiOr is not due to any unanticipated impacts on drug absorption or excretion, thereby countering the proposal put forth by Smith et al. in 1979 (23). If the inequitable abilities of LiOr and LiCO to affect hyperlocomotion are not due to off-target effects of the orotic acid carrier, then it is probable that differential transport- and/or dissociation-related characteristics are responsible for the observed discrepancies in the AIH model.

### 3.5.4 Conclusions

LiOr is more potent in the AIH model of mania, and the recapitulation of these results in *ex vivo* experiments suggests that this is not due to alteration of $dA/Li^+$ absorption/excretion. This hypothesis is explored further in chapter 5. Also, the predictive validity of the model is supported by the minimal effective concentrations of LiCO mirroring the lower doses used to treat BD.
**Transition statement**

Following establishment of LiOr’s improved potency and efficacy in the AIH model, the next logical step is to assess the tolerability of the compound relative to LiCO at therapeutically relevant concentrations. In 1979, Smith et al. raised concerns regarding the potential renal toxicity of LiOr, and cautioned against use of the compound in BD (23). As our findings in chapter 3 suggest that the elevations in brain Li⁺ proposed by Kling et al. (14) can be leveraged to reduce dosing, we propose that these reduced dosage requirements will improve the tolerability of LiOr (Fig. 3.8) and thereby quell the concerns raised by Smith and associates (23).

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**Rationale for chapter 3**

**The problem with LiCO**

The narrow therapeutic window of LiCO contributes to high rates of treatment non-compliance.

BD requires treatment throughout the life-span, thus necessitating discovery of more tolerable alternatives.

**What about LiOr?**

1973 Nieper advocates for the use of LiOr in mania  
Nieper, 1973

1978 LiOr yields brain Li⁺ levels 3x greater than LiCO  
Kling et al., 1978

**Central question**

Can the putative capacity of LiOr to elevate central Li⁺ expression be leveraged to reduce dosing requirements?

**Major findings in chapter 3**

The potency of LiOr was compared to that of LiCO in the mania-mimetic AIH model.

LiOr is more potent and efficacious than LiCO

**Moving forward into chapter 4**

Given that the incidence and severity of lithium-induced adverse outcomes correlate with serum Li⁺ content, we next explored whether the improved potency of LiOr translates to enhanced tolerability relative to LiCO.

---

**Fig 3.8. Diagrammatic representation of the how the results of chapter 3 set the stage for chapter 4.** The narrow therapeutic window of LiCO contributes to high rates of treatment non-compliance. LiOr, which may result in brain Li⁺ levels greater than those achieved by LiCO, may represent a more potent and efficacious alternative. This supposition was explored by using the mania-mimetic AIH model to contrast the efficacies of LiOr and LiCO. As we found LiOr to blunt hyperleomotion at reduced concentrations relative to LiCO, we next explored whether this improved potency translates to a superior safety profile; the toxicity of lithium correlates with its expression within sera. LiOr - lithium orotate; LiCO - lithium carbonate; AIH - amphetamine-induced hyperlocomotion.
CHAPTER 4: ASSESSING THE TOLERABILITY OF LITHIUM OROTATE

4.1 Rationale

Current lithium therapy relies chiefly on the use of LiCO, which dissociates readily in solution and requires administration of doses large enough to force Li$^+$ across the BBB and into cells via sodium channels. The main problems with this treatment are two-fold: the requisite dosages result in serum Li$^+$ levels that can instigate significant adverse reactions (272, 273), which in turn contributes to high rates of treatment non-compliance (257).

The side effects of short-term LiCO use typically involve some combination of polydipsia, polyuria, nausea, diarrhea, or tremor. More severe side effects, including cognitive impairment, hypothyroidism and hyperparathyroidism, may be observed during the intermediate stages of treatment (273). End-stage renal failure is a possible but largely unlikely outcome of long-term therapy, e.g., >10-15 years (Fig. 4.1) (275).

The issues associated with LiCO therapy are believed to be caused by reduced thyroid hormone synthesis and release (manifests as increased serum TSH) (276, 277); increased parathyroid hormone signaling (leading to impaired water balance courtesy of hypercalcemia) (278, 279); and disrupted interactions between antidiuretic hormone and its receptors on the collecting ducts of the nephron (causing polyuria and secondary polydipsia). Given enough time, these actions may precipitate hypothyroidism, which can occur within months of starting therapy, and/or renal insufficiency, which is an uncommon late-stage outcome (276).

In chapter 4, 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 minimal effective concentration (minimal effective concentration was 1.5 mg/kg for LiOr and 15 mg/kg for LiCO) delivered once daily for 14 consecutive days. When allometrically scaled, the LiCO concentrations employed roughly correlate to the therapeutic range utilized in human therapy, i.e., 15-45 mg
Li⁺/kg in mice translates to ~400-1500 mgs of total LiCO in an adult patient (Fig. 4.2). The human equivalent dose is determined by dividing the animal dose by 12.3, which is the correction factor ratio for human to mouse scaling; the correction factor is determined by dividing the body weight (kg) of a species by its surface area (m²) (372). All mice were sacrificed on the 15th day, 24 hours after receiving their final dose of lithium.

4.2 Objectives and hypotheses
Considering the observed lesser dosage requirements of LiOr relative to LiCO (12, 14), and the associations between serum Li⁺ content and toxicity, I hypothesize that…

LiOr displays improved tolerability relative to LiCO.

In brief, the objective of chapter 4 was to contrast the safety profiles of LiOr and LiCO by measuring markers of thyroidal and renal health after 14 consecutive days of once daily administration. The concentrations employed for each compound reflect 1x, 2x or 3x the minimal effective concentrations determined in chapter 3.

4.3 Experimental groups and methodological modifications
4.3.1 Experimental groups
Male and female C57Bl/6 mice aged 3-4 weeks were used for the main toxicity probe, whereas only males were employed for the behavioural studies. For the primary toxicity probe, mice were randomly assigned to one of 7 treatment groups, with each group receiving either saline, LiCO at 15, 30, or 45 mg Li⁺/kg, or LiOr at 1.5, 3.0, or 4.5 mg Li⁺/kg. All drugs were delivered via oral gavage. For the behavioural experiments, mice were treated with one of saline, LiCO (30 mg Li⁺/kg), or LiOr (3.0 mg Li⁺/kg).

4.3.2 Toxicity protocol
For both the behavioural studies and main toxicity probe, all mice received their allocated treatments once daily via oral gavage for 14 consecutive days. Whole blood was harvested on day
15, 24 hours after final drug administration, for analysis of serum markers of kidney and thyroidal health. Regarding the behavioural studies, the Barnes maze acquisition trials started on day 3 and ended on day 10, followed by the probe trial 48 hours later (day 12). The y-maze tests were run on day 14 of the protocol.

4.3.3 Statistics
For the assessment of polydipsia, long-term spatial recall, and short-term spatial recall, all treatment groups were compared to the saline control via one-way ANOVA with Dunnett’s post-hoc testing. Two-way ANOVA coupled with Bonferroni’s post-hoc analysis was used to determine the contributions of sex to the effects of LiCO and LiOr on serum TSH, creatinine, BUN, and AST.

4.4 Results
4.4.1 Lithium orotate shows no effect on water intake or kidney/thyroidal function
The potential early adverse effects of LiOr and LiCO on kidney and thyroid health – characterized, in part, by elevated serum TSH, AST, BUN and/or creatinine levels – were contrasted in male and female mice at concentrations of 1x, 2x or 3x the minimal effective concentration (minimal effective concentration 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 human therapy (Fig. 4.2). In other words, 15-45 mg Li+/kg in mice translates to ~400-1200 mg of total LiCO in an adult patient (373). All mice were sacrificed on the 15th day, 24 hours after receiving their final lithium dose.

Given that polydipsia is a frequent adverse effect of lithium use, we compared the water intake of mice treated with either compound; intake was measured every 5th day and is reported as ml/animal/day. LiCO, but not LiOr, elicited polydipsia when administered at concentrations greater than or equal to 2x the minimal effective concentration, with the first signs of excessive water intake observed on day 5 for the 3x dose and day 10 for the 2x dose (Fig. 4.3A). While the 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 tested concentrations (Fig. 4.3A, male), whereas water intake plateaued on days 10-through-15 in female mice treated with the 3x dose (Fig. 4.3A, female). As was the case for the male cohort, female water intake was unaffected by the ingestion of LiOr. Neither compound had any impact on body weight (Fig. 4.3B).
Next, we assessed treatment effects on serum BUN and creatinine, which are metabolic waste products used to estimate kidney function. Treatment with lithium significantly altered creatinine expression (two-way ANOVA treatment, P=0.023), with post-hoc analyses revealing marked differences in mice treated with LiCO versus LiOr. Consistent with the lack of effect on polydipsia, LiOr did not alter serum creatinine levels relative to control, even when employed at doses three-fold greater than its minimal effective concentration (Table 1). In contrast, the 3x dose of LiCO significantly elevated creatinine levels in the male cohort (Table 1). Despite the sex of the animal influencing total variance (two-way ANOVA sex, P=0.020), no significant interactions between sex and treatment were noted (two-way ANOVA interaction, p=0.112). Neither drug affected serum BUN expression (Table 1).

Serum AST content, which can indicate kidney and/or liver damage when increased, was also measured. At this early time-point, neither LiCO nor LiOr substantially affected AST (Table 1); however, when considered alongside the relatively large (albeit not significant) contribution of treatment type to the total variance (two-way ANOVA treatment, p=0.151), the general trend toward increase observed in each group treated with LiCO (1x, 2x, or 3x the minimal effective concentration) suggests that the lack of significance may be due to insufficient study power.
As the thyroid is also known to be impacted by lithium, we probed the impacts of each drug on lithium-induced hypothyroidism via quantification of serum TSH. In brief, elevated release of TSH from the pituitary is a compensatory response to insufficient thyroid hormone output (276, 277). While LiOr once again had no effect, both the 2x and 3x doses of LiCO substantially elevated serum TSH expression (Table 1). Intriguingly, these effects of LiCO were only observed in the female cohort (two-way ANOVA sex, p<0.0001; interaction, p<0.001).

Table 1. Measures of kidney and thyroid toxicity

<table>
<thead>
<tr>
<th>Sex</th>
<th>Drug</th>
<th>Conc.</th>
<th>Creatinine</th>
<th>BUN</th>
<th>AST</th>
<th>TSH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>xMEC*</td>
<td>mg/dl</td>
<td>mg/dl</td>
<td>pg/ml</td>
<td>ng/ml</td>
</tr>
<tr>
<td>Male</td>
<td>Control</td>
<td>0x</td>
<td>1.64 ± 0.16 (7)</td>
<td>23.17 ± 1.70 (6)</td>
<td>5284 ± 909 (6)</td>
<td>5.59 ± 0.57 (7)</td>
</tr>
<tr>
<td></td>
<td>LiCO</td>
<td>1x</td>
<td>1.88 ± 0.24 (6)</td>
<td>23.77 ± 0.94 (6)</td>
<td>5847 ± 701 (5)</td>
<td>5.34 ± 0.65 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2x</td>
<td>1.72 ± 0.13 (6)</td>
<td>21.81 ± 0.80 (6)</td>
<td>3970 ± 167 (5)</td>
<td>4.97 ± 0.70 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3x</td>
<td>2.43 ± 0.13 (6)</td>
<td>24.22 ± 1.74 (6)</td>
<td>5722 ± 150 (5)</td>
<td>5.06 ± 0.34 (7)</td>
</tr>
<tr>
<td>Female</td>
<td>Control</td>
<td>0x</td>
<td>1.52 ± 0.07 (6)</td>
<td>20.87 ± 1.53 (6)</td>
<td>5190 ± 503 (4)</td>
<td>7.87 ± 1.17 (6)</td>
</tr>
<tr>
<td></td>
<td>LiCO</td>
<td>1x</td>
<td>1.62 ± 0.10 (6)</td>
<td>21.62 ± 1.11 (6)</td>
<td>7007 ± 1427 (5)</td>
<td>10.09 ± 1.10 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2x</td>
<td>1.71 ± 0.22 (6)</td>
<td>21.02 ± 1.15 (6)</td>
<td>6988 ± 1077 (5)</td>
<td>18.86 ± 3.79 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3x</td>
<td>1.68 ± 0.10 (7)</td>
<td>18.48 ± 0.79 (6)</td>
<td>7119 ± 656 (4)</td>
<td>13.09 ± 1.87 (7)</td>
</tr>
<tr>
<td></td>
<td>LiOr</td>
<td>1x</td>
<td>1.86 ± 0.12 (6)</td>
<td>18.53 ± 1.32 (6)</td>
<td>5134 ± 477 (4)</td>
<td>9.82 ± 1.21 (7)</td>
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<tr>
<td></td>
<td></td>
<td>2x</td>
<td>1.33 ± 0.08 (6)</td>
<td>18.07 ± 1.02 (6)</td>
<td>4384 ± 498 (5)</td>
<td>9.57 ± 2.55 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3x</td>
<td>1.63 ± 0.14 (7)</td>
<td>19.70 ± 0.83 (6)</td>
<td>5386 ± 758 (4)</td>
<td>8.65 ± 0.93 (6)</td>
</tr>
</tbody>
</table>

* Concentrations for LiOr and LiCO are based on 1x, 2x or 3x the MEC determined using the AIH model
* P < 0.05 via two-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; MEC – minimal effective concentration
The numbers enclosed within parentheses represent sample size

Finally, the effects of LiCO and LiOr on lithium-induced vasopressin resistance and/or pathological alterations to kidney structure were explored via assessment of epithelial aquaporin-2 water channel expression and renal morphology, respectively. No changes relative to control were noted for either compound (Box 4).
Box 4. Hematoxylin and eosin staining and IHC-mediated visualization of aquaporin-2 channels within kidney cortex. A) Kidneys were sectioned through the cortex at 300 µm, then stained with hematoxylin and eosin for visualization of morphology. Although quite uncommon, long-term LiCO therapy has been linked to substantial alterations to kidney morphology, including glomerulosclerosis, tubulointerstitial fibrosis, and tubular atrophy. No such morphological changes were observed in any of the treatment groups. B) Aquaporin-2 water channels were visualized in the collecting ducts through DAB-based IHC. Nephrogenic diabetes insipidus (NDI) is a common side effect of lithium therapy that is believed to arise through resistance to vasopressin. As one of the chief downstream consequences of lithium-induced vasopressin resistance is a reduction in luminal aquaporin-2 (AQP2) expression, the effects of LiCO and LiOr on luminal AQP2 density within the nephron were contrasted via DAB-IHC and light microscopy. No alterations in AQP2 density were elicited by either treatment, despite the significant differences in water intake and urination noted earlier (Fig. 4.3A). This lack of effect may be an artifact of our method's limitations, i.e., it is possible that a more robust imaging technique, such as transmission electron microscopy, could reveal differences that our methods were unable to detect. All kidneys were harvested after 14 days of once daily treatment with either water, LiCO (15 or 45 mg/kg) or LiOr (1.5 or 4.5 mg/kg). All LiCO/LiOr concentrations represent mg of elemental lithium per kg of animal body weight. All kidneys were harvested after 14 days of once daily treatment with either water, LiCO (15, 30 or 45 mg/kg) or LiOr (1.5, 3.0 or 4.5 mg/kg). LiCO - lithium carbonate; LiOr - lithium orotate. DAB - 3,3'-Diaminobenzidine; IHC - immunohistochemistry.
4.4.2 Neither short-term nor long-term spatial recognition memory were affected

While the effects of LiCO on memory are uncertain, some patients report experiencing memory-related issues (374). Thus, the modified visual cue Y-maze and Barnes maze were employed to contrast the effects of LiCO and LiOr on short-term and long-term spatial recognition in male mice, respectively. Mice were treated once daily for 14 consecutive days with either water, LiCO (30 mg/kg), or LiOr (3.0 mg/kg). All behavioural work commenced precisely 1.5 hours after lithium administration. The intermediate lithium doses were chosen for these experiments based on their frequent use in human patients (370).

For the Barnes maze, mice were assessed for their usage of various search strategies on each day of the 7-day long acquisition phase, followed by observation of escape latency and escape zone residency time during a probe trial conducted 48 hours after the final acquisition session. Search scores reflect the average quality of the strategies employed by each group over time, with random, serial, corrected and direct granting numerical values of 0, 1, 2, or 3, respectively. Treatment with LiOr significantly improved search strategy utilization by day 7 of the acquisition trials (Fig. 4.4C), as revealed by repeated measures ANOVA. This enhancement is characterized by a progressive adoption of direct escape strategies (visual) relative to serial or random searching patterns (non-visual) (Fig. 4.4B). Similar effects were observed in the 48-hour probe trial, where treatment with either LiCO or LiOr significantly improved both escape latency (Fig. 4.5C) and time spent within

![Figure 4.4. Effects of LiOr and LiCO on spatial memory acquisition. A) Male mice were treated once daily with water, LiOr (3.0 mg/kg), or LiCO (30 mg/kg) for 14 days. The acquisition phase ran from the 3rd day of treatment to the 10th. The probe commenced 48 hours later. B) Search strategies used during escape from the Barnes maze were assessed over 7 consecutive days. C) Search scores reflect the average quality of the strategies employed by each group over time, with random, serial, corrected and direct granting numerical values of 0, 1, 2, or 3, respectively. Acquisition trials commenced 1.5 hours after drug administration on each testing day. Mean ± SEM. Groups were compared to control via two-way repeated-measures ANOVA with Bonferroni's post-hoc testing. *P < 0.05, **P < 0.01, n = 4-6 per group. LiOr - lithium orotate; LiCO - lithium carbonate; OG - oral gavage.](image)
the escape area (Fig. 4.5D).

As for the Y-maze, the mice were allowed to explore the arena with the novel arm closed for 5 minutes, followed by a second 5-minute trial 30 minutes later with the novel arm left open. The effects of each treatment on short-term spatial memory were assessed by monitoring exploration of the novel arm as well as interactions with the visual cue located at its end. Both LiOr and LiCO significantly reduced latency to the first nose-point interaction with the novel arm visual cue; however, the frequency of these interactions was unaffected (Fig. 4.6C). No statistically significant impacts on the frequency of entry into or duration within the novel arm were induced by either compound (Fig. 4.6B). Each group displayed a similar degree of total locomotor activity, which suggests that these results are not confounded by differences in exploratory behaviour.
4.5 Discussion

4.5.1 Summary of findings

- No adverse effects were noted for LiOr within its effective range
- LiCO induced polydipsia in both sexes
- LiCO elevated creatinine levels in males, and TSH expression in females
- Neither compound adversely impacted short-term or long-term spatial memory

4.5.2 The improved tolerability of Lithium orotate may improve treatment outcomes

The narrow therapeutic window of LiCO results in emergence of adverse effects at therapeutically relevant concentrations (272, 273). Given the positive correlation between lithium-induced toxicities and serum Li$^+$ content (13), a lithium-based compound with similar efficacy but reduced dosing requirements should mitigate side effect incidence.

While we found LiOr to display lesser dosing requirements than LiCO in chapter 3, Smith et al. previously suggested that the heightened central expression of LiOr, and thereby its efficacy, could be attributable to reduced glomerular filtration rates (14, 23), which would consequentially worsen renal health outcomes through elevation of serum Li$^+$ exposure (13). Intriguingly, LiOr proved to be capable of blunting AIH at doses which yield serum Li$^+$ levels below 0.1 mM, indicating that the improved efficacy of LiOr is likely not due to any off-target effects on Li$^+$ excretion (Fig. 3.4C).
Following up on these observations, we sought to determine whether the ability of LiOr to affect AIH at doses which result in serum Li⁺ expression well below the traditional therapeutic range could be leveraged to improve tolerability. In brief, the improved potency of LiOr translated to an absence of adverse effects in a 14-day toxicological probe when employed at concentrations up to three-fold greater than its minimal effective concentration. In contrast, LiCO precipitated deleterious renal and thyroidal health outcomes, which is not surprising given our observation of detectable levels of Li⁺ within the serum following administration of LiCO at doses that are inefficacious in the AIH model. Additionally, as polydipsia and polyuria are some of the earliest side effects encountered during lithium therapy, the absence of any sort of water-balance related side effects elicited by LiOr suggests that it is highly tolerable when employed within its therapeutic range. This lack of alteration to urinary concentrative capacity (Fig. 4.3) – which was not the case for LiCO – should dispel the concerns raised by Smith et al. in 1979 regarding the potential deleterious influence of LiOr on kidney function (23).

Regarding thyroidal health, the improved tolerability of appropriately-dosed LiOr could be of particular benefit to women with BD; female patients are known to be at greater risk for the development of lithium-induced hypothyroidism than their like-aged male counterparts (375).

Lastly, both compounds improved performance in both the Barnes maze and Y-maze, indicating that disruption of short-term or long-term spatial memory acquisition and recall is not a concern, at least over the short-to-intermediate term. While the reasons for this enhancement are indeterminate, suppression of LTD and disinhibition of LTP consequent to GSK3 antagonism is a potential mechanism (376). Whether this effect of LiOr/LiCO would be maintained during multi-year prescription requires elucidation.

Overall, our results are consistent with a recent 28-day toxicological probe that found no evidence of adverse effects in rats treated with LiOr at doses up to 15 mg Li⁺/kg/day (377). Given the paucity of reported adverse reactions to LiOr despite decades of largely unsupervised and unregulated use in North America (378), the translational potential of the enhanced safety profile of LiOr demonstrated herein appears promising.

4.5.3 The emergence of toxicity after 14 days in lithium carbonate-treated mice confirms its narrow therapeutic window relative to lithium orotate
While 14 days is an early time point for the assessment of toxicity in man, the time course over which pathologies develop in mice relative to humans is far from 1 to 1 (379). In terms of lifespan, 14 days in sexually mature mice is analogous to 5 years in humans (380); however, the full influence of the diminutive murine lifespan on clinically relevant pathologies is indeterminate (379), and unless the differential rate at which all biological changes occur is linearly correlated with senescence, a 14-day toxicological evaluation in mice is likely not truly equivalent to 5 years of prescription in humans. Nonetheless, 14 days represents a far more substantial duration in murine models than in human patients (380, 381). Consequently, the deleterious effects of LiCO on renal function, as evidenced by polydipsia, polyuria, and elevated serum creatinine, and the thyroid, as demonstrated by increased serum TSH, are particularly concerning, and mirror the observations of emergent LiCO-induced toxicities at therapeutic doses in human lithium therapy.

In contrast to LiCO, the absence of any adverse effects on TSH, BUN or creatinine elicited by LiOr indicates a strong safety profile; and the lack of polydipsia suggests that even minor and readily inducible side effects could be avoided which, if true, would dramatically improve tolerability during therapy. While the dose of LiOr employed was lower than that of LiCO, it is important to note that each test point captured either 1x, 2x, or 3x of the compound’s minimal effective concentration (determined in the AIH model). Thus, the emergence of toxicities in the 2x and 3x groups for LiCO, but not LiOr, strongly suggests that the therapeutic window of LiOr is expanded relative to LiCO.

4.5.3 Conclusions
The superior potency of LiOr in the AIH model translates to improved tolerability in both male and female mice, as evidenced by its lack of effect on measures of renal and thyroidal health. Thus, it appears that the increased central expression of Li⁺ elicited by LiOr (14) enables reductions in dosing (Fig. 3.3) that translate to improved safety (Fig. 4.3; Table 1) concurrent with maintenance of efficacy, at least in rodents.
**Transition statement**

With the improved efficacy, potency, and tolerability of LiOr confirmed, the next question to be addressed is obvious: why does LiOr differ from LiCO? Moving forward, our aim was to characterize the transport- and dissociated-related properties of LiOr that set it apart from LiCO (Fig. 4.7). First, the supposition that LiOr does not readily dissociate in solution needs to be confirmed, as a fully dissociated compound would not be expected to differ from LiCO in any meaningful way. Following this, LiOr’s potential utilization of various channels and transporters can be assessed using pharmacological disruption of lithium sensitive experimental outputs. As Li⁺ must eventually separate from its carrier, the process responsible for the eventual dissociation of LiOr must also be elucidated.

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**Fig 4.7. Diagrammatic representation of the how our findings in chapters 3 and 4 influenced the design of chapter 5.** LiOr is more potent and efficacious in the AIH model; this improved potency translated to enhanced tolerability. Given that no differences would have been noted if the two compounds had identical pharmacokinetic and pharmacodynamic properties, our final set of experiments sought to elucidate the differential dissociative and transport-related properties of LiOr and LiCO. LiOr - lithium orotate; LiCO - lithium carbonate; AIH - amphetamine-induced hyperlocomotion; Poly - polydipsia; Cre - creatinine; TSH - thyroid stimulating hormone; MEC - minimal effective concentration.
CHAPTER 5: EXPLORING THE BIODISTRIBUTION OF LITHIUM OROTATE

5.1 Rationale

The BBB gates the ingress of sodium into the CNS, which in turn is believed to limit the entry of Li⁺ due its reliance on sodium transporters (17, 18, 297, 298). As LiCO dissociates fully upon ingestion into Li⁺ and CO₃²⁻, its movement throughout the body likely is influenced by the regulation of sodium dynamics. Orotic acid has been theorized to more readily transport inorganic ions – such as lithium, magnesium, or calcium – across biological membranes as part of a non-dissociated mineral-orotate complex (11, 12). If true, then perhaps the improved potency and efficacy of LiOr relative to LiCO is attributable to differential dissociative and transport-related properties that allow for bypass of the limitations placed upon sodium-dependent transport mechanisms.

First, unlike LiCO, LiOr may not readily dissociate at physiological pH, thereby necessitating utilization of transport pathways which differ from those employed by LiCO. While several intriguing options exist, we propose that the movement of LiOr throughout the body is facilitated by OATPs, a subclass of transporters with affinity for large, hydrophobic anionic and/or neutral molecules (302) that are abundantly expressed within neurons, glia, and the endothelium of the BBB.

Second, given that Li⁺ must eventually separate from its carrier to affect cellular functions, it is probable that some interaction or enzymatic process triggers the dissociation of LiOr. One possible explanation is that UMPS of the de novo pyrimidine biosynthesis pathway (for which orotic acid is a substrate) liberates Li⁺ from its carrier via decarboxylation of the side group to which it is bound. This idea stems from Hans Nieper’s claims that LiOr preferentially targets cells with high rates of activity within the pentose phosphate pathway (12), which provides ribose sugar substrates to pyrimidine biosynthesis (313), and the structure of LiOr itself; Li⁺ associates with the carboxyl group that is eventually cleaved from orotic acid during the formation of uridine monophosphate. Thus, a UMPS-mediated dissociation of LiOr could explain how the compound comes to dissociate and why it theoretically accumulates to a greater degree within select tissues (12, 14).

In this chapter, the dissociation- and transport-related properties of LiOr were explored using pharmacological inhibitors of OATPs/UMPS in lithium-sensitive in vitro (GSK3β activity assay,
resistivity to current), *in vivo* (AIH), and *ex vivo* (hippocampal LTP) experimental paradigms.

5.2 Objectives and hypotheses

Relative to LiCO, LiOr has been proposed to yield greater levels of Li\(^+\) within the brain (14) and to display fewer side effects during therapy (12). Given the improved potency in the AIH model (Fig. 3.3) and enhanced tolerability in the toxicological evaluations (Fig. 4.3; Table 1) reported herein, *I hypothesize that…*

*Utilization of OATPs (transport) and incorporation into pyrimidine biosynthesis (intracellular dissociation) underpin the improved efficacy of LiOr.*

The objectives for chapter 5 were to a) demonstrate that LiOr does not dissociate under physiologically relevant conditions; b) determine the role of OATPs in the passage of LiOr across membranes; and c) identify the role of UMPS in the eventual dissociation of LiOr.

5.3 Experimental groups and methodological modifications

5.3.1 Experimental groups

Male C57Bl/6 mice were used for all studies. AIH trials utilized mice aged 3-4 weeks, whereas live slice experiments were performed on mice aged 14-40 days post-natal. For the AIH study, mice were randomly assigned into groups to receive LiOr (2.5 mg Li\(^+\)/kg), LiCO (20 mg Li\(^+\)/kg) or 0.9% saline either alone or in combination with one of PEG-400 (50%), naringin (100 mg/kg), or 6-azauracil (30 mg/kg); all groups were injected with \(dA\) (6 mg/kg).

5.3.2 Adjusted AIH protocol

PEG-400 (50%), naringin (100 mg/kg), or 6-azauracil (30 mg/kg) were administered 30 minutes prior to IP injection of LiOr or LiCO. 30 minutes later, mice were injected IP with \(dA\) (6 mg/kg) or saline, placed into an open field arena (35 x 35 x 35 cm) for 120 minutes, and scored for total locomotion offline using Ethovision XT 11 software (Noldus, Wageningen, The Netherlands). Drug efficacy was measured as the ability of the tested lithium compound to diminish the hyperlocomotion induced by the injection of amphetamine. The contributions of OATPs and UMPS to the efficacy of LiOr and LiCO were measured by the degree to which the blockade of hyperlocomotion elicited by each lithium compound was attenuated in the presence of OATP/UMPS inhibitors.
5.3.3 Statistics

LiOr and LiCO were compared using an unpaired t-test in the resistivity, GSK3β activity, and PAMPA experiments. To assess the effects of OATP/UMPS inhibitors on the efficacy of LiOr/LiCO in the AIH and LTP models, a two-way ANOVA statistical analysis was employed with Bonferroni’s post-hoc testing.

5.4 Results

5.4.1 Lithium orotate does not readily dissociate within solution

One of the ways in which LiOr is proposed to differ from LiCO is in its lack of dissociation within solution (12, 382). As low resistivity is indicative of a solution that readily allows current flow (383), it can be used to assess the degree of a compound’s dissociation/ionization; ions carry charge more easily than neutral molecules. When we contrasted the dissociation of LiOr and LiCO in distilled water, we found the electrical resistivity of a 20 mM LiCO solution to be markedly lower than that of a 20 mM LiOr solution, indicating that LiCO was ionized to a greater degree (Fig. 5.1A). The 20 mM concentration was selected to enable the detection of altered current flow, which is difficult to achieve when using lesser concentrations. Next, these results were confirmed using a more physiologically relevant GSK3β activity assay, where 2 mM LiCO, but not 2 mM LiOr, blocked 50% of enzymatic activity (Fig. 5.1B). GSK3β inhibition is an established outcome of lithium therapy (41, 42, 384, 385), and is strongly believed to have clinical relevance in BD (39, 41, 42, 366). This inhibition is made possible by the similar ionic radii of Li+ and magnesium allowing for Li+ to displace magnesium (a required cofactor) from the catalytic core of the enzyme, which means that Li+ must first be liberated from its carrier before it can interact with GSK3β. As the IC50 for lithium-induced inhibition of GSK3β in vitro is ~2 mM (386), a 50% attenuation of
enzymatic activity is expected for a fully dissociated lithium compound (whereas a non-dissociated molecule should have no effect).

### 5.4.2 Neither compound is lipophilic

Lipophilic compounds can cross biological membranes via passive diffusion (387). To determine whether the improved efficacy of the non-dissociated LiOr compound was due to increased lipophilicity relative to LiCO, the lithium content of solutions containing either compound was assessed before and after filtration through a mesh coated with a lipid bilayer. We found that neither compound demonstrated any degree of lipophilicity, as evidenced by the lack of lithium detected in the post-membrane chamber (Fig. 5.2).

5.4.3 Lithium orotate and lithium carbonate utilize differing transport pathways

Owing to their broad affinity for large, hydrophobic molecules and abundant localization within neurons, glia, and the BBB endothelium, OATPs present an intriguing target for the transport of LiOr. To explore the potential role of OATPs in the uptake and subsequent efficacy of LiOr, we probed the ability of PEG-400 and naringin – non-selective and selective inhibitors of OATPs and OATP1A2/Oatp1a1/Oatp1a4 (26, 27), respectively – to affect the efficacy of LiOr and LiCO in the attenuation of AIH. The application of either 50% PEG-400 (OG) or 100 mg/kg naringin (IP injection) 30 minutes prior to IP injection of lithium completely prevented the blockade of AIH that is ordinarily induced by 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. 5.3). Given the results of our post-hoc analyses, the interactions between PEG-400/naringin and lithium (two-way ANOVA interaction Li⁺ x PEG, p = 0.0002; two-way ANOVA interaction Li⁺ x naringin, p = 0.0474) strongly suggest that the inhibitors selectively interfere with the ability of LiOr to blunt hyperlocomotion. The 2.5 mg/kg LiOr and 20 mg/kg LiCO treatments were chosen because they
represent the doses at which the maximum attenuation of amphetamine-induced hyperlocomotion was observed for each respective compound in male mice.

To generalize our findings to an additional experimental context as well as assess the contributions of the BBB, we repeated the above experiments using an ex vivo live brain slice platform. As we have previously determined that 0.6 mM LiOr and 0.6 mM lithium chloride (LiCl) strengthen LTP within the CA1 hippocampal subfield post TBS, we assessed whether 15-minute pre-treatment of slices with 1% PEG-400 or 50 µM naringin in aCSF would blunt the LTP enhancing effects of either compound. Consistent with our findings in the AIH model, interactions were observed between the lithium treatments and the inhibitor drugs (two-way ANOVA interaction Li⁺ x PEG, p = 0.0607; two-way ANOVA interaction Li⁺ x naringin, p = 0.0271), with post-hoc analyses revealing that pre-application of either PEG-400 or naringin attenuates the LTP promoting actions of LiOr while sparing the effects of LiCl (Fig. 5.4). These results indicate that the impacts of these drugs on LiOr’s efficacy extend to multiple experimental paradigms, and that their effects are not limited to the BBB and/or peripheral vasculature. LiCl was used in place of LiCO because of its nearly identical pharmacokinetic profile (388) and lack of effect on the pH of aCSF solutions.

5.4.4 Functional UMPS is required for the efficacy of lithium orotate

To affect cellular functions, Li⁺ must first separate from its carrier. Given that orotic acid is a substrate for pyrimidine biosynthesis, we theorized that the dissociation of LiOr may be mediated by UMPS, an enzyme which facilitates the production of uridine monophosphate from orotic acid. In short, UMPS catalyzes the decarboxylation of orotic acid after the addition of phosphoribosyl pyrophosphate (389), and may thus liberate Li⁺ from its carrier by cleaving the carboxyl group to
which it is bound. To assess this possibility, the UMPS inhibitor 6-azauracil (29) 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. In the AIH model, pre-application of 30 mg/kg 6-azauracil (IP) 30 minutes prior to lithium (IP) abolished the suppression of hyperlocomotion ordinarily induced by 2.5 mg/kg LiOr while sparing the effects of 20 mg/kg LiCO (Fig 5.3). When added to the perfusate during live slice recordings (30 minutes prior to wash-in of lithium), 1.25 µM 6-azauracil diminished the effects of 0.6 mM LiOr, but not 0.6 mM LiCl, on hippocampal LTP (Fig. 5.4).

![Diagram](image_url)

Although the interactions between 6-azauracil and lithium treatment failed to reach significance (two-way ANOVA interaction in the AIH model, p = 0.0705; two-way ANOVA interaction in the LTP model, p = 0.1235), our post-hoc analyses revealed that the LiOr-treated groups were significantly affected by 6-azauracil. Given that 6-azauracil had no individual impacts on either control or LiCO/LiCl-treated mice (as discussed above), it appears as though the contributions of the antagonistic influence of 6-azauracil against LiOr were obscured in the tests of overall interaction. Regardless, the effects of LiOr were abolished while LiCO/LiCl were entirely unaffected, which heavily suggests that 6-azauracil selectively blunts the efficacy of LiOr.

### 5.5 Discussion

#### 5.5.1 Summary of findings
- LiOr does not dissociate within solution
• Neither LiOr nor LiCO are lipophilic, which rules out direct diffusion across membranes as the mechanism behind LiOr’s improved transport
• LiOr appears to utilize OATPs for transport across biological barriers
• UMPS may trigger the dissociation of LiOr

5.5.2 The role of OATP1A2 in the transport of lithium orotate

In the early 1970s, Hans Nieper claimed that LiOr passed through membranes with greater efficiency than other lithium compounds (11, 12). To our knowledge, any direct evidence to back these assertions is either nonexistent or lost to time, thus necessitating characterization of the transport- and dissociation-related properties of LiOr.

We first addressed the dissociation, or lack thereof, of LiOr, for if it readily dissociates into Li$^+$ and orotic acid, then its ability to affect AIH should not have been appreciably different from that of LiCO. This becomes especially clear when considering the inability of sodium orotate to alter hyperlocomotion (Fig. 3.3B), which rules out the possibility that orotic acid in some way alters the absorption, metabolism, or excretion of either lithium or amphetamine. In our opening experiments where a current was applied to solutions containing equimolar concentrations of either LiOr or LiCO, we found that LiOr displayed lesser conductivity than LiCO (Fig. 5.1A), indicating a relative lack of ionization; in general, ions are more conducive to current flow than neutral molecules. These findings were confirmed under more physiologically relevant conditions involving lithium-induced inhibition of GSK3β activity. Because lithium and magnesium share similar ionic radii, the lithium ion can inactivate GSK3β by displacing magnesium from the enzyme’s catalytic core (386); resultantly, a lithium salt that does not dissociate into its constituent ions should not affect GSK3β activity during the assay. Thus, our findings that LiOr fails to attenuate enzymatic activity (Fig. 5.1B) heavily suggests that it does not readily dissociate. Given the size of LiOr relative to Li$^+$ alone, it is almost certain that it can not make use of the sodium-dependant transport mechanisms employed by LiCO (17, 18, 195), thereby necessitating the utilization of alternative transport pathways.

Regarding potential transporters, OATPs display a high degree of affinity for large, hydrophilic molecules and are abundantly expressed within glial cells, neurons, and the endothelium of the BBB (21), making them ideally situated to mediate the passage of LiOr across biological membranes. Intriguingly, we observed that the non-selective OATP inhibitor PEG-400 (390)
robustly attenuates the effects of LiOr on AIH and LTP (Figs. 5.3 and 5.4), which strongly supports the involvement of OATPs in the transport of LiOr. Following up on these initial observations, we narrowed down the exact OATP involved by using naringin, an inhibitor selective for OATP1A2 (Oatp1a1 and Oatp1a4 in mice) (26). Like PEG-400, naringin completely abolishes the effects of LiOr on both AIH and LTP (Figs. 5.3 and 5.4), indicating that OATP1A2 is likely the primary OATP subtype responsible for facilitating the movement of LiOr throughout the body. The generalizability of these findings across two different lithium-sensitive measures suggests that the effects of OATP inhibition are in fact due to blockade of LiOr transport as opposed to any unanticipated effects on drug absorption, excretion, or metabolism.

We propose that the consequences of LiOr’s reliance on OATPs for transport are likely to be quite diverse and impactful. For example, the distribution of Li⁺ within the brain following ingestion of LiCO is highly heterogeneous and displays a great degree of interindividual variability between patients (391). Although unconfirmed, this heterogeneity is believed to be due to the differential localization patterns of sodium channel/transporter subtypes among the different cell populations of the brain parenchyma and BBB (19, 392, 393), with some proposing that variations in BBB transporter composition are responsible for the inconsistent responses to treatment observed during lithium therapy (18). Consequently, the reliance of LiOr on OATPs for transport, rather than on sodium channels a la LiCO, will likely have major impacts on a) where in the brain LiOr accumulates as well as b) the response rate of different patient populations to treatment (will it be better or worse?). While the exact influence of these differences over treatment outcomes is indeterminate at present, our observations concerning the improved potency, efficacy, and tolerability of LiOr are quite promising.

5.5.3 Lithium orotate may target cells with high rates of pyrimidine biosynthesis

Given that the therapeutic mechanisms of lithium are known to be centered on its ionic form, Li⁺ must eventually separate from its carrier to affect cellular functions. If LiOr does not readily dissociate, as we have shown (Fig. 5.1), then how and where does its eventual dissociation occur? After all, the influence of lithium in the AIH model is due to interactions between ionic lithium and GSK3β (347, 354, 355), which means that LiOr would be ineffective against hyperlocomotion if it did not eventually separate into Li⁺ and orotic acid.

As previously discussed, Hans Nieper proposed that LiOr targets tissues with high rates of pentose
phosphate pathway activity (12). While intriguing, no evidence was provided as to why this might be the case. Taking into consideration that LiOr is a pyrimidine-precursor analog, we theorized that it may be taken up into the de novo pyrimidine biosynthesis pathway, which is in close association with the pentose phosphate pathway (20), rather than the pentose phosphate pathway itself. Of the numerous enzymes responsible for catalyzing the different stages of pyrimidine biosynthesis, one seems to be of particular import: UMPS. As UMPS facilitates the formation of uridine monophosphate from orotic acid (310, 389), a process which includes the decarboxylation of the functional group to which Li⁺ is bound, the abolishment of LiOr’s effects following inhibition of the enzyme strongly suggests that its activity is required to trigger the dissociation of LiOr (Fig. 5.5).

An intracellularly situated dissociation mediated by UMPS could have dramatic consequences on the function and localization of LiOr relative to LiCO. Ordinarily, non-proliferative cell populations generate pyrimidines chiefly through salvage pathways that mediate the uptake and subsequent conversion of uridine and cytidine to uridine monophosphate and cytidine monophosphate, respectively, while de novo biosynthesis plays a lesser role (310); however, neurons display relatively high levels of activity within the de novo pathway despite being post-mitotic (394), which raises the possibility that LiOr could target neurons more readily than other post-mitotic cell types. This uptick in de novo synthesis may be a compensatory response to satisfy the need for pyrimidine nucleotides created by heightened neuronal energy demands and the constant membrane remodelling which occurs during axonal and dendritic arborization (394, 395). In support, comparative evaluations into the relative density between brain regions of the enzymes
involved in *de novo* pyrimidine biosynthesis reveal that highly active populations – such as those within the locus coeruleus – demonstrate increased protein expression (394).

Considering that the reliance of LiCO on sodium transport (19, 392, 393) results in a quite heterogeneous distribution of Li⁺ throughout the brain parenchyma (391), it is possible that LiOr’s utilization of OATPs for transport and UMPS for dissociation will result in the two compounds targeting differential cell types, neuronal populations, and brain regions. While it is difficult to predict what the consequences of these differences might be, the potential contributions of OATPs and UMPS to the improved potency and tolerability of LiOr are discussed in chapter 6.

**5.5.4 Conclusions**

In contrast to LiCO, which dissociates readily in solution, LiOr remains in its non-dissociated state post-administration. These differential dissociative characteristics give rise to differences in the transport pathways employed by each compound, with LiCO relying upon sodium transporters while LiOr utilizes OATPs. Finally, the incorporation of LiOr into the *de novo* pyrimidine biosynthesis pathway appears to underly its eventual dissociation. As the Li⁺ derived from LiCO/LiCl is subject to the regulation of sodium dynamics that occurs within the periphery, BBB, and brain parenchyma, the reliance of LiOr on OATPs for transport and UMPS for dissociation may alter its pharmacokinetic and pharmacodynamic properties. In chapter 6, we explore how these alterations might underpin the improved potency and tolerability of LiOr relative to LiCO.
CHAPTER 6: GENERAL DISCUSSION

6.1 Major thesis findings
Despite early evidence of increased central expression, the use of LiOr in psychiatric applications has gone largely unexplored over the past few decades (12, 14, 15). Using the AIH model of mania – which was selected for its high-throughput, mania-mimetic characteristics, 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. 3.3). It is possible that these differences are attributable to the utilization of OATPs for transport coupled with an intracellularly situated dissociation mediated by UMPS (Fig. 5.5), as evidenced by the loss of effect for LiOr, but not LiCO, on both AIH and LTP in the presence of OATP or UMPS inhibitors (Figs. 5.3 and 5.4). The improved potency of LiOr translated to an absence of adverse effects on markers of renal and thyroidal health (Figs. 4.3; Table 1), supporting the notion that the lesser dosage requirements of LiOr improve its safety profile relative to LiCO.

6.2 Are the differential effects of lithium carbonate and lithium orotate due to differences in biodistribution?
Because LiCO readily dissociates into Li\(^+\) and CO\(_3^{2-}\), it chiefly relies on sodium channels for transport throughout the body (17-19, 195, 198). Resultantly, LiCO is subject to the regulation of CNS sodium dynamics exerted by the BBB (17-19, 298, 396), as evidenced by the suppression of Li\(^+\) influx into brain endothelial cells following inhibition of sodium transport (17). Like other inorganic cations, sodium is slow to equilibrate between the blood and brain (397), which suggests that Li\(^+\) influx is similarly limited (17, 18, 398, 399). Thus, it is possible that the tight control over central sodium content maintained by the BBB (19) gates the entry of LiCO into the brain (17, 18, 297, 298); in support, some have proposed that the inconsistent responses to lithium observed among BD patients are partially due to differences in BBB composition (18, 400). To overcome this restriction, high concentrations of LiCO are required to overcome the limitations on Li\(^+\) ingress imposed by the BBB. Unfortunately, these doses lead to rapid induction of side effects upon acute administration, followed by precipitation of more deleterious outcomes over the long-term (Fig. 6.1) (272, 273). If the reliance of LiCO on sodium-dependent transport mechanisms is responsible for the compound’s narrow therapeutic window, then perhaps a lithium formulation that utilizes sodium-independent pathways would be able to circumvent this issue. Owing to the unique
transport-related characteristics identified in chapter 5, LiOr appears to represent such a compound.

In contrast to LiCO, LiOr relies on OATPs rather than sodium transporters for entry into the CNS, as evidenced by the abolishment of its effects on AIH and LTP in the presence of OATP inhibitors (Figs. 5.3 and 5.4). Given that the entry of LiCO-derived Li\(^+\) into the brain parenchyma is likely limited by its reliance on sodium transporters (17-19, 297, 298), it is probable that the sodium-independent pathways utilized by LiOr account for its reduced dosing requirements in the AIH model (Fig. 3.3). Conceivably, OATP-dependant transport could increase the ease with which LiOr crosses biological barriers and (Fig. 6.2A), in conjunction with its unique dissociative characteristics (Fig. 5.1), enable the direct delivery of Li\(^+\) to intracellular target sites (Fig. 6.2B). This theory is in partial agreement with Hans Nieper’s original proposal that LiOr crosses biological membranes unchanged before dissociating within the intracellular compartment of cells that display high rates of pentose phosphate pathway activity (12); however, rather than the pentose phosphate pathway, LiOr may instead show a bias toward cells which exhibit a high degree of \textit{de novo} pyrimidine biosynthesis, as discussed in chapter 5 (recall that inhibition of UMPS robustly attenuates the actions of LiOr, but not LiCO, on both AIH and LTP). Thus, as the therapeutic efficacy of lithium is heavily linked to its impacts on intracellular enzymes (e.g., GSK3\(\beta\)), it is reasonable to suppose that the increased entry of LiOr into the CNS afforded by utilization of OATPs (perhaps courtesy of circumventing the BBB-mediated regulation over sodium flux) coupled with a UMPS-mediated intracellular dissociation results in enhanced blockade of enzymatic activity, which in turn leads to improved attenuation of hyperlocomotion in the murine
AIH model and, presumably, superior control over BD symptomatology in man.

Alternatively, the improved efficacy of LiOr could have more to do with where it is compartmentalized in the brain rather than the degree to which it accumulates inside of the CNS. This supposition is buttressed by our observation that brain Li\(^+\) levels do not necessarily correlate with the strength of blockade elicited in the AIH model (Fig. 3.4B). Similar trends are reported in clinical settings, where brain Li\(^+\) levels remain relatively uniform until serum concentrations exceed 0.85 mEq/L (401). Thus, LiOr and LiCO may target different cell populations and display differing temporal influx/efflux patterns due to their separate utilization of OATPs and sodium.
transporters, respectively. It is also possible that an intracellular dissociation triggered by UMPS, which is located near mitochondria (402), could lead LiOr to exert most of its influence on signaling pathways situated near its site of liberation deep within the cytosol, whereas the Li\(^{+}\) derived from LiCO might act primarily on membrane-associated pathways (e.g., the PI cycle) due to their proximity to the sodium transporters through which ionic Li\(^{+}\) enters the cell. While intriguing, it must be noted that the lack of robust correlation between the degree of AIH attenuation and brain Li\(^{+}\) concentration in our studies was confounded by the inability of our chosen detection method to isolate Li\(^{+}\) situated in the intracellular compartment from that found within the interstitial space, cerebrospinal fluid, and brain microvasculature.

In summary, we propose that the improved potency of LiOr is attributable to improved CNS entry and/or enhanced cellular uptake courtesy of OATPs, followed by an intracellular dissociation triggered by the UMPS-mediated decarboxylation of orotic acid (Fig. 6.2).

### 6.3 The potential relationship between the unique properties of lithium orotate and its improved tolerability

As previously discussed, current lithium therapy is plagued by high rates of treatment non-adherence, largely due to the prevalence of adverse effects elicited by LiCO at therapeutically relevant concentrations (272, 273). LiOr has long been proposed to enter cells more readily than LiCO (12), theoretically resulting in lesser dosing requirements and an improved safety profile; however, satisfactory explanations as to why this might be the case are largely absent. We propose that utilization of OATPs allows LiOr to cross membranes more readily than LiCO, thereby reducing the requisite dose for symptom control and subsequently limiting the amount of Li\(^{+}\) that off-target organs are exposed to; and that the intracellular dissociation of LiOr – mediated by UMPS – further contributes to the tolerability of LiOr by preventing spikes in serum Li\(^{+}\) concentration.

In line with our predictions, the safety profile of LiOr was markedly improved relative to LiCO, as discussed in chapter 4. While speculative, the improved tolerability of LiOr may be attributable to its reliance on OATPs for transport. As the movement of LiCO is limited by the regulation of sodium dynamics (17, 18, 198, 297, 298), the utilization of a sodium-independent transport pathway by LiOr could increase its potency courtesy of improved CNS ingress and subsequent cellular entry (Fig. 6.2A). Given that plasma Li\(^{+}\) levels positively correlate with toxicity (13), the
reduced dosing requirements of LiOr – perhaps due to the improved ease of transport afforded by OATPs – likely diminishes the incidence of adverse outcomes by limiting serum Li⁺ expression. It is also possible that the reliance of LiCO on sodium-dependant transport contributes to thyroidal/kidney toxicity courtesy of the differential permeabilities of central versus peripheral capillary walls; the permeability of BBB-associated endothelial cells to sodium is rather limited relative to that of the highly fenestrated capillaries located within the systemic compartment (19). Resultantly, the Li⁺ derived from traditional therapeutics, such as LiCO, likely accesses lithium-sensitive systemic organs (e.g., thyroid, parathyroid, kidneys) more readily than the brain parenchyma. In contrast, LiOr could bypass this issue via use of sodium-independent transport pathways: by relying on OATPs for transport, LiOr is not subject to the potential issues caused by discrepancies in the rate of sodium flux across central versus peripheral blood-tissue interfaces. Thus, the utilization of OATPs might improve the safety profile of LiOr by increasing its ability to cross biological barriers, thereby reducing the dose required for therapeutic efficacy and subsequently limiting the concentration of Li⁺ within the blood; and by enabling a more equitable expression of Li⁺ within the brain relative to the thyroid/parathyroid/kidney through circumvention of the peripheral bias for accumulation that is potentially a consequence of sodium-dependent transport (Fig. 6.3) (19).

In concert with the differential transport mechanisms utilized by each compound, it is possible that the contrasting dissociative properties of LiCO and LiOr further contribute to their differing toxicity profiles. While LiCO dissociates immediately upon administration and precipitates a rapid influx of Li⁺ into the blood (403), the UMPS-mediated intracellular dissociation of LiOr could result in a prolonged trickle of Li⁺ from individual cells back into the bloodstream, thereby reducing the relative concentration of Li⁺ that organs are exposed to per unit of time (Fig. 6.3). As Li⁺-associated toxicities are worsened by acute spikes in systemic exposure (13), circumvention of the rapid influx of Li⁺ into the vasculature that follows the ingestion of LiCO (403) might explain, in part, why LiOr proved far more tolerable in our toxicological evaluations (Fig 4.3; Table 1).

Opposing our submission of improved safety, Smith et al. proposed that the elevated central expression of Li⁺ elicited by LiOr (14) is attributable to GFR impairments which may ultimately contribute to worsened renal health outcomes (14, 23). While concerning, our present results
showing improved tolerability are supported by a recent 28-day toxicological evaluation which found no adverse effects of LiOr at doses up to 400 mg/kg/day in rats (elemental Li\(^+\) ~ 15 mg/kg/day) (377). Also, arguably the most well-known case of LiOr-induced toxicity serves to highlight its safety: in a 2007 case report, Pauze and Brookes detailed a scenario where a female patient ingested 18 LiOr tablets (3.83 mg Li\(^+\)/tablet), which is roughly 6-to-9 times more than what is recommended based on our findings in chapter 3. Despite this excessive intake, the patient displayed nothing more than some minor tremor and nausea sans emesis. Her vital signs remained normal throughout the incident, and all symptoms resolved after 3 hours of observation without any sort of medical intervention (404). Finally, our LiOr-treated mice did not demonstrate any evidence of polydipsia, polyuria, or elevated serum creatinine, thereby indicating that LiOr does not suppress GFR when dosed appropriately.

In closing, it is possible that the improved safety profile of LiOr relative to LiCO is attributable to reductions in serum Li\(^+\) expression and diminution of blood Li\(^+\) spikes facilitated by the reliance
of LiOr on OATPs for transport and UMPS for dissociation within the intracellular compartment, respectively.

### 6.4 Final summary: Putting it all together

Although highly efficacious, the narrow therapeutic windows of LiCO and lithium citrate (10) contribute to excessive rates of treatment regimen non-adherence. Given the intractable and lifelong nature of BD, a compound which displays similar efficacy to LiCO coupled with an expanded therapeutic window would be of tremendous benefit to patients.

In the early 1970s, Hans Nieper argued that orotic acid conjugates pass through biological membranes more readily than carbonate-based therapeutics, target tissues which display high rates of pentose phosphate pathway activity, and dissociate within the intracellular compartment (11, 12). In support, Nieper cited a reduction in the requisite dosage for symptom management in manic patients treated with LiOr rather than LiCO (12). The improved potency of LiOr was seemingly confirmed in 1978 when Kling et al. observed that brain Li$^+$ levels are ~3-fold greater in rats when administered as an orotate as opposed to as a carbonate (14). Unfortunately, work by Smith and associates in 1979 highlighted that LiOr may have more deleterious effects on the kidney than LiCO when employed at equivalent (albeit high) concentrations (23). While concerning, we hypothesized that the putative transport-related properties of LiOr and its consequent ability to enter the CNS more readily than LiCO could be leveraged to reduce the dosage employed during therapy, which in turn would limit side effect incidence and improve treatment compliance. To study these proposals, we first explored if LiOr demonstrates superior potency relative to LiCO in the AIH model, and if so, whether it translates to improved tolerability. Next, we sought to determine the transport-related properties responsible for the differential characteristics of the two compounds.

To begin, we first contrasted the effectiveness of LiOr and LiCO in the mania-mimetic AIH model. In line with the early suppositions of Kling (14) and Nieper (11, 12), we found LiOr to be both more potent and efficacious in the attenuation of hyperlocomotion. Next, the relative toxicity profiles of the two compounds were compared over their effective ranges. In contrast to LiCO, no adverse effects were noted for LiOr on measures of thyroidal or kidney health, which was expected given its ability to maintain efficacy even when the serum Li$^+$ levels elicited by a given dose were quite low; lithium-induced toxicities correlate with serum Li$^+$ exposure (13). These findings are
consistent with both recent toxicological evaluations of LiOr (377) and the apparent absence of serious side effects despite over 50 years of unsupervised use in North America. To further our understanding of why these two compounds differ, their respective transport- and dissociation-related properties were explored. We found that unlike LiCO – which dissociates readily in solution and largely relies on sodium transporters for movement throughout the body (17, 18) – LiOr remains undissociated in physiological media, thereby necessitating utilization of alternative transport pathways. The use of the OATP1A2 inhibitors PEG-400 and naringin (26, 27) in the lithium sensitive AIH and hippocampal LTP experimental models revealed that OATP1A2 facilitates the transport of LiOr. As discussed previously, OATP-mediated transport may allow for LiOr to bypass the limited CNS ingress of Li⁺ (derived from LiCO) that is imposed by the BBB-mediated regulation over sodium flux (17-19). Finally, as Li⁺ must eventually separate from orotic acid to affect cellular functions, we employed the UMPS inhibitor 6-azauracil to assess the role of the de novo pyrimidine biosynthesis pathway in the intracellular dissociation of LiOr; as anticipated, 6-azauracil abolished the effects of LiOr on both AIH and LTP while sparing those of LiCO. Given the close association of the pentose phosphate pathway and pyrimidine biosynthesis pathway (20), it appears that LiOr may target cells which display high rates of pentose phosphate pathway activity, as was originally proposed by Hans Nieper in 1973 (12).

In closing, the unique dissociation- and transport-related properties of LiOr increase its potency in the AIH model. These reduced dosing requirements translate to improved tolerability when appropriate concentrations are employed, which should dispel the concerns regarding renal toxicity raised by Smith et al. in 1979 (23). While the translational potential of these studies is indeterminate (although promising), the improvements in efficacy, potency, and tolerability displayed by LiOr in the AIH model suggests that use of LiOr in BD may limit emergence of the dose-dependent, compliance-disrupting side-effects encountered during lithium therapy (Fig. 6.4). Given the potential face, mechanistic, and predictive validity of the AIH model (25, 58, 60, 74, 353, 367), the findings reported herein indicate that the use of LiOr in human trials is worthy of consideration.
**The problem with LiCO**

While highly effective, LiCO has a narrow therapeutic window, which leads to high rates of treatment non-compliance.

**Could LiOr represent a more tolerable alternative?**

1978

LiOr yields brain Li+ levels 3x greater than LiCO

Kling et al., 1978

1979

LiOr more harmful to kidney health at high doses

Smith et al., 1979

**Central questions**

1. Can the putative capacity of LiOr to elevate central Li+ expression be leveraged to reduce dosing requirements?
2. If so, will these reduced dosing requirements alloy the toxicity concerns raised by Smith et al. in 1979?

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**First, the efficacies of LiOr and LiCO were compared in the mania-mimetic AHI model**

LiOr is more potent than LiCO against AHI

The improved efficacy of LiOr is not due to any effects on exploratory activity or locomotor capacity

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**Next, the toxicity profiles of LiOr and LiCO were contrasted over 14 consecutive days**

<table>
<thead>
<tr>
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<th>Female</th>
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**XMEC**

- 1x
- 2x
- 3x

**Poly**

- 1x
- 2x
- 3x

**Cre.**

- 1x
- 2x
- 3x

**BUN**

- 1x
- 2x
- 3x

**TSH**

- 1x
- 2x
- 3x

**AST**

- 1x
- 2x
- 3x

The improved potency of LiOr allows for maintenance of efficacy even at low serum Li+ levels

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**Finally, the dissociation- and transport-related properties responsible for the differential effects of LiOr and LiCO were elucidated**

**A. LiOr does not readily dissociate**

As LiOr differs substantially from LiCO in the AHI model, it likely does not dissociate immediately upon administration. If it did, then its effects wouldn’t differ from LiCO

**B. LiOr uses OATPs for transport**

If LiOr does not dissociate, then it must use alternative transport pathways

**C. UMPS mediates the intracellular dissociation of LiOr**

LiOr must eventually separate into Li+ and orotic acid in order to affect cellular functions

---

Fig. 6.4. Summary of the rationale and major findings for each study objective. LiOr and LiCl were washed over the slices via addition to the aCSF perfusate for 15 minutes prior to induction of LTP via TBS (A) or LTD via LFS (B). Lithium compounds remained in the perfusate for the full duration of the experiment. The amplitudes of each of the final 10 sweeps of the 30-minute post-LTP/LTD recording were expressed as a percentage of the amplitudes of the final 10 sweeps of the baseline recording. LFS involved stimulation at 1 Hz for 10 minutes. TBS involved 8 bursts (at 5 Hz) of 4 pulses (at 100 Hz) delivered 3 times, 60 seconds apart and repeated a second time 300 seconds after the first. Stimulation was applied to the Schaffer collaterals (30% max amplitude), and fEPSPs were recorded within the CA1 at the synapses between the dendrites of the pyramidal cell layer and the axonal terminals of the Schaffer collaterals. Error bars represent the mean ± SEM. All groups were compared to the appropriate control condition via one-way ANOVA with Tukey’s post-hoc testing. *P < 0.05, **P < 0.01. Sample sizes are enclosed within parentheses. LiOr - lithium orotate; LiCl - lithium chloride; aCSF - artificial cerebrospinal fluid; TBS - theta burst stimulation; LFS - low-frequency stimulation; fEPSP - field excitatory post-synaptic potential; LTP - long-term potentiation; LTD - long-term depression. This work was performed by Hannah Wark as part of her Master of Science Degree project (data currently unpublished).
CHAPTER 7: LIMITATIONS AND FUTURE DIRECTIONS

7.1 Limitations

7.1.1 Stereotyped behaviours are difficult to objectively quantify

As discussed in 3.4.1, minutes 35-70 after the administration of DA were characterized chiefly by motor stereotypies, such as repetitive licking and gnawing. Similar to AIH, the emergence of motor stereotypies are a known consequence of hyperdopaminergia (346). However, unlike AIH, some groups have found DA-induced stereotypic behaviours to be refractory to lithium treatment (405), which suggests that these phenotypes are either the result of differential activation of disparate neuronal populations or divergence downstream of dopamine receptors within the same cell (25). While hyperactivity is considered a rodent behavioural correlate for mania (360), motor stereotypies are not, which calls into question the interpretability of results gathered during the 35-70 minute window.

7.1.2 Unknown degree of generalization

Generalizability pertains to the degree to which results from one study can be applied to a broader context (406). Consequently, for our AIH results concerning the differential dosing of LiCO and LiOr to be considered generalizable, they would need to be observed across several different experimental paradigms.

In support of the generalizability of our findings, we have observed stark differences in the dose-responsiveness of hippocampal LTP and LTD to LiOr and LiCO, with LiOr demonstrating increased potency (Box 3). However, as these plasticity-related findings have no relation to BD, it would be prudent to determine whether the observed differential dose response characteristics of LiOr and LiCO against AIH are maintained in other mania-mimetic models, such as those induced by repeated intracerebroventricular administration of ouabain (407).

7.1.3 Sub-optimal timing for analysis of serum and brain Li+ concentrations

Serum and brain Li+ analyses were performed on mice sacrificed after the completion of the AIH protocol. This presents a few issues: the Li+ levels of our analytes are not reflective of the values that would be observed during the heart of the AIH protocol (~1.5 hours after LiOr/LiCO injection); and fail to capture the effects of chronic dosing (i.e., what might Li+ concentrations be after multiple days of dosing?). We elected to harvest the blood and brain tissues post-AIH as a
measure of extracting as much information as possible from a single animal. Optimal Li⁺ analyses performed at the most appropriate time-points would necessitate the use of a large number of additional mice (well over 100), which was simply not feasible given budgetary constraints.

7.1.4 Potential issues affecting translation

7.1.4.1 How significant is the absence of a cyclical phenotype?
BD is incredibly difficult to model for myriad reasons. First, the symptomatology of BD is extremely heterogenic and mood-state dependent (408); second, diagnostic classifications are phenomenological, meaning that disparate illnesses with unique etiologies but similar symptomatic expression may fall within the same class (albeit unknowingly); third, no unifying theory of BD currently exists (409); and fourth, the cyclical nature of BD is difficult to capture (410), as models which solely exhibit manic-like or depressive-like characteristics are inherently incomplete. In truth, development of a comprehensive model will likely not be possible until a much more thorough understanding of the underlying pathology of BD is reached.

7.1.4.2 How well does AIH capture mania?
The AIH model seeks to recapitulate elements of dopamine dysregulation (43) by utilizing amphetamine (25) to disrupt DAT-mediated reuptake and increase vesicular dopamine release. The resultant increase in synaptic dopamine elicits a hyperdopaminergic state potentially associated with manic symptomatology (43, 58, 411). While several lines of evidence support the validity of the model (25, 55, 56, 68, 356, 409, 412), it fails to consider the totality of processes implicated in BD pathology, and does not display the characteristic cycling between mood states emblematic of the illness. While models which can transition from a manic to depressive state do exist (413), this switch is neither reciprocal nor repeatable, and thus is not indicative of true cycling.

Further, the primary behavioural output of the AIH model is hyperactivity. Unfortunately, this phenotype presents a number of limitations, including: in addition to mania, AIH has been interpreted as a model of schizophrenia and tardive dyskinesia; manic states involve a broad and diverse set of symptoms which do not always include hyperactivity; and hyperdopaminergic models elicit hyperactivity acutely, whereas BD is a chronic condition characterized by long-term behavioural alterations (360).

7.1.4.3 The response to amphetamine and lithium differs by murine strain
The induction of AIH and response to lithium are strain dependent (25). For example, C57Bl/6 strains display the expected hyperlocomotor output and subsequent cessation in response to dA and lithium plus dA, respectively, whereas lithium increases hyperlocomotion in the C3H/HeJ strain and has no impact whatsoever on dA responses in FVB/NJ mice (25). Thus, the translational potential of our findings relies on the phenotypes displayed in C57Bl/6 being more representative of the human condition than those observed in non-responsive strains.

7.1.4.4 Discrepancies between the acute and chronic effects of lithium
It is generally acknowledged that the primary therapeutic benefits of lithium in BD are only observed after many weeks of treatment, with the full breadth of its therapeutic effects often requiring months to finally emerge (370). This means that the impacts of lithium on AIH are best viewed as consequential to the initial actions of the drug as opposed to the more long-term neuroprotective and plastic effects believed to be partly responsible for its efficacy in maintenance therapy. Thus, the influence of lithium in the AIH model may be more representative of its therapeutic benefits in the cessation of an acute manic episode.

7.2 Future directions
7.2.1 Dose-response determination in the ouabain-induced model of mania
While the AIH model of mania is considered to have strong face and predictive validity (25, 345), it is not the only model that exists. To establish the generalizability of our findings, it would be prudent to recapitulate the differential potency and efficacy of LiOr in the ouabain-induced model of mania. Unlike amphetamine, which elicits hyperlocomotion through increased dopaminergic tone (25, 346), ouabain triggers hyperactivity through inhibition of Na⁺/K⁺-ATPase (335). Contrasting LiCO and LiOr in the ouabain model would be preferable to utilizing an additional hyperdopaminergic protocol (e.g., DAT knock-out) because it mimics a different process implicated in BD pathogenesis (336); thus, confirming the differential effects of the two compounds in this model would strengthen the translational potential of our findings.

7.2.2 Assessment of LiOr’s efficacy in a model of depression (e.g., chronic unpredictable stress)
Given that our evaluations focussed exclusively on the efficacy of LiCO and LiOr against mania-mimetic phenotypes, the next logical step would be to assess their antidepressant potential either
alone or in adjunctive therapy. While some have argued that select models display both manic and depressive phenotypes, the evidence is rather surface level, and it is highly debatable as to whether the transition from the manic to depressive state represents cycling (413). With this in mind, I propose that the depressive state would be best modelled by social defeat or chronic unpredictable mild stress (414-418). Although it is unlikely that these paradigms reflect true bipolar depression, some important phenotypes are present, including loss of BDNF (96, 123, 419, 420), altered neuronal activity in the hippocampus and prefrontal cortex (421-423), and GSK3β-associated neuroinflammation (99).

7.2.3 Lengthier toxicological evaluations (e.g., multiple months)

While our 14-day toxicological evaluation of LiCO and LiOr yielded intriguing differences between the two compounds, it would be interesting to see if a dramatic increase in treatment duration could precipitate morphological alterations indicative of kidney pathology. Structural damages, such as tubulointerstitial fibrosis, tubular atrophy, or glomerulosclerosis are usually only observed after 10-15 years of therapy (275), which suggests that our study duration was likely insufficient for the emergence of major renal pathologies (380, 381). Additionally, the possibility that the improved transport of LiOr may lead to progressive accumulation within off-target organs during chronic dosing warrants consideration, particularly within the context of colorectal cancer (lithium has pro-growth characteristics) where uptake of 5-fluoruracil (structurally similar to lithium orotate) is enhanced (424). Lastly, prolonged inhibition of GSK3α and GSK3β signaling may have deleterious impacts on cognition, raising the possibility that an increased length of exposure could reveal adverse effects on memory that were not observed during our 14-day protocol.

7.2.4 LiOr supplementation in neurodegeneration prophylaxis

Owing to their reported neuroprotective properties (425), lithium salts are increasingly employed in basic research and clinical studies for the treatment and/or prevention of neurodegeneration (e.g., Alzheimer’s disease) (426). Unfortunately, currently employed lithium compounds, such as LiCO, have a narrow therapeutic window resulting in common thyroid, parathyroid and renal complications (276). Given the improved efficacy, potency, and tolerability of LiOr presented in this dissertation, it may be ideal for use at supplemental dosages in neurodegenerative disease prophylaxis.
Alzheimer’s disease (AD) presents a particularly intriguing candidate for LiOr-based prophylactic treatment. While the etiopathogenesis of AD is currently unsettled, a bevy of evidence links excessive GSK3β signaling to disease progression. First, GSK3β hyperactivity is able to precipitate formation of the hallmark characteristics of AD, which are tau protein hyperphosphorylation (427) (contributes to development of neurofibrillary tangles) and β-amyloidogenic processing of amyloid precursor protein (428) (linked to Aβ plaque deposition). Second, GSK3β is a central component of numerous risk factors for AD, such as brain insulin resistance (429), neuroinflammation (430), and major depressive disorder (431).

7.2.5 Replication of resistivity experiments in media matching the internal environment of the stomach and duodenum

While the resistivity experiments and GSK3β activity assay results indicate that LiOr does not readily dissociate in solution, they do not provide information as to what might happen in a low pH environment akin to the stomach. Thus, replication of the resistivity experiments in media matching the pH of the stomach (1.5-2; gastric acid) and duodenum (~6) would provide valuable information regarding the dissociative characteristics of LiOr following oral ingestion.

7.2.6 Confirmation of the role of UMPS in mediating the dissociation of LiOr

Although the results of our 6-azauracil trials in the AIH and hippocampal LTP models are highly encouraging, replication using a second UMPS inhibitor, such as pyrazofurin, would increase our confidence in the data. Additionally, ion chromatography could be used to confirm the dissociation of LiOr, or lack thereof, by allowing for the direct assessment of Li⁺ concentrations in cell cultures with and without functional UMPS; UMPS could be inhibited either pharmacologically or via genetic knock-out.

7.2.7 Characterization of OATP1A2 expression within target regions

If the distribution of OATP1A2 and OATP2B1 within the brain is heterogeneous, dual immunolocalization experiments performed on fixed brain slices would allow for observation of the overlap between OATP signatures and neuron subtype-selective markers (e.g., D2 medium spiny neurons within the ventral striatum), thereby allowing for characterization of which neuronal populations are most strongly affected by LiOr.
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