LITHIUM OROTATE SELECTIVELY INHIBITS GSK3β WITHOUT IMPACT ON INOSITOL SIGNALLING

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By

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Abstract

Introduction:

Lithium Carbonate (LiCO) has been the standard pharmacological treatment for bipolar disorder (BD) for over half a century because it successfully reduces the manic and depressive characteristics of BD. Unfortunately, patients often discontinue LiCO treatment due to adverse physiological and cognitive side effects. LiCO treatment is limited by the narrow therapeutic window where high doses lead to toxicity or increased risk of side effects while lower doses are considered ineffective. Lithium orotate (LiOr) is an alternative treatment suggested to possess superior uptake properties compared to LiCO, which may reduce dosing requirements and lessen cognitive side effects. As LiOr yields higher brain lithium (Li^+) than LiCO due to different transport mechanisms and is only liberated intracellularly distant from the cell membrane, we hypothesize that **1)** LiOr will be more potent than LiCl and **2)** LiOr will selectively inhibit GSK3β to facilitate long-term potentiation (LTP) while LiCl acts closer to the membrane on N-methyl-D-aspartate (NMDA) receptors and the phosphatidylinositol cycle.

Methods:

Dose-dependent LiCl and LiOr effects on synaptic plasticity were assessed in the hippocampal Schaffer collateral-CA1 synapse in male C57BL/6 mouse slices. LTP was induced by theta burst stimulations (TBS) (8 bursts at 5 Hz of 4 high-frequency pulses at 100 Hz repeated three times, 60 seconds apart) at 32℃. Long-term depression (LTD) was induced using a low-frequency stimulation (LFS; 1 Hz) for 15 minutes at room temperature. We used typical therapeutic Li^+ concentrations between 0.2-1.0 mM to assess Li^+ effects on synaptic plasticity. We compared the effects the phosphoinositol cycle had on Li^+-mediated effects on LTP by incubating slices in myo-inositol for 2-3 hours. We used basic pharmacology to determine the effects LiCl and LiOr had on GSK3β and NMDA receptors with 3.5 μM AZD2858, a non-specific GSK3B inhibitor, and 5 μM DNQX to isolate NMDA currents.

Results:

We found that LiCl and LiOr differentially affect synaptic plasticity by mediating different secondary messenger pathways. All concentrations of LiOr influenced synaptic plasticity, but only higher concentrations of LiCl altered LTP and LTD. We determined that LiCl had a dose-dependent effect on the phosphatidylinositol pathway and NMDA receptors, whereas LiOr consistently increased LTP through inhibition of GSK3β.

Conclusion:

Therapeutic LiCl concentrations demonstrate a dose-dependent response on synaptic plasticity, whereas LiOr has a consistent effect at both high and low concentrations. This differential effect could explain the contrasting findings on the effects LiCO has on cognition, as slight changes in Li^+ concentration drastically change the synaptic response. This provides clinical relevancy to BD research as it indicates LiOr is a more beneficial treatment for BD because it can be prescribed at a lower dose than LiCO with the same effect. This study could influence clinical Li^+ application and may lessen the adverse physiological and cognitive impact associated with Li^+ treatment.

Table of Contents

List of Figures

Chapter 1.0 Introduction

Chapter 3.0 Results

List of Abbreviations

BD – bipolar disorder

LiCO – lithium carbonate

LiOr – lithium orotate

Li^+ – elemental lithium

NMDA - N-methyl-D-aspartate

AIH – amphetamine-induced hyperlocomotion

OATP – organic anion polypeptide

UMPS -uridine monophosphate synthase

DMS-5 - Diagnostic and Statistical Manual for Mental Disorders, 5^{th} edition

GSK3β – glycogen synthase kinase-3β

PKA – protein kinase A

CNS – central nervous system

PI cycle - phosphatidylinositol cycle

PIP_2 - phosphatidylinositol 4,5-bisphosphate

SMITs - sodium-myo-inositol transporters

BBB – blood-brain barrier

PLC - phospholipase C

IP_3 - inositol-1,4,5 triphosphate

DAG - 1,2-diacylglycerol

IMPase- inositol monophosphatase

IPPase- Inorganic pyrophosphatase

PKC – protein kinase C

SNRIs- selective serotonin-norepinephrine reuptake inhibitors

SNRIs - selective serotonin reuptake inhibitors

PSD-95- postsynaptic density protein 95 kDa

NKCC - sodium potassium chloride cotransporter

NBC - sodium-bicarbonate cotransporters

NHE- sodium proton exchanger

LTD – long-term depression

LTP – long-term potentiation

TBS - theta-burst stimulation

LFS- low-frequency stimulation

AMPA - α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

aCSF – artificial cerebrospinal fluid

fEPSPs - field excitatory post synaptic potentials

1.0 Introduction

Bipolar Disorder (BD) is an umbrella term for a chronic mental illness category consisting of depressive and manic mood cycles[1–5]. Lithium (Li^+) salts, such as lithium carbonate (Li_2CO_3; LiCO henceforth and LiCl), have been the standard pharmacological treatment for BD for over half a century because it successfully reduces the manic and depressive characteristics of BD. Although other antipsychotics have gained popularity in recent years, LiCO remains one of the main treatment options for its efficiency in preventing mood episodes and its role in suicide prevention[3]. Unfortunately, patients often discontinue LiCO treatment due to adverse physiological and cognitive side effects that range from inconvenient to life-threatening. LiCO treatment is limited by a narrow therapeutic window where high doses lead to toxicity or increased risk of side effects, while lower doses are considered ineffective.

Lithium orotate ($LiC_5H_3N_2O_4$; LiOr henceforth) is an alternative treatment suggested to possess superior uptake properties compared to LiCO, which may reduce dosing requirements and lessen cognitive side effects relative to LiCO. Hans Nieper was one of the earliest supporters of orotic acid in the 1970s, suggesting orotic acid conjugates were more readily transported across cellular membranes compared to conjugate counterparts. In support, Kling *et al.* (1978) demonstrated that greater concentrations of brain Li^+ exist in the brain when administered as an orotate instead of a carbonate compound[6]. In line with this study, previous work in the Bekar lab found that lower doses of LiOr blocked hyperlocomotion in an amphetamine-induced hyperlocomotion (AIH) model, and the strength of blockade elicited was greater than LiCl [7]. These results indicate that the potency and efficacy of LiOr are superior to LiCl or LiCO, likely due to LiOr's superior pharmacokinetic and pharmacodynamic properties.

Recently, Pacholoko and Bekar (2023) discovered differential transport- and dissociation-related characteristics between LiOr, LiCl and LiCO, which offers a possible explanation for why LiOr has lower dosage requirements than other Li^+ compounds [7]. Previous research in the lab found that, in contrast to LiCO, which dissociates readily in solution, LiOr remains in its non-dissociated state post-administration [7]. As LiCO and LiCl readily dissociate, it primarily uses sodium channels for transport throughout the body[8–11]. As a result, CNS sodium dynamics limit the entry of LiCO and LiCl into the CNS, which, much like other inorganic cations, makes sodium slow to equilibrate

between the blood and brain[8,9,11]. To overcome these restrictions, high concentrations of LiCO are required to overcome the limitations of Li^+ entry to the CNS[12–15]. Unfortunately, these high doses of LiCO lead to adverse side effects upon acute administration, including polydipsia, polyuria, nausea, diarrhea and tremors[16,17]. More severe side effects that occur following long-term LiCO treatment include cognitive impairments, nephrotoxicity, hypothyroidism, and hyperparathyroidism [16,17]In contrast, Pacholko and Bekar (2023) provided evidence that LiOr is intercellularly transported as a compound through Organic anion-transporting polypeptides (OATS), suggesting an alternative transport mechanism than sodium transporters could increase the ease with which LiOr crosses biological barriers to enable the direct delivery of Li^+ to intracellular target sites[7]. The difference in dosing requirements between LiOr and LiCl makes LiOr a better treatment option because the decreased dose needed for therapeutic LiOr treatment may reduce any adverse side effects associated with LiCO due to the high dose requirements of the drug [6,16,18–20].

Despite recent pharmacological discoveries, the question of whether LiOr and LiCO have different effects on synaptic mechanisms remains unknown. With this in mind, the objectives of this thesis were to **1)** compare the effect therapeutically relevant doses of low (0.2 and 0.4 mM) and high (0.6, 0.8, 1.0 mM) concentrations of LiCl and LiOr have on synaptic plasticity. **2)** determine the effect LiOr and LiCl have on the phosphatidylinositol cycle and GSK3β during synaptic plasticity. **3)** determine the effect LiOr and LiCl have on N-methyl-D-aspartate (NMDA)-receptor-mediated currents.

1.1.Bipolar Disorder

1.1.1. An Introduction to Bipolar Disorder

BD is an umbrella term for a severe, lifelong mental illness category consisting of depressive and manic mood cycles linked with extreme rates of suicide[1–5]. Suicidality is estimated to be 20 times higher in people with BD than in the general population, with approximately 30% of people with BD attempting suicide at least once in their lifetime[21–23]. Given the severity of symptoms, BD impairs psychosocial and cognitive functioning and causes a 10–20-year reduction in the anticipated lifespan[24–26].

BD is diagnosed by the presence of altering manic and depressive episodes over two years, with the severity and duration of these cycles characterized into classes BD I, BD II, and cyclothymia (Fig. 1.1) [23,25–27]. The essential features of manic or hypomanic episodes are elevated or irritable mood states with associated symptoms of increased self-esteem, impaired judgment, and reduced need for sleep [23,25–27]. The difference between manic and hypomanic are differentiated in the Diagnostic and Statistical Manual for Mental Disorders, 5th edition (DMS-5) by duration, stating manic states last for one week, whereas a hypomanic episode lasts for a period of 4 days[2,26]. Depressive episodes associated with BD are characterized by low mood states with related symptoms of anhedonia, sleep disturbance, and fatigue[2,26]. BD 1 is distinguished from BD II by more extreme manic episodes compared to the more hypomanic episodes observed in BD II[26,27]. However, the depressive states between BD I and BD II are similar; thus, BD II should not be considered a milder form of the disease[2,22–24,26,28]. Cyclothymic disorder is a milder subset of BD that displays frequent and rapid mood cycling of hypomania and depression states that does not meet the full requirements of BD[26]. BD is a life-long condition that requires consistent medical treatments to manage mental disturbances that cause social and occupational impairment, making it necessary to research the disease and identify treatment options [23–25,28].

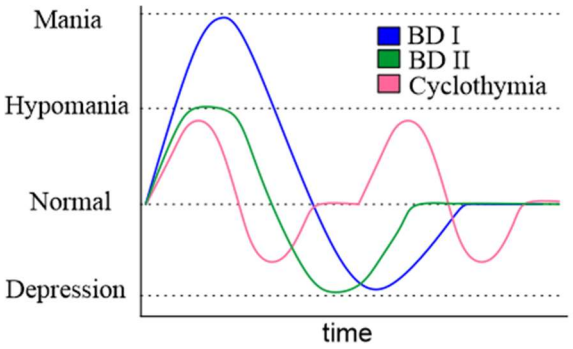

Fig. 1.1. Mood cycling states in BD classes. BD is diagnosed by the presence of altering manic and depressive episodes, with the severity and duration of these cycles characterized into classes BD I, BD II, and cyclothymia. BD I is distinguished from BD II by more extreme manic episodes compared to the more hypomanic episodes observed in BD II. Cyclothymic disorder is a milder subset of BD that displays frequent and rapid mood cycling of hypo-mania and depression states.

1.1.2. Pathophysiology of Bipolar Disorder

Although the exact pathophysiology of BD is unknown, the understanding of BD has progressed rapidly over the last few decades, with research finding environmental, genetic, and physiological connections between people with BD [2,29,30]. Traditionally, BD pathophysiology is connected to the dysregulation of monoaminergic neurotransmitter systems, including serotonergic, noradrenergic, and, particularly, the dopaminergic neurotransmitter system [2,29,30]. The dopaminergic system is implicated in BD pathophysiology due to increased dopamine transport levels and density of D2/3 dopamine receptors in BD patients [2,30,31]. However, changes

to other dopaminergic functions are inconsistent, indicating other pathophysiological functions' involvement in those with BD[30,31].

1.1.2.1. Dopamine dysregulation hypothesis of bipolar disorder

The role of dopamine in BD is one of the earlier hypotheses of the disease that focused on the relationship between mania and increased dopamine levels in the CNS, supported by parallels between decreased manic episodes observed with antidopaminergic drugs and altered behaviour following dopaminergic stimulants [30,31]. More recently, the dopaminergic system has been implicated in BD due to increased dopamine transport levels and density of D2/3 dopamine receptors (D2R) in BD patients[2,30,31]. The modern dopamine dysregulation hypothesis proposes that dopaminergic neurotransmission is altered due to the lack of homeostatic mechanisms that regulate dopaminergic function [30–32]. It proposes that hyperdopaminergia occurs during mania, eventually transitioning to a depressive and hypodopaminergic state through downregulating D2R sensitivity and increased dopamine transporters (DAT) activity. The manic and hyperdopaminergic phase is restored following the hypodopaminergic state by upregulation of D2R sensitivity and decreased dopamine transport (Fig. 1.2).

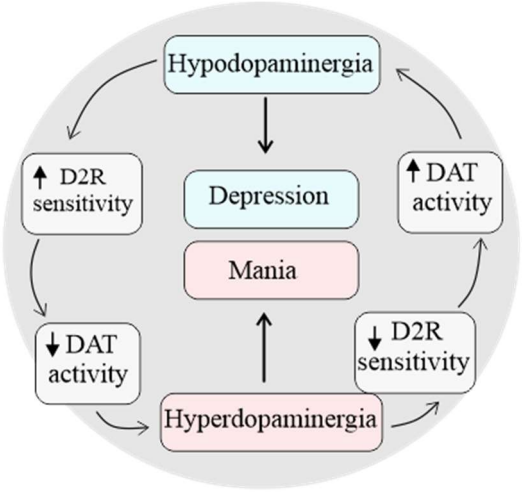

Fig. 1.2. The dopamine hypothesis of BD. The modern dopamine dysregulation hypothesis proposes altered homeostatic regulation of dopaminergic function underlies cyclic manic and depressive characteristics of BD. This failure of homeostatic mechanisms leads to hyperdopaminergia during mania, eventually transitioning to a depressive and hypodopaminergic state through downregulating D2 dopamine receptor (D2R) sensitivity and increased dopamine transporters (DAT) activity. The manic and hyperdopaminergic phase is restored following the hypodopaminergic state by upregulation of D2R sensitivity and decreased DAT activity.

In summary, the relationship between the cycling of mood episodes and dopamine levels may underline BD's cyclical nature. This hypothesis is supported by altered expression of D2R sensitivity, D2R availability, and expression of DAT in Bd patients [30–32]. However, the changes to other dopaminergic functions are inconsistent, which indicates the involvement of other pathophysiological functions in those with BD[30,31].

1.1.2.2. GSK3β dysregulation hypothesis of bipolar disorder

Glycogen synthase kinase-3-beta (GSK3β) has been implicated in BD pathology since the discovery that Li^+, a mood stabilizer used to treat BD, selectively inhibits GSK3β [15,33]. GSK3β is regulated by the phosphorylation status of tyr216 and Ser9 by downstream cellular pathways, including Akt and protein kinase A (PKA). The phosphorylation of tyr216 is required for basal activity, and high phosphorylation of this residue causes GSK3β to be active in resting cells [34]. The phosphorylation of Ser9 overrides activation induced by phosphorylation tyr216, which inhibits GSK-3β [35–37]. Conversely, dephosphorylation of Ser9 by Ser/Thr proteases leads to further activation of GSK-3β [35]. The dysregulation of GSK3β is implicated in BD pathology due to heightened GSK3β expression and genetic changes to GSK3β observed in human BD patients [33,38]. As GSK3β regulates many cell signalling mechanisms, abnormal GSK-3β activity could cause changes in brain neurophysiology that lead to BD[39].

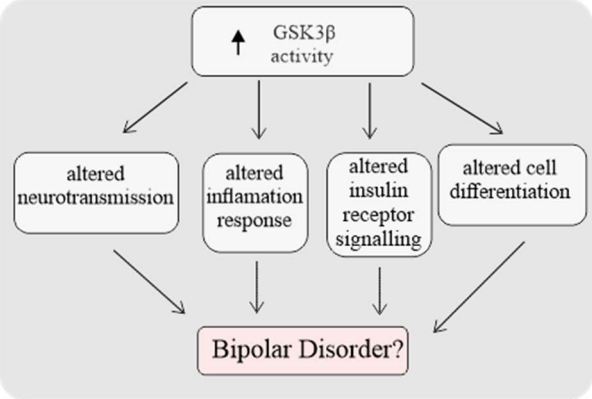

Fig. 1.3.GSK3β dysregulation hypothesis of bipolar disorder. The dysregulation of GSK3β has been implicated in BD pathology due to heightened GSK3β expression and genetic changes to GSK3β observed in human BD patients.GSK3β is critical for typical central nervous system functioning because it regulates neurotransmission, neurite outgrowth, growth, cone dynamics, cytoskeleton, synaptic plasticity, apoptosis and neurogenesis through phosphorylation of neuronal substrates through its own excitatory and inhibitory phosphorylation. Given the widespread regulatory abilities of GSK3β, it stands to reason that any dysfunction in activity will disrupt the homeostatic state of CNS functioning. As the disruption of GSK3β activity is observed in both BD patients and animal models of BD symptoms, this suggests GSK3β hyperactivity is central in BD pathophysiology.

GSK3β is a highly conserved, multifunctional serine/threonine kinase ubiquitously expressed in all mammalian tissues and organs that regulate many cell signalling pathways, such as including insulin receptor signalling, cell differentiation during embryonic development, immunity and inflammation responses, and neurotransmission, making it crucial for cellular homeostasis (Fig. 1.3) [39–41]. It is critical for the typical central nervous system (CNS) functioning because it regulates neurotransmission, neurite outgrowth, growth, cone dynamics, cytoskeleton, synaptic plasticity, apoptosis, and neurogenesis through phosphorylation of neuronal substrates through its own excitatory and inhibitory phosphorylation. Given the widespread regulatory abilities of GSK3β, it stands to reason that any dysfunction in activity will disrupt the homeostatic state of CNS functioning.

The dysregulation of GSK3β has been implicated in BD pathology due to heightened GSK3β expression and genetic changes to GSK3β observed in human BD patients [33,38]. For instance, genetic polymorphisms in the GSK3β promoter region are significantly associated with BD. This defect is connected to an early onset of the disease, the therapeutic response to Li^+, and the structure of white matter in the brain[42]. Specifically, the *DISC1* gene, a gene associated with mood disorders, is known to directly regulate GSK3β by inhibiting phosphorylation of β-catenin, which directly impacts the Wnt pathway [42]. The GSK3β hypothesis is supported by rodent studies that connect GSK3β to behaviour associated with BD. In mice overexpressing GSK3β, specific GSK3β inhibitors replicate the behaviour-modulating effects of Li^+[43]. Several studies connect the dysregulation of GSK3β, and manic and depressive states associated with BD. For instance, mice with only one copy of the *GSK3β* gene behaved similarly to mice chronically treated with Li^+[44–47]. On the other hand, mice that overexpress GSK3β show increased locomotion that mimics manic-like behaviour observed in BD patients[43,44,48].

In summary, GSK3β is a key regulator of many CNS pathways necessary for CNS homeostasis, including insulin receptor signalling, cell differentiation during embryonic development, immunity and inflammation responses, and neurotransmission, indicating that disruption of GSK3β may lead to altered CNS functioning. The disruption of GSK3β activity is observed in both BD patients and animal models of BD symptoms, suggesting GSK3β hyperactivity is central in BD pathophysiology.

1.1.2.3.The IMPase hypothesis of bipolar disorder

The phosphatidylinositol cycle (PI cycle) is an intracellular cycle where the primary outcome is to provide free myo-inositol for the biosynthesis of phosphatidylinositol 4,5-bisphosphate (PIP_2), which is a precursor for secondary cellular pathways that regulate a diverse array of processes ranging from cell growth to neural transmission [4,49,50]. The PI cycle is implicated in BD due to correlations between BD and altered myo- inositol and phosphoinositide levels in the brain [4,51,52]. Interestingly, myo-inositol levels fluctuate with manic and depressive cycling associated with BD with magnetic resonance spectroscopy revealing higher levels of myo-inositol detected during manic phases and significantly lower levels identified in the frontal cortex during depressive phases [51–54]. Furthermore, dietary supplementation of inositol correlated to improvements in depression in both human and animal models of depression [53,55,56]. Given that

changes in the availability of inositol in the CNS are connected to altered brain cell signalling pathways, it stands to reason that dysfunction within the PI cycle affects a plethora of synaptic mechanisms associated with myo-inositol availability, such as neurotransmission and synaptic plasticity [4,51,57].

As myo-inositol is the precursor for all inositol lipids and phosphates, eukaryotic cells obtain myo-inositol through three different routes to ensure consistent myo-inositol availability[4,57]. The PI cycle obtains myo-inositol from the surrounding environment by sodium-myo-inositol transporters (SMITs), derived from *de novo* synthesis of glucose-6-phosphate and recycled from various phosphatidylinositol molecules[57]. It is important to note that levels of myo-inositol are significantly higher in the brain than in blood and other tissues, and, as the blood-brain barrier (BBB) limits myo-inositol uptake, the primary forms of myo-inositol in the CNS are derived from *de novo* synthesis and recycling of inositol phosphates [58,59].

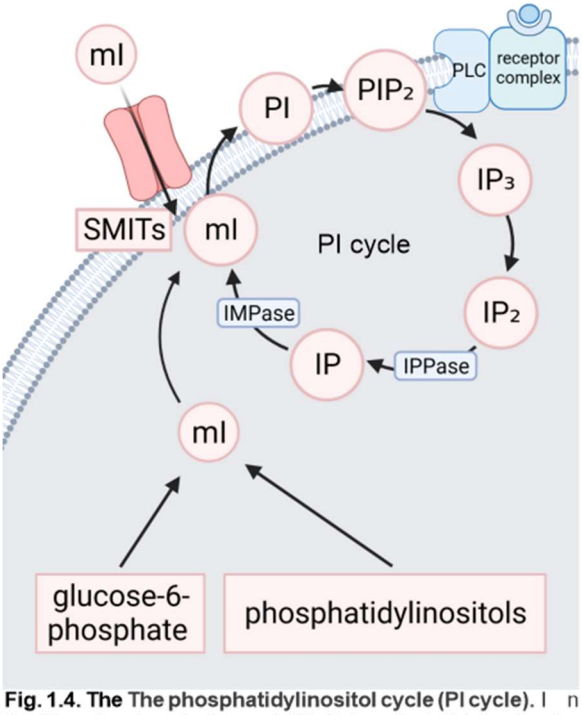

Fig. 1.4. The The phosphatidylinositol cycle (PI cycle). In the PI cycle, phospholipase C (PLC) is activated following the stimulation of the cell receptor complex consisting of a receptor, Gq-protein and phospholipase (PLC) [55,57]. This leads to the hydrolysis of PIP2 to secondary messengers' inositol-1,4,5 triphosphate (IP3) and 1,2-diacylglycerol (DAG). These secondary messengers activate downstream signalling pathways where IP3 ultimately increases free cytosolic calcium, and DAG activates protein kinase C (PKC) to affect cholinergic, noradrenergic, serotonergic, and other neurotransmitter systems[55]. To regenerate myo-inositol, IMPase and IPPase are critical enzymes in the PI cycle that facilitate the recycling of IP3 back to myo-inositol to allow for the PI cycle to continue [4,16,58]. The PI cycle obtains myo-inositol from the surrounding environment by sodium-myoinositol transporters (SMITs), derived from de novo synthesis of glucose-6-phosphate and recycled from various phosphatidylinositol molecules. Created with BioRender.com.

In the PI cycle, phospholipase C (PLC) is activated following the stimulation of the cell receptor complex consisting of a receptor, Gq-protein, and PLC [57,59]. This leads to the hydrolysis of PIP_2 to secondary messengers' inositol-1,4,5 triphosphate (IP_3) and 1,2-diacylglycerol (DAG). These secondary messengers activate downstream signalling pathways where IP_3 ultimately increases free cytosolic calcium, and DAG activates protein kinase C (PKC) to affect cholinergic, noradrenergic, serotonergic, and other neurotransmitter systems[57]. To regenerate myo-inositol, inositol monophosphates (IMPase) and inorganic pyrophosphatase (IPPase) are critical enzymes in the PI cycle that facilitate the recycling

of IP_3 back to myo-inositol to allow the PI cycle to continue (Fig. 1.4) [4,15,60]. Thus, regulation of the PI cycle is essential for normal CNS functioning, which suggests dysregulation can result in changes to cellular activities mediated by enzymes in the PI cycle [61,62].

1.1.3. Treatment of Bipolar Disorder

Given the prevalence, morbidity and mortality associated with BD, managing BD disorder through psychiatric and pharmaceutical treatment is an essential goal within psychiatry. However, BD is difficult to treat due to the cycling of manic and depressive episodes and different expression of the disease between patients. The approach to treatment varies depending on whether the patient is presenting with hypomania, mania, depression or euthymia. Moreover, the treatment method is determined based on medical and psychiatric comorbidities, previous or current treatments, response to treatment or adverse effects in patients and relatives, and the patient's willingness to be treated. Generally, BD patients first receive acute management, with the ultimate goal of complete remission, and, later, chronic management to prevent reoccurrences of mood episodes [29]. BD is generally treated with a wide range of medications, including antipsychotics, antidepressants, and mood stabilizers [29].

1.1.3.2.Antipsychotics

Antipsychotic drugs have been used for over half a century to treat various psychiatric disorders, including BD, through dopamine and serotonin receptor antagonism. In recent years, new antipsychotic drugs, known as "second-generation antipsychotics," have replaced phenothiazine, thioxanthene and butyrophenone neuroleptics for treating acute psychotic, manic and psychotic-depressive disorders. As a class, the newer drugs, including aripiprazole, clozapine, olanzapine, quetiapine, risperidone, and ziprasidone, have been promoted as being broadly clinically superior. The neuropharmacology of second-generation antipsychotics is more complex than conventional antipsychotics; however, the general dopamine and serotonin receptor antagonism is similar. As antipsychotics normalize dopaminergic activity, the clinical success of antipsychotics supports the dopamine dysregulation hypothesis of BD (see section 1.2.2.1).

1.1.3.3.Antidepressants

The use of antidepressants to treat depressive phases in BD is controversial among clinicians due to concerns about switching depressive phases to mania or hypomania episodes. Additionally, the chronic effect antidepressants have on preventing reoccurrence and managing

BD symptoms are unknown. The use of antidepressants is particularly controversial as a monotherapy, and if antidepressants are used, they are recommended in conjunction with a mood stabilizer or antipsychotic. Moreover, specific types of antidepressants, such as selective serotonin-norepinephrine reuptake inhibitors (SNRIs) and tricyclics, are not recommended due to concerns about a manic switch. Instead, selective serotonin reuptake inhibitors (SSRIs) or bupropion are recommended because they are less likely to cause a manic switch. In general, antidepressants are typically not prescribed to those with BD as a monotherapy; however, some BD patients can be successfully treated with them.

1.1.3.4.Mood stabilizers

Since BD is characterized as a series of cycling major mood episodes, psychiatric treatment and neurobiological studies focus on stabilizing mood fluctuations in BD patients. Discovered by John Cade in 1949 [63], Li^+ salts were the first pharmacological treatment for BD due to their role in controlling mania and preventing mood-episode recurrence. Although Li^+ still has an essential role in BD management today, other mood stabilizers (e.g., valproate, lamotrigine, and carbamazepine) and antipsychotics are often prescribed instead of Li^+ due to the physiological and cognitive side effects associated with Li^+ therapy [64–67]. However, this reduction in Li^+ treatment may be ill-advised, particularly in maintenance therapy, due to results from a meta-analysis performed by Miura *et al.* (2014) that found only Li^+ and quetiapine prevented manic-and depressive relapses and recurrences. While mood stabilizers have declined in recent years, Li^+ still maintains a central role in preventing and treating BD due to its role in preventing mood episode recurrence.

1.2. The Role of Lithium Therapy

Discovered by John Cade in 1949 and brought to popularity by Samuel Gershon in 1960, Li^+ salts play a central role in preventing manic and depressive episodes associated with BD. In addition to its role as a mood stabilizer, Li^+ is often temporarily prescribed in conjunction with other anti-depressants or antipsychotics as stabilizing medication because, along with its prevention of manic and depressive episodes, it is established to prevent suicide [68–70]. As the mortality of BD patients is 2-3 times that of the general population, suicide prevention is crucial in BD management due to the high suicide statistics associated with BD disease. While the exact mechanism that makes Li^+ different from other BD medications is unknown, Li^+ compounds are

believed to address BD symptomology through various secondary mechanisms, including antagonism of GSK3β, IPPase, and IMPase, linked to BD pathophysiology [14,15].

1.2.2. Lithium pharmacokinetics

Li^+ is a commonly prescribed medication for treating BD due to its success in preventing acute manic and depressive episodes and its action in suicide prevention. Unlike other mood-stabilizing medications, Li^+ appears to exert its therapeutic actions by modifying intracellular second messengers, downstream metabotropic receptors, and inhibiting enzymes [12,15]. Given the similar ionic radii of Li^+ and magnesium, Li^+ competitively binds to the catalytic core of proteins that have magnesium as a cofactor, resulting in the inhibition of these proteins [39,61,71]. While the exact mechanism of action is unknown, Li^+ appears to act on multiple cellular pathways, including GSK3β, PI cycle, and NMDA receptors, to modulate BD [15].

1.2.2.1. Lithium decreases NMDA receptor function.

NMDA receptors are a subtype of glutamate receptor regulated by protein phosphorylation of NR2 subunits crucial for development, neuroplasticity, and excitotoxicity in the central nervous system [7273]. The activity of NMDA receptors is regulated by interactions between NR2A subunits, Src-family tyrosine kinases, Fyn, and postsynaptic density protein 95 kDa (PSD-95) that form a ternary complex that facilitates NR2A tyrosine phosphorylation [73]. While NMDA receptors' role in BD pathophysiology is currently unknown, with genetic and post-mortem studies yielding conflicting results, Li^+'s inhibitory effect on NR2A tyrosine phosphorylation implicates NMDA receptors as a potential therapeutic target for managing BD (Fig. 1.5A) [74]. Recently, the neuroprotective effects Li^+ had on excitotoxicity

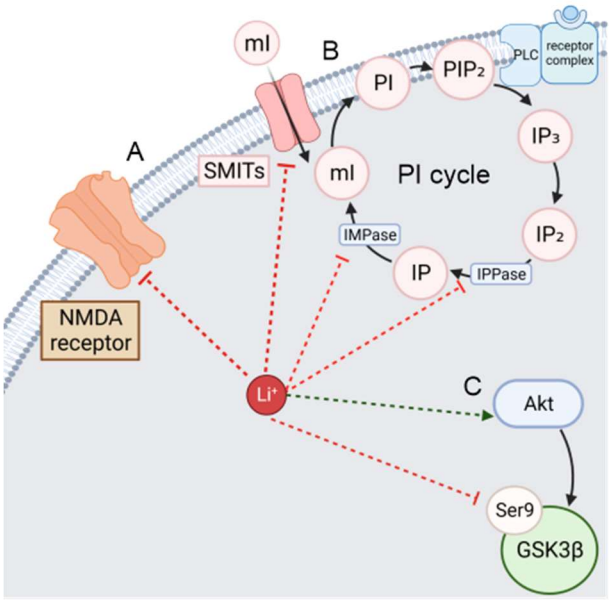

Figure 1.5. Known effects of lithium on PI cycle, GSK3β and NMDA receptors. A) Lithium is observed to cause downregulation of NMDA receptor expression and signalling through inhibition of NR2A tyrosine phosphorylation and its interactions with Src and Fyn mediated by PSD-95. **B)**Lithium alters IP3 signalling by inhibiting sodium myo-inositol transporters (SMITs), IPPase and IMPAse, which causes a decrease in cellular myo-inositol (mI). **C)** Lithium directly decreases GSK3β signalling by competitive binding of magnesium and indirectly inhibits GSK3β by activating Akt to cause an increase in serine phosphorylation. C) Lithium is assumed to inhibit NMDA mediated currents to alter synaptic plasticity.Created with BioRender.com.

mediated by NMDA receptors have been demonstrated, which suggests NMDA receptors may be a therapeutic target for the treatment of BD. Li^+ is observed to cause downregulation of NMDA receptor expression and signalling through inhibition of NR2A tyrosine phosphorylation and its interactions with Src and Fyn mediated by PSD-95 [73,75–78]. Although NMDA receptors' role in BD is conflicting, the Li^+-induced downregulation of NMDA receptor function may be pivotal in managing BD symptoms.

Li^+-mediated downregulation of NMDA receptors has given Li^+ the reputation of having neuroprotection against excitotoxicity. In brain ischemia studies, cerebral ischemia increased tyrosine phosphorylation of NR2 subunits, causing upregulation of NMDA receptor function. Ma and Zhang (2003) observed Li^+ -inhibited changes in tyrosine phosphorylation of NR2 subunits caused by cerebral ischemia, resulting in down-regulation of NMDA receptor activity and decreased excitotoxicity [73]. Similarly, long-term treatment of cultured neurons with therapeutic concentrations of Li^+ (1 mM) decreased apoptotic cell death associated with glutamate excitotoxicity mediated by NMDA receptors, suggesting Li^+-mediated neuroprotective effects against excitotoxicity represents part of the molecular mechanisms by which Li^+ exerts its therapeutic efficacy against BD [79]

1.2.2.2.Lithium decreases intracellular myo-inositol.

Li^+ inhibits critical enzymes in the PI cycle through the displacement of the magnesium cofactor, leading to decreased free cellular myo-inositol and disrupting secondary messenger pathways derived from PIP_2 (Fig. 1.5B) [61,62]. As dysfunction in the PI cycle is connected to BD pathophysiology (see section 1.1.), the normalization Li^+ has on the PI cycle may explain the therapeutic efficiency Li^+ displays on BD symptomology. In this process, Li^+ suppresses myo-inositol production with an IC_{50} of 0.5 mM by inhibiting IMPase and IPPase, which causes a substantial decrease in free intracellular myo-inositol in the brain. In contrast, chronic therapeutic Li^+ has been found to increase the activity of IMPase, which alters any neurochemical processes regulated by myo-inositol [80]. However, chronic therapeutic Li^+ treatment has also been observed not to affect IMPase activity, suggesting that Li^+'s long-term effects on the PI cycle are poorly understood [81].

As myo-inositol availability is central to normal cell signalling, it stands to reason that the disruption of myo-inositol availability leads to pathophysiological side effects. Many side effects

reported with Li^+ therapy (see section 1.2.2), including weight gain, hypothyroidism, insulin resistance, hyperandrogenism, amenorrhea, and polyuria/polydipsia, can be traced back to reduced levels of myo-inositol in related tissues[4,50,82]. For this reason, inositol supplementations in conjunction with Li^+ therapy are observed to induce beneficial changes in side effects observed with Li^+ therapy [55,56,83,84]. Specifically, the dosage of 3-4 grams/ daily of myo-inositol has been observed to decrease the side-effects of Li^+ without creating any adverse side effects of its own use[85]. Using myo-inositol as a therapeutic strategy for BD may counteract many side effects caused by Li^+ without disrupting the central therapeutic efficiency of Li^+ treatment. In summary, Li^+ decreases intracellular myo-inositol by inhibiting key enzymes in the PP cycle that control myo-inositol availability; however, the downstream consequences of inositol depletion are poorly understood.

1.2.2.3. Lithium inhibits GSK3β.

GSK3β is a downstream regulator of many cell-signalling pathways, including insulin receptor signalling, cell differentiation during embryonic development, immunity and inflammation responses and neurotransmission [86–90]**.** Li^+ directly decreases GSK3β signalling by competitive binding of magnesium and indirectly inhibits GSK3β by activating Akt to cause an increase in serine phosphorylation (Fig. 1.5C). BD patients display aberrant GSK3β activity, the inhibition of GSK3β is suggested to be central to the therapeutic effects Li^+ has on BD[33]. Although the exact mechanism of action remains largely unknown, Li^+ is noted to be a rapid and direct inhibitor of GSK3β with an IC_{50} value of approximately 2 mM[33,91]. Given that therapeutic concentrations of Li^+ are less than 1 mM and effects are only noticed after many weeks of use, it is unlikely therapeutic concentrations of Li^+ mediate BD solely through inhibition of GSK3β[33,46,91]. In contrast, partial inhibition of GSK3β observed with therapeutic Li^+ concentrations may be helpful in mediating BD symptomology.

As tissues collected from BD patients display elevated activity of GSK3β, Li^+'s normalization of GSK3β may be central in managing BD symptomology[38]. Li^+ directly inhibits GSK3β through competitive inhibition of the magnesium binding and indirectly inhibits GSK3β by increasing the inhibitory serine phosphorylation of GSK3β. In support, Li^+ administered chronically to mice causes a significant increase in Ser9 phosphorlation[33,92]. Similarly, animal models with overexpression of GSK3β elicit manic-like activity similar to BD, which can be

reversed by Li^+ treatment [93]. In contrast, GSK3β haploinsufficiency mimics behaviours associated with chronic Li^+ treatment[44,94]. Taken together, it suggests that the inhibition of GSK3β is central to Li^+**'s** therapeutic efficiency in managing BD.

1.2.3. Lithium carbonate

LiCO has been the standard pharmacological treatment for BD for over half a century because it successfully decreases and reduces manic and depressive episodes associated with BD [15,64,68,69,95–98]. LiCO treatment is limited by a narrow therapeutic window where high doses lead to toxicity or increased risk of side effects, and lower doses are considered ineffective [15]. In general, side-effects of those prescribed LiCO experience range from uncomfortable, such as thirst and excessive urination, nausea and, diarrhea and tremors, disabling, such as cognitive dullness, to life-threatening or altering. The more severe side effects that typically lead to patient incompliance include thyroid, renal and cognitive disturbances [99]. Managing these side effects remains critical to psychiatrists' optimal treatment of BD, as not taking medication may lead to increases if early affective mortality[17,100].

1.2.3.1.LiCO dosing protocol optimized to prevent patient non-adherence.

The adverse effects of Li^+ on cognition, thyroid, and liver can be minimized by obtaining a dose of Li^+ concentration that is high enough to treat BD symptoms but not too high to induce Li^+ toxicity [17]. As LiCO and LiCl readily dissociate, it primarily uses sodium channels and transporters, including sodium potassium chloride cotransporter (NKCC), sodium-bicarbonate cotransporters (NBC), and sodium proton exchanger (NHE) for transport throughout the body (Fig. 1.6)[8–11]. As a result, CNS sodium dynamics slow the entry of Li^+ from LiCO and LiCl into the CNS because Li^+ is in competion with sodium for entry

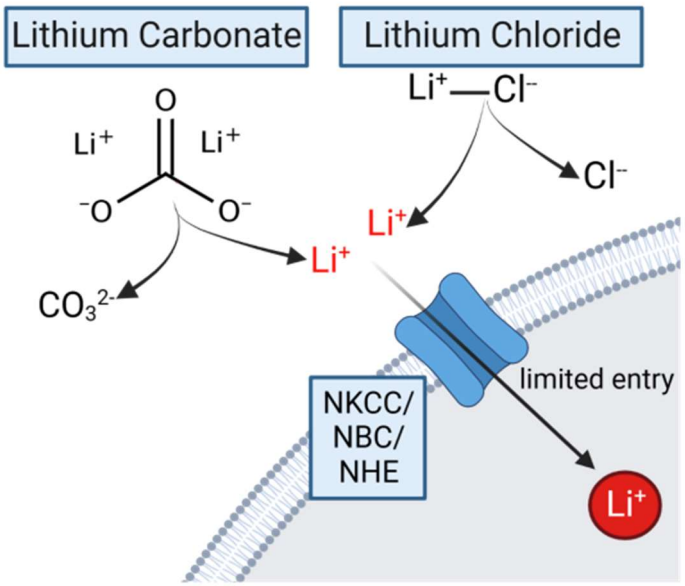

Figure 1.6. LiCO and LiOr primarily uses sodium channels for transport throughout the body. As LiCO and LiCl readily dissociate, it primarily uses sodium channels, including sodium potassium chloride cotransporter (NKCC),sodium-bicarbonate cotransporters (NBC), and sodium proton exchanger (NHE) for transport across cellular membranes.Created with BioRender.com.

through sodium channels, which, much like other inorganic cations, makes sodium slow to equilibrate between the blood and brain[8,9,11]. To overcome CNS sodium restrictions, high concentrations of LiCO are required to overcome the limitations of Li^+ entry to the CNS[12–15]. Unfortunately, these high doses of LiCO lead to adverse side effects upon acute administration, which contribute to high rates of nonadherence[101].

When beginning LiCO therapy, Li^+ levels are recommended to be assessed regularly to ensure plasma Li^+ levels remain between 0.5-1.2 mmol/L [102]. Given that higher Li^+ levels increase the likelihood of instigating adverse side effects and inducing Li^+ toxicity, the optimal range of plasma Li^+ ranges from 0.5-0.8 mmol/L to avoid acute toxicity. Li^+ levels are recommended for chronic treatment between 0.4-0.8 mmol/L; however, the dose may vary given illness polarity and gravity of symptoms. The optimal Li^+ dose is generally determined by comparing mood episode recurrences to the severity of side effects, prioritizing minimizing patient non-adherence.

1.2.3.2.LiCO physiological side effects

Although LiCO remains a gold standard for BD treatment due to its success in decreasing BD symptoms, patients often discontinue LiCO treatment due to negative physiological side-effects observed in up to 90% of patients [5,17,99,103]. Li^+ adversely affects the kidneys, thyroid gland, and parathyroid glands, necessitating monitoring of these organ functions through periodic blood tests [98]. In short-term treatment, patients often experience some combination of polydipsia, polyuria, nausea, diarrhea, or tremors [104–106]. More severe side effects include hypothyroidism and hyperparathyroidism, with the possibility of renal failure occurring in long-term therapy. While Li^+ toxicity is a concern when the Li^+ level exceeds 1.2 mmol/L, it is essential to note that these side effects described below occur in the therapeutic range of treatment.

The most common physiological side effects associated with long-term LiCO affect the thyroid gland and parathyroid gland to cause hypothyroidism and hyperparathyroidism, respectively[17,107]. Hypothyroidism occurs in approximately 20% of people taking Li^+, with symptoms including lethargy, depression, weight gain, and mental slowing. It is suspected to be caused by inhibiting thyroid hormone synthesis and release and reductions in glandular iodine trapping. Kraszewska et al. (2015) found that the concentration of the thyroid-stimulating hormone and the volume of the thyroid gland was significantly higher in patients receiving Li^+[98,106]. Interestingly, hypothyroidism is more common in women in both control and Li^+-treated groups,

suggesting women are at risk of developing hypothyroidism even before beginning Li^+ therapy. Less commonly, LiCO induces hyperparathyroidism by elevating calcium reabsorption in the kidney and increasing the release of parathyroid hormone from the parathyroid gland. The main symptoms of hyperparathyroidism include weakness, fatigue, and renal insufficiency. While hypothyroidism and hyperparathyroidism can be managed through hormone replacement, the symptoms are uncomfortable and contribute to patient non-compliance.

As high concentrations of LiCO are necessary to overcome the limitations on Li^+ entry to the CNS, up to 70% of patients prescribed LiCO often experience adverse side effects to the kidneys, including excessive urination and thirst, polydipsia, vasopressin resistance, and, in extreme cases, renal failure[108,109]. LiCO profoundly affects renal function because Li^+ is absorbed in the gastrointestinal tract, filtered by the glomeruli, and absorbed in the kidney's proximal tubule [109]. The adverse side effects LiCO has on the kidney have been extensively studied, with many studies concluding that Li^+ interferes with antidiuretic hormone release by interfering with the capacity of the cortical portion of the collecting tubule in the kidneys. The consequences of increased antidiuretic hormone release reduce the capacity of the kidneys and lead to excessive urination and thirst. The adverse effects LiCO has on the kidneys are often treated by monitoring plasma Li^+ levels to ensure the lowest possible dose that ensures efficacy; however, a lower dose may not alleviate manic and depression symptoms as effectively.

1.2.3.3.LiCO cognitive side effects

It is difficult to measure the role side effects, especially complaints of cognitive dulling, have in Li^+ nonadherence because the information is mainly self-reported[17,103]. However, Li^+ prescription has declined, suggesting patients stop taking Li^+ due to the side effects experienced while taking this medication [17,98,110,111]. The literature on LiCO treatment's effects on cognition, such as psychomotor speed and memory, is unclear and contradictory; however, reports of cognitive dulling have followed LiCO treatment since early LiCO studies. It is difficult to determine the effect Li^+ has on cognition because there is no ideal methodology to determine the effects of LiCO on cognition definitively. Specifically, the comparison between LiCO-treated BD patients and healthy controls is misleading because those with BD may respond differently than their control counterparts. Due to the lack of methodology for investigating LiCO's cognitive impact, it is not easy to make a definitive conclusion about the effects LiCO has on cognition.

While measuring the subjective effects on cognitive dulling is complex many studies compare cognitive functioning in LiCO-treated BD patients and regular volunteers[103]. In addition to complaints of cognitive dulling, LiCO has been reported to cause various cognitive deficits, including attention, concentration, memory, and psychomotor speed [103,112,113]. On the other hand, LiCO has been reported to enhance learning and memory, likely due to the neuroprotective qualities of Li^{+}[97,98,103,114,115]. In an extensive analysis of 12 LiCO studies, Wingo et al. (2009) found that Li^{+} only negatively affected cognition in verbal learning and memory [116]. Recently, Bersani et al. (2016) and Burdick et al. (2016) found that there was no difference in cognition in groups taking LiCO and those that were not, which signifies LiCO does not impair cognition [111,117]. In summary, the effects LiCO has on cognition contradict, suggesting that the effects LiCO has on cognition vary between patients or that there is no ideal methodology to determine the effect LiCO has on cognition.

1.2.4. Lithium Orotate

LiOr is an alternative treatment suggested to possess superior uptake properties compared to LiCO, which may reduce dosing requirements and lessen cognitive side effects in Li^{+} therapy [7,16]. In the 1970s, Hans Nieper was of the earliest supporters of the superior transport mechanisms of orotic acid conjugates compared to other conjugate counterparts [18]. In support of the superior potency of orotate compounds, Kling *et al.* (1978) observed greater concentrations of brain Li^{+} in the brain when administered as an orotate instead of a carbonate compound[6]. Despite the early success of LiOr in animal and clinical trials, research was halted following concerns raised in 1979 by Smith *et al*. about potential adverse effects on kidney function, and only recently, interest in LiOr as a treatment in BD has re-sparked. In connection to the superior potency of orotate compounds, , Pacholoko and Bekar (2023) observed lower doses of LiOr blocked hyperlocomotion in an AIH model, suggesting the potency and efficiency of LiOr are superior to LiCl and LiCO[7].

1.2.4.1.LiOr and LiCO are differentially transported across cell membranes.

LiOr is a mineral orotate compound that, unlike other lithium salt counterparts, does not dissociate in solution and is directly transported by OATPs to metabolically active tissues[7] . Pacholoko and Bekar (2023) observed distinct transport and dissociation characteristics between LiOr, LiCl and LiCO, indicating the direct transport of orotates explans the superior potency of LiOr compared to other Li^+ compounds [7].

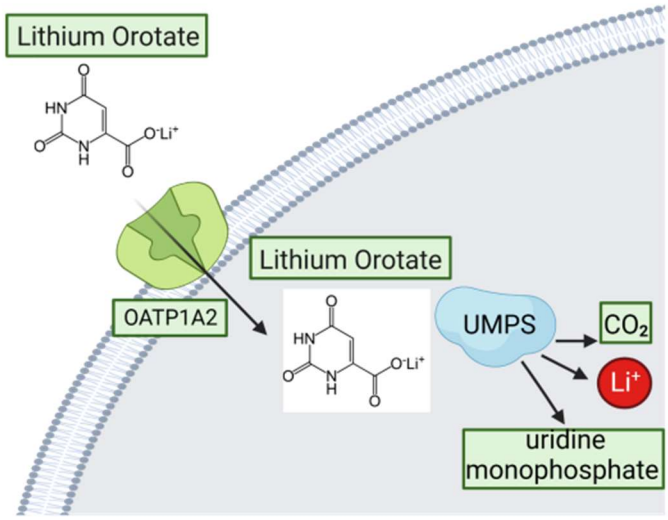

Figure 1.7. LiOr is transported across cellular membranes as a compound through organic anion-transporting polypeptides (OATS). Unlike LiCO and LiCl, which dissociates readily in solution, LiOr remains in its non-dissociated state post- administration. LiOr utilizes OATPs for passage across cellular membranes and uridine monophosphate synthase (UMPS) for intracellular dissociation . Created with BioRender.com.

Pacholko and Bekar (2023) provided evidence that LiOr utilizes OATS for intracellular transport and uridine monophosphate synthase (UMPS) for intracellular dissociation (Fig. 1.7), suggesting an alternative transport mechanism than sodium transporters could increase the ease with which LiOr crosses biological barriers to enable the direct delivery of Li^+ to intracellular target sites[7]. In summary, if LiOr demonstrated therapeutic potential at reduced doses relative to other Li^+ salts due to the direct transport of orotates, than these reduced dosing requirements may lesson cognitive side effects and relieve long-term toxicity concerns [6,16,18–20].

1.3. Hippocampal Synaptic Plasticity

The hippocampus is located within the medial temporal lobe of the brain and is widely regarded to be involved in learning and memory[118–120]. Hippocampal synaptic plasticity is suspected to be involved in many pathways in the CNS, including encoding and retrieval of memory, and is considered central to episodic and semantic long-term memory[121]. Due to its simple, laminar structure of neuronal pathways, the hippocampus has long been used as a model of synaptic plasticity mechanisms, such as long-term potentiation (LTP) and long-term depression (LTD). The hippocampus is comprised of several distinct regions, including the CA1, CA2, and CA3, where synaptic plasticity can be induced by stimulating along the by stimulating Schaffer

collateral of the hippocampus, which consists of a thick band of pyramidal cells in the CA1 region [121]. Research of hippocampal synaptic plasticity is motivated by the idea that long-term synaptic plasticity is the synaptic mechanism responsible for processing, encoding, and storing information [118,122,123].

1.3.2. Hippocampal Physiology

The simple trisynaptic loop of neurons and neuronal pathways in the hippocampus makes *in vivo* extracellular recordings the standard method to study cellular plasticity mechanisms[121,124,125]. The hippocampus is a bulb-like shaped structure embedded within the medial temporal lobe of the brain consisting of three subdivisions: CA3, CA2, and CA1[120]. The other region of the hippocampal formation is a group of brain areas consisting of the dentate gyrus, subiculum, presubiculum, parasubiculum, and entorhinal cortex (Fig. 1.8) [120]. Using electrophysiology, synaptic plasticity mechanisms can be induced by stimulating along the Schaffer collateral comprised of axons from the pyramidal cells in the CA3 and recording from any pyramidal cells in the CA1[118,119,121].

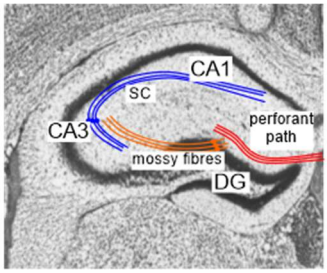

Figure 1.8.Hippocampal physiology. The hippocampus is a bulb-like shaped structure consisting of 3 subdivisions: CA3, CA2, and CA1. The primary input to the hippocampus is carried from the entorhinal cortex by axons of the perforant path to granule cells of the dentate gyrus (DG). In turn, the mossy fibres of granule cells of the dentate gyrus project to the proximal apical dendrites of the CA3 pyramidal cells, which project along the Schaffer collateral (SC) to CA1 pyramidal cells.

1.3.3. Long-term hippocampal synaptic plasticity

Synaptic plasticity is observed in the CNS, where activity-dependent changes in neuronal strength in response to neuronal activity and many other influences alter synaptic behaviour patterns[118,122]. Synaptic changes can arise from plasticity mechanisms ranging from milliseconds to minutes and, in some cases, even several years [126]. Most short-term synaptic plasticity mechanisms alter the amount of neurotransmitter release from presynaptic terminals in response to an action potential, causing a quick modification in synaptic transmission [126]. In contrast, long-term synaptic plasticity mechanisms are responsible for brain function changes that persist for 30 minutes or longer [126].

Long-term synaptic plasticity has been identified within the mammalian brain, in which patterns of synaptic activity improve efficiency, known as long-term potentiation (LTP), or lower synaptic strength, known as long-term depression (LTD) [126,127]. As LTP and LTD are the only mechanisms in the CNS known for persistent regulation of synaptic strength, long-term synaptic

plasticity is suspected to be the synaptic mechanism responsible for processing, encoding, and storing information [118,122,123]. In the hippocampus, long-term synaptic plasticity is believed to have a critical role in the refinement of neuronal circuits during development, learning, and memory in the mature CNS [118,122]. This theory is supported by the combination of activity-dependent synaptic plasticity being a prominent feature of the hippocampus and lesions in the hippocampus preventing the formation of episodic memories [118].

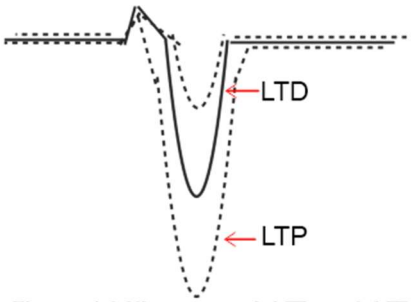

Figure 1.9.Hippocampal LTP and LTD. Hippocampal LTP can be induced using a high-frequency, theta-burst stimulation (TBS) protocol to the Schaffer collateral. LTP is observed as an increase to the post-synaptic response that is maintained for at least 30 minutes. LTD can be induced using a repetitive, low-frequency stimulation of 900 pulses at 1-3 Hz to the Schaffer collateral. This low-frequency stimulation protocol induces LTD, which is observed as a decrease in the post-synaptic response maintained for at least 30 minutes

Much research into synaptic plasticity concerns the role LTP has in learning and memory, with most of the experimental work focusing on NMDA receptor-dependent forms of LTP [118,122,123]. However, it would be an oversimplification to state that LTP is the synaptic mechanism responsible for memory. Instead, the role LTP has in memory has moved from a simple hypothesis, in which induction of LTP in the hippocampus requires synchronous presynaptic activity and postsynaptic activity, as described by Hebb's rule, to a more complex and dynamic idea of synaptic plasticity and memory [122]. In short, the synaptic plasticity and memory hypothesis is rooted in the idea that activity-dependent synaptic plasticity occurs at the appropriate synapses for memory formation [122].

1.3.3.1. Long-term potentiation

Hippocampal LTP can be induced using a high-frequency, theta-burst stimulation (TBS) protocol that mimics complete-spike discharges of pyramidal neurons and rhythmic modulation of excitability observed in the hippocampus. Using extracellular recordings, LTP is observed in CA1 synapses of rodents when brief bursts of high-frequency stimulation at 100 Hz are applied to the Schaffer collateral in a theta rhythm (5-7 Hz) [128–132]. This intense stimulation protocol induces LTP, which is observed as an increase in the post-synaptic response that is maintained for at least 30 minutes (Fig. 1.9). Induction of LTP in the hippocampus requires synchronous presynaptic activity and postsynaptic activity, as described by Hebb's rule, allowing an influx of calcium ions to pass through NMDA receptors to trigger biochemical changes that improve the efficiency of active synapses [129].

1.3.3.2.Long-term depression

While LTD has not been as extensively studied as LTP in the hippocampus, LTD can be induced using repetitive, low-frequency stimulation (LFS) of 900 pulses at 1-3 Hz along the Schaffer collateral[126,127,133,134]. This LFS protocol induces LTD, which is observed as a decrease in the post-synaptic response that is maintained for at least 30 minutes (Fig. 1.9) [134]. Unlike LTP, Kirkwood and Bear (1994) concluded that LTD is dependent on the frequency of stimulation given to the hippocampus because they observed 900 pulses at 1-5 Hz causing LTD, 900 pulses at 10 Hz caused no net effect, and 50 Hz caused LTP [135]. The differential effect varying frequencies have on synaptic plasticity supports the model that the voltage-dependent NMDA receptor channel converts the correlation of presynaptic and postsynaptic activity into a graded postsynaptic calcium signal. This calcium signal triggers LTP when it exceeds some critical value, known as the modification threshold, and triggers LTD when it falls below this level [135].

1.3.4. Long-term synaptic plasticity mechanisms

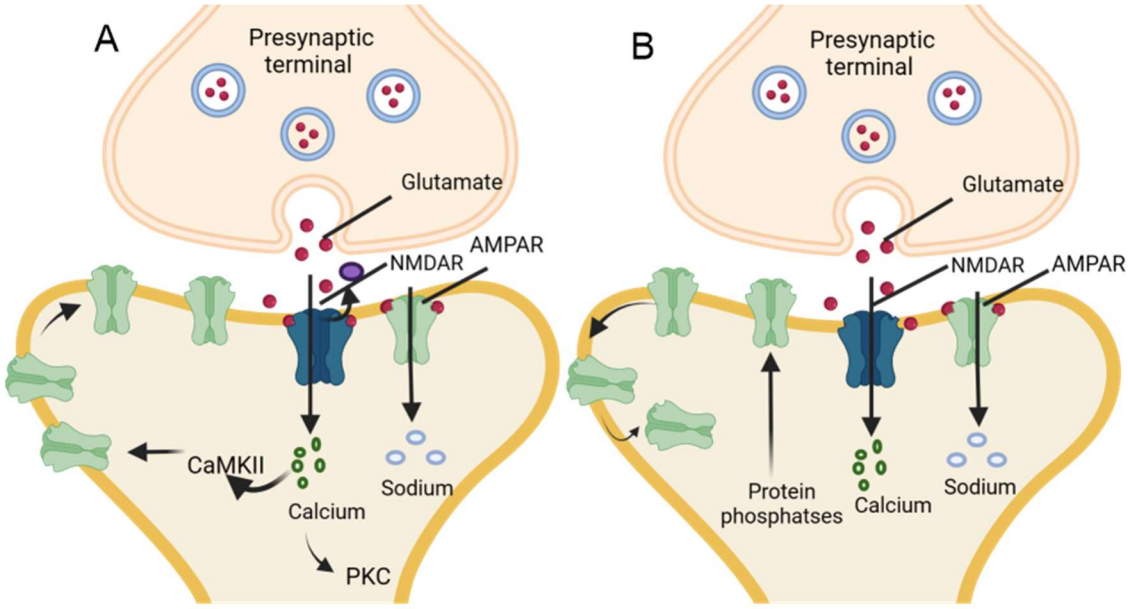

Figure 1.10.Long-term synaptic plasticity mechanisms. A) TBS-induced LTP at CA1 synapses is generally believed to be induced by a rapid increase in post-synaptic calcium through activated NMDA receptors and voltage-gated calcium receptors, which activate CaMKII in post-synaptic neurons to increase AMPA) receptor in the post-synaptic membrane. The increase in AMPA receptors increases the excitatory synaptic response in the CNS. **B)** LTD is generally believed to be induced by a slower influx of calcium through activated NMDA receptors to activate protein phosphatases that decrease AMPA receptor density in post-synaptic neurons. Created with BioRender.com.

1.3.4.1. NMDA-dependent synaptic plasticity

The primary forms of LTP and LTD are triggered by synaptic changes to NMDA receptors [86,136,137]. Synapses undergo a form of bidirectional plasticity where different patterns of synaptic activation can result in NMDA receptor signals that cause either LTP or LTD [36,138]. NMDA receptors are well established to abide by Hebb's rule because they require simultaneous activation of the membrane potential to relieve the voltage-dependent membrane block and binding of both glycine and glutamate to be activated[139].

Synaptic plasticity at CA1 synapses is induced by changes to postsynaptic intracellular calcium concentrations through NMDA receptors and voltage-dependent calcium receptor channels [86] First, NMDA activation causes an increase in postsynaptic calcium that activates calcium-dependent kinases and phosphatases that regulate synaptic plasticity [136,140,141]. Hippocampal synapses can undergo a form of bidirectional plasticity where different patterns of synaptic activation result in calcium-mediated kinases and phosphatases that mediate either LTP or LTD [36,138]. For example, TBS-induced LTP at CA1 synapses is generally believed to be induced

by a rapid increase in post-synaptic calcium through NMDA receptors and voltage-gated calcium receptors, which activate CaMKII in post-synaptic neurons to increase α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor density (Fig. 1.10A). In contrast, LTD is generally believed to be induced by a slower influx of calcium through NMDA receptors to activate protein phosphatases that decrease AMPA receptor density in post-synaptic neurons (Fig. 1.10B). The cellular processes regulating NMDAR-dependent LTP and LTD remain an essential target for understanding learning and memory's cellular activity.

1.3.4.2.AMPA receptors in synaptic plasticity

The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor is a ligand-gated ion channel that mediates most excitatory synaptic responses in the CNS [142]. Following NMDA receptor activation, the insertion or deletion of AMPA receptors into the post-synaptic membrane is an important mechanism that mediates synaptic plasticity. In this mechanism, NMDA receptors are activated following sufficient depolarization to displace the magnesium block, which causes an influx of calcium that activates downstream calcium-dependent kinases and phosphatases that regulate synaptic plasticity [136,140,141]. These downstream messengers, including CaMKII and PKA, promote the trafficking of AMPA receptors to the postsynaptic membrane [143]. The increase in AMPA receptors increases the excitatory synaptic response in the CNS, which induces LTP (Fig. 1.10A) [143,144]. In contrast, LTD is induced following a decrease in calcium influx that activates protein phosphatases that internalize AMPA receptors, lowering the excitatory postsynaptic response (Fig. 1.10B).

1.3.4.3.GSK3β is a key regulator of synaptic plasticity.

GSK-3β is thought to be involved in mediating NMDAR-dependent LTP and LTD because it is highly enriched in the brain, constitutively active, and synaptic changes can be inhibited upstream by regulators, including AKT and PKA, that reduce GSK-3β activity. Regulated by the phosphorylation status of Ser9 and tyr216 (see 1.1.2.2.), GSK3β is considered a synaptic plasticity mediator because increased activity of GSK3β induces LTD and decreased activity of GSK3β increases the likelihood of inducing LTP (Fig. 1.11). In the rat hippocampus, induction of NMDAR-mediated LTD resulted in further activation of GSK3β through dephosphorylation, while induction of LTP resulted in the downregulation of GSK3β through phosphorylation [86,145]. Collingridge et al. (2010) observed that NMDA receptor-dependent LTP inhibits the activity of GSK3β and determined that GSK-3β is required for NMDA receptor LTD [146].Similarly, Peineau et al. (2008) observed that an increased expression of GSK-3β had an inhibitory effect on LTP, and the activation of GSK-3β is involved in NMDA receptor-dependent LTD [86]. In a later paper, they found that inhibition of GSK-3β prevented the induction of NMDA receptor-dependent LTD [86]. These findings suggest that LTP and LTD regulate each other, with GSK3β acting as the molecular switch.

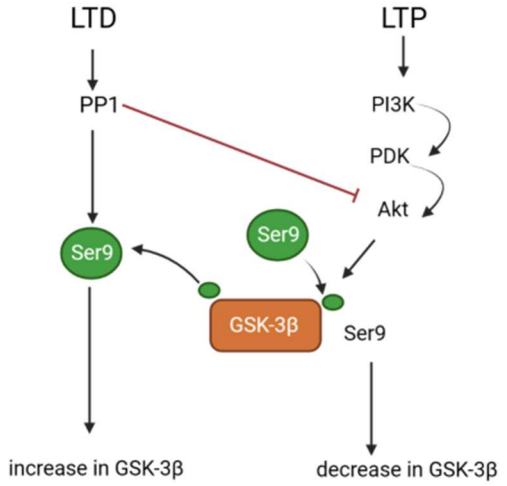

Figure 1.11. GSK3β regulates LTP and LTD. GSK3β is considered a synaptic plasticity mediator because increased activity of GSK3β induces LTD and decreased activity of GSK3β increases the likelihood of inducing LTP.GSK3β is regulated by phosphorylation status of Ser9, where phosphorylation of Ser9 by the Akt pathway decreases activity and dephosporylation of Ser9 through protein phosphatase 1 (PP1) increases acitivity of GSK3β. Pp1 actively inhibits the Akt pathway, indicating LTP and LTD regulate each other, with GSK-3β acting as the molecular switch

1.3.5. Lithium alters Synaptic plasticity.

1.3.5.1.Lithium inhibits LTD through GSK3β phosphorylation.

LiCO inhibits several enzymes in secondary messenger pathways to exert its therapeutic effects, but the exact mechanism of action remains largely unknown [68,147–149]. LiCO is observed to inhibit GSK3β, which is a multifunctional serine/threonine kinase thought to mediate to NMDA receptor-dependent synaptic plasticity (see 1.2.1.2) [86,146,147]. GSK-3β is thought to be

involved in mediating NMDA receptor-dependent LTP and LTD because it is highly enriched in the brain, constitutively active, and synaptic changes can be inhibited upstream by regulators that reduce GSK-3β activity. GSK3β is considered a synaptic plasticity mediator because increased expression induces LTD, and decreased activity increases the likelihood of inducing LTP. In the rat hippocampus, induction of NMDAR-mediated LTD resulted in further activation of GSK3β through dephosphorylation, while induction of LTP resulted in the downregulation of GSK3β through phosphorylation [86,145]. Recent findings have implicated Li^+ as a GSK3β inhibitor through competitively binding Mg^{2+} to decrease the expression of GSK3β [150].

The effect LiCO has on LTP is not as researched as its effect on LTD, but Li^+'s inhibitory effect on GSK3β is well documented in the literature [15,33,60,86,151,152]. Both Zhu et al. (2007) and Hooper et al. (2007) performed similar brain surgery experiments on rats where they placed stimulating and recording electrodes in the CA3 region of the hippocampus to record changes in LTP following Li^+ treatment. They found that LTP deficits are similarly rescued following treatment with Li^+ and by the downregulation of GSK3β in transgenic mice, indicating GSK3β plays a pivotal role in the mediation of synaptic plasticity [145,153]. Although the exact mechanism of action of LiCO remains largely unknown, the inhibition of GSK3β and the disruption of myo-inositol is thought to be involved in Li^+'s effects on stabilizing manic and depressive episodes associated with BD[33,154].

1.3.5.2.Lithium may mediate LTP through changes to the phosphoinositol cycle.

The PI cycle is an intracellular cycle that regulates many cellular functions through secondary messenger functions that ultimately activate downstream signalling pathways where IP_3 ultimately increases free cytosolic calcium, and DAG activates PKC to affect cholinergic, noradrenergic, serotonergic, and other neurotransmitter systems[57]. Li^+ inhibits key enzymes in the PI cycle through the displacement of the magnesium cofactor, which ultimately leads to the decrease in free cellular myo-inositol and disrupts secondary messenger pathways derived from PIP_2 (see section 1.2.1.1.) [61,62]. The literature on the effect a Li^+-mediated decrease in intracellular myo-inositol has on synaptic plasticity is nonexistent; however, IP_3-mediated changes in post-synaptic calcium are believed to be involved in synaptic plasticity. The IP_3 receptor acts as an IP_3-gated calcium release channel in a variety of cells, indicating that the PI cycle may be involved in synaptic plasticity. Recently, LTP suppression was attenuated in the mutant mice lacking the IP_3

receptor compared to the wild-type mice, suggesting the PP cycle mediates the direction of LTP expression.

1.3.5.3.Lithium may alter synaptic plasticity through NMDA receptors.

While the effects NMDA receptors have on Li^+-mediated changes to synaptic plasticity are unknown, Li^+ is observed to decrease the phosphorylation of NMDA receptors. The activity of NMDA receptors is regulated by interactions between NR2A subunits, Src-family tyrosine kinases, Fyn and PSD-95 that form a ternary complex that facilitates NR2A tyrosine phosphorylation [73]. Li^+ is observed to cause downregulation of NMDA receptor expression and signalling through inhibition of NR2A tyrosine phosphorylation and its interactions with Src and Fyn mediated by PSD-95 [73,75–78]. Given that NMDA receptors mediate synaptic plasticity and that Li^+ down-regulates NMDA receptors, we hypothesize that Li^+ may interact with NMDA receptors to alter NMDA-dependent synaptic plasticity [73,76,136,155].

1.4. Hypotheses and Objectives

LiCO is a key treatment option for preventing mood episodes associated with BD. Unfortunately, patients often discontinue LiCO treatment because of the negative side-effects associated with LiCO's therapeutic range. LiOr is an alternative treatment observed to yield higher concentrations of Li^+ in the brain and possesses superior uptake properties compared to LiCO. This may reduce dosing requirements and lessen side-effects typically observed with LiCO. The overall goal of my master's project is to compare the effects therapeutic concentrations of LiOr and LiCl have on long-term synaptic plasticity. ***I hypothesize that…***

1. As LiOr yields higher brain Li^+ than LiCO due to different transport mechanisms, LiOr will be more potent than LiCl.
2. As LiOr is only liberated intracellularly distant from the cell membrane, LiOr will selectively inhibit GSK3β to facilitate LTP, while LiCl acts closer to the membrane on NMDA receptors and the phosphatidylinositol pathway.

The project objectives are to **1)** compare the effect therapeutically relevant doses of low (0.2 and 0.4 mM) and high (0.6, 0.8, 1.0 mM) concentrations of LiCl and LiOr have on LTP and LTD. **2)** determine the effect LiOr and LiCl have on the phosphatidylinositol pathway and GSK3β during synaptic plasticity. **3)** determine the effect LiOr and LiCl have on NMDA-mediated currents.

Hypothesis: As LiOr yields higher brain Li^+ than LiCO due to different transport mechanisms and is only liberated intracellularly distant from the cell membrane, we hypothesize that **1)** LiOr will be more potent than LiCl and **2)** LiOr will selectively inhibit GSK3β to facilitate LTP while LiCl acts closer to the membrane on NMDA receptors and the phosphatidylinositol pathway.

2.0 Materials and Methods

2.1 Slice Preparation

The experiments described in this paper were performed on transverse slices prepared from the hippocampus of 40–70-day old male C57BL/6 mice. The mice were housed two-to-four per cage and kept on a 12-hour light/dark program. Single mice were never left in a cage for more than a day to avoid stress. The mice were anesthetized with isoflurane and then sacrificed by decapitation. The brain was quickly removed and placed in ice-cold artificial cerebral spinal fluid (aCSF) consisting of (in mM)): 130 NaCl, 3 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 2 $MgSO_4$, 0.1 Na-ascorbate, 24 $NaHCO_3$, 10 dextrose, and 1 lactate. After cooling, the cerebellum and olfactory bulb were removed to reveal a transverse brain slice containing the hippocampus. The brain was sliced at the mid-line, glued to a plate, and inserted into the stage of the vibratome (Leica VT1200), which contained cold aCSF bubbled with 95% oxygen 5% CO_2. The stage was surrounded by an ice-water solution to maintain temperature. The brain sliced coronally to a thickness of 300 μM. The first slices were not taken to ensure the proper thickness of the slices. Slices were placed in a chamber containing the room temperature aCSF bubbling with 95% oxygen and 5% CO_2 for two hours before experiments began to ensure recovery. Experiments were done up to six hours after slicing or until slices showed signs of death (maximum amplitude < 2.0 mV). All experiments were performed in accordance with the guidelines of the Canadian Council of Animal Care and approved by the University of Saskatchewan Committee on Animal Care and Supply.

2.2 Electrophysiology

Slices were placed in a perfusion chamber (~2ml volume) containing aCSF bubbled with 95% O_2 and 5% CO_2. The bath was perfused at a rate of ~4 mL/min at 32°C for LTP experiments and room temperature for LTD experiments. A Zeiss Axioscope upright microscope was used to visualize the slices and position electrodes. Recording electrodes were pulled as needed using a vertical Narishige PC-10 two-step puller, and a new recording electrode filled with 0.9% saline was placed every day before experiments started. The resistance of the electrodes was between 4-6 MΩ. The recording electrode was placed in the stratum radiatum of the CA1 region of the hippocampus to record from a population of pyramidal synapses. The stimulating electrode was placed along the Schaffer collateral compared of axons from pyramidal cells in the CA3 to stimulate the Schaffer collateral using a differential amplifier (DP311; Warner Instruments)

connected to Digidata 1440A (Molecular Devices). The excitatory postsynaptic potentials (EPSP) caused by stimulation were captured at 2 KHz using a high-pass filter of 1 Hz and a low-pass filter of 300 Hz. Using PClamp 10.2 software, we found a maximum EPSP using a 50 ms paired-pulse protocol by increasing stimulation until the EPSP no longer changed. We only used slices that had a maximum EPSP > 2.0 mV to ensure consistent slice health. After establishing the maximum EPSP, the amplitude was reduced to 30% of the maximum EPSP and allowed to rest for one minute before the baseline was recorded. The baseline was recorded using a 20s paired-pulse protocol where the first pulse (P1) was observed to be relatively stable for ten sweeps. After the baseline was established, the LTP or LTD stimulation protocol began. LTP was induced by theta burst stimulations (TBS) (8 bursts of 4 high-frequency pulses at 5 Hz repeated 3 times, 60 seconds apart) at 32℃. LTD was induced by 1 Hz stimulation for 15 minutes at room temperature. After the LTP or LTD stimulation, the EPSP was recorded for 30 minutes using a 20s paired-pulse protocol.

2.3 Drugs

We used basic pharmacology to determine the effects LiCl and LiOr had secondary signalling mechanisms and NMDA receptors. LiCl was purchased as a 8 M solution from Sigma-Aldrich (ON,CA) and diluted in aCSF to therapeutic serum Li^+ ranges. LiOr was created by combining lithium hydroxide (Sigma-Aldrich; ON, CA), and orotic acid (Sigma-Aldrich; ON, CA) in a 1:1 M ratio in distilled water before adding salts and sugars to make aCSF. The other drugs purchased from Sigma-Aldrich (ON, CA) include 6,7-dinitroquinoxaline-2,3-dione (DNQX) and myo-inositol. AZD2858 was purchased from MedChemExpress (NJ,USA), and D-(-)-2-Amino-5- phosphonopentanoic acid (D-AP5) was purchased from Cayman Chemicals (MI, USA). We compared the effects the phosphoinositol pathway had on Li^+-mediated effects on synaptic plasticity by incubating slices in 1 mM myo-inositol for 2-3 hours. We used basic pharmacology to determine the effects LiCl and LiOr had on GSK3β and NMDA receptors with 3.5 μM AZD2858, a non-specific GSK3B inhibitor, 5 μM DNQX to isolate NMDA currents, and 50 μM D-AP5 to inhibit NMDA currents. All drugs were mixed with otherwise normal aCSF and perfused over the slices into the bath chamber after a suitable baseline had been established.

2.4 Statistical Analyses

After recordings, field excitatory post synaptic potentials (fEPSPs) were analyzed using Clampex. In this application, the baseline, and the post-stimulation EPSPs were analyzed. The

baseline was established as 10 sweeps, where P1 amplitude was observed to be relatively stable. Drug effects were usually taken three or more sweeps after application to allow time for the drug to reach perfusate and bind receptors. To determine our drug effect, ten sweeps where P1 amplitude was maintained close to baseline were averaged. All data is expressed as the mean +/- standard error. Additionally, all data is expressed after being normalized to the baseline. To determine statistical significance two-way ANOVAs were performed on all experiments in GraphPad Prism. Additionally, we performed Tukey HSD post-hoc tests to evaluate the conditions under which any significant difference in our ANOVAs was found. Statistical significance was set at $p < 0.05$ for ANOVAs. GraphPad Prism and Excel were used for further analysis and the making of graphs.

3.0 RESULTS

3.1. An overview of hippocampal LTP and LTD

It has previously been established that non-therapeutically high Li^+ concentrations affect hippocampal synaptic plasticity (see section 1.3.4). LiCO inhibits several enzymes in secondary messenger pathways to exert its therapeutic effects, but the exact mechanism of action remains largely unknown [68,147–149]. LiCO is observed to inhibit GSK3β to mediate LTD and LTP, which is a multifunctional serine/threonine kinase thought to mediate NMDA receptor-dependent synaptic plasticity [86,146,147]. As Li^+ was previously shown to inhibit GSK3β, researchers found that induction of NMDAR-dependent LTD was inhibited by Li^+ because it decreased GSK3β activity (see section 1.3.4) [145,153]. In this research project, we decided to use Li^+'s inhibitory effect on LTD as a control to ensure Li^+ had a consistent effect with the results reported in the literature. In order to compare the dose-dependent effects LiCl and LiOr have on synaptic plasticity, we induced LTP and LTD in the mouse hippocampus by stimulating along the Schaffer collateral comprised of axons from the pyramidal cells in the CA3 and recording from a population of pyramidal synapses in the stratum radiatum of the CA1 following acute and long-term incubation of LiOr and LiCl. The stimulating electrode was placed in the CA1 region of the hippocampus, and the recording electrode was placed along the Schaffer collateral to measure the potential change in the voltage (Fig. 3.1A). LTP was induced by TBS (8 bursts at 5 Hz of 4 high-frequency pulses at 100 Hz repeated three times, 60 seconds apart) at 32℃ (Fig. 3.1B). LTD was induced using a LFS (1 Hz) for 15 minutes at room temperature (Fig. 1C). We measured the percent change in baseline amplitude following the LTP and LTD stimulation protocol using a 20-second paired-pulse protocol for 30 minutes following either LTP or LTD stimulation (Fig. 3.1D).

3.2. LiOr affects synaptic plasticity differently than LiCl.

3.2.1. Both LiCl and LiOr inhibit LTD

As previous studies showed that Li^+ inhibits LTD (non-therapeutically high concentrations), we chose to explore and characterize the effect therapeutically relevant brain Li^+ concentrations have on LTD (see 1.3.4). To achieve this, we placed a stimulating and recording electrode along the Schaffer Collateral in the mouse hippocampus to induce LTD using a LFS protocol (Fig. 3.1). We found that, although both LiOr and LiCl attenuate LTD, LiOr inhibits LTD

at lower concentrations than LiCl (interaction p = 0.0013) (Fig. 3.2). We observed that 0.2 mM and 0.4 mM LiOr completely reversed LTD, whereas corresponding concentrations of LiCl had no effect (Fig. 3.2A). Thus, LiOr is more potent than LiCl. We did not observe a difference between higher concentrations of LiCl and LiOr (Fig. 3.2B). These results show that therapeutically relevant Li^+ concentrations have an inhibitory effect on hippocampal LTD.

3.2.2. LiOr affects LTP differently than LiCl.

Our finding that LiOr and LiCl inhibited LTD at therapeutically relevant concentrations suggests that 0.2 mM – 1.0 mM LiCl and LiOr would also affect LTP. Next, we investigated the effects of therapeutically relevant Li^+ concentrations on LTP by inducing LTP along the Schaffer Collateral in the mouse hippocampus using a TBS protocol (Fig. 3.1). We observed an interaction between the form and concentration of Li^+ used (interaction $p < 0.0001$), supporting the notion that the two forms of Li^+ act differently on LTP. All concentrations of LiOr consistently increased LTP by approximately 25% from the control. In contrast, LiCl potentiated LTP at low concentration (0.6 mM) and inhibited LTP at high concentration (1.0 mM; Fig. 3.3). These differences suggest LiOr and LiCl mediate LTP using different cellular mechanism and further supports that LiOr is more potent than LiCl.

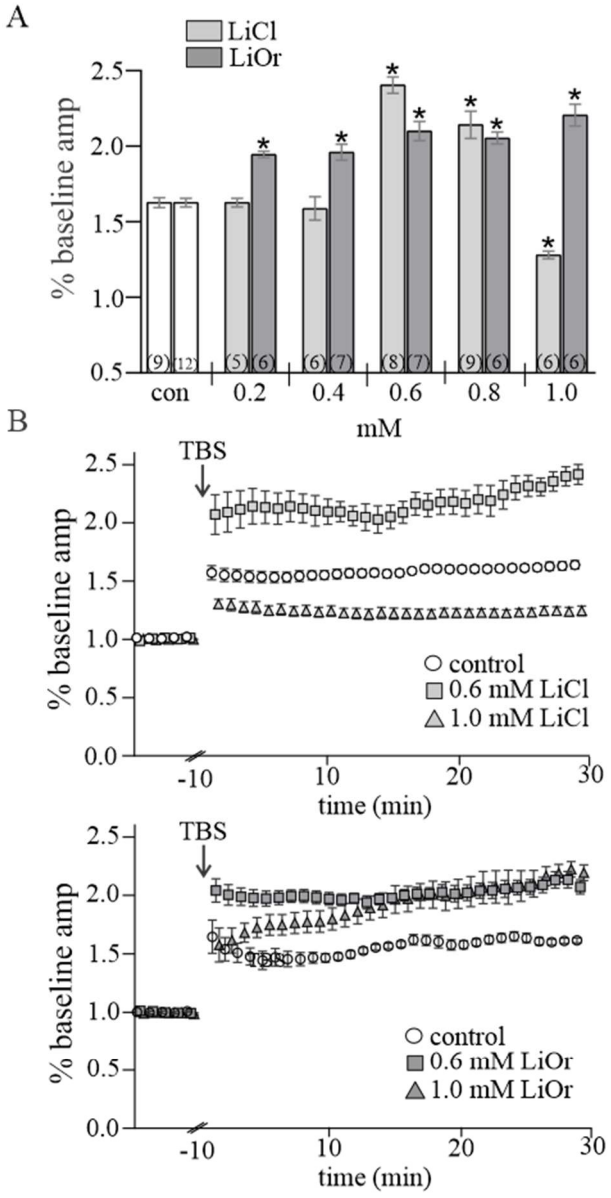

Figure 3.3. LiOr is more potent than therapeutic concentrations of LiCl and does not show inhibition of hippocampal LTP. A) Summary graph on the effects LiOr and LiCl have on LTP. Average pulse 1 amplitude (mV) during the last 10 minutes after a theta burst stimulation (TBS) (8 bursts at 5 Hz of 4 high-frequency pulses at 100 Hz repeated 3 times, 60 seconds apart) protocol was applied along the Schaffer collateral of male C57BL/6 mouse slices at 32°C. Slices were acutely treated with therapeutic LiCl and LiOr concentrations between 0.2-1.0 mM and compared to controls. All concentrations of LiOr increased LTP while LiCl had a biphasic effect. **B)** Effects 0.6 mM and 1.0 mM LiCl and LiOr had on LTP over time. Each recording was normalized to a baseline of 1 following LTP stimulation protocol with each bar representing SEM, n = 5-12. * LiCl or LiOr LTP compared to corresponding control <0.05 by two-way ANOVA with Tukey HSD post-hoc.

3.2.3. LiCl, but not LiOr, displays a time-dependent effect.

Given that BD patients typically take Li^+ for an extended period, we decided to determine if there is a change in LTP following a long-term incubation of slices in LiCl or LiOr. To assess this, we incubated brain slices in 0.6 mM and 1.0 mM LiOr and LiCl for 4-6 hours before experiments. We observed that 0.6 mM LiCl had a time-dependent interaction on LTP whereas 0.6 mM LiOr did not have a different effect between acute and long-term incubation (Li^+ x time interaction $p < 0.0001$) (Fig. 3.4A). Higher concentrations (1.0 mM) of LiCl and LiOr did not cause a time-dependent interaction on LTP (Li^+ x time interaction $p = 0.9444$) (Fig. 3.4B). Interestingly, long-term incubation of 0.6 mM LiCl had a similar effect on LTP as acute 1.0 mM LiCl whereas LiOr consistently increases LTP, suggesting LiOr is more potent than LiCl. Although the differences between acute and long-term incubation of LiCl and LiOr may be the effect of experimental design, it is essential to note that there is an interaction between acute and long-term incubation of LiCl but not LiOr. The time-dependent effects LiCl has on LTP provide further evidence that LiOr and LiCl affect synaptic plasticity using different mechanisms.

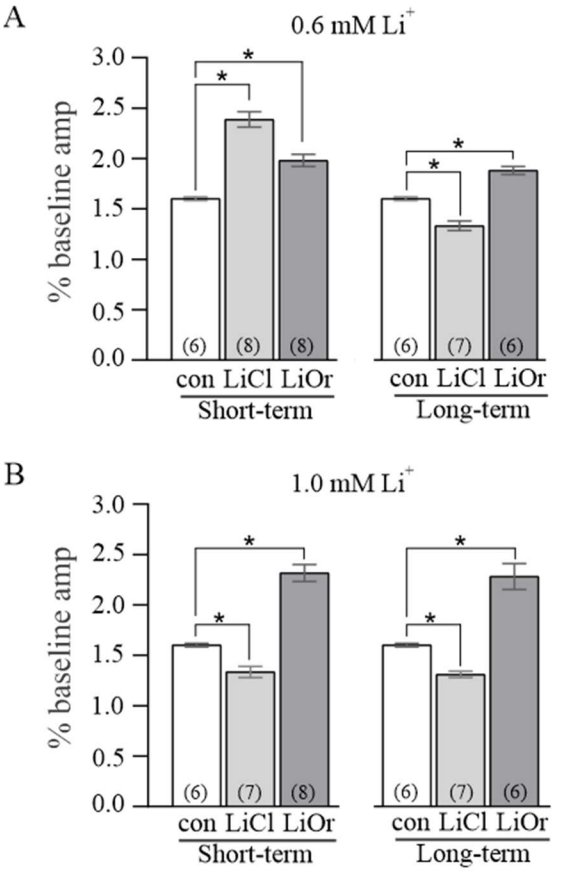

Figure 3.4. Long-term LiCl incubation effects LTP whereas LiOr consistently increases LTP. A) Summary graph on the effects acute and long-term incubation of 0.6 mM LiOr and LiCl have on LTP. Long-term LiCl incubation decreases the effect acute 0.6 mM LiCl has on LTP whereas acute and long-term LiOr incubation similarly affected LTP. **B)** Summary graph on the effects acute and long-term incubation of 1.0 mM LiOr and LiCl mediated effects on LTP. Long-term incubation of both 1.0 mM LiCl and LiOr were not different from the acute affects. Each graph displays average pulse 1 amplitude (mV) during last the 10 minutes after LTP stimulation was applied to the Schaffer collateral-CA1 synapse of male C57BL/6 mouse slices. Slices were incubated in 1 mM myoinositol and LiCl or LiOr for 4-6 hours prior to experiments. Each recording was normalized to baseline of 1 following LTP stimulation protocol with each bar representing SEM, n = 6-8. * compared to control <0.05 by two-way ANOVA with Tukey HSD post-hoc.

3.3. LiCl and LiOr mediate synaptic plasticity using different cellular mechanisms.

3.3.1. LiCl-mediated inhibition of LTP is inhibited by myo-inositol incubation whereas LiOr-mediated enhancement of LTP is occluded by GSK3B inhibition.

Given our observation that LiOr and LiCl have different effects on LTP, we next sought to assess the impact LiOr and LiCl have on GSK3β activity and phosphoinositol signalling. To achieve this, we used a GSK3β inhibitor, AZD2858, to assess the role LiCl and LiOr have on GSK3β inhibition during LTP. We incubated slices in 1 mM myo-inositol before acute Li^+ treatment to investigate the role LiCl and LiOr have on phosphoinositol signalling during LTP. It is important to note that AZD2858 increased LTP by 25% from control, indicating the importance GSK3β inhibition has on LTP induction. Additionally, we did not observe a difference between the control LTP with normal aCSF and the addition of 1 mM myo-inositol, suggesting the addition of myo-inositol does not disrupt cell signalling (Fig. 3.5). We observed an interaction between the form of Li^+ and drug used (interaction $p < 0.0001$), supporting the notion that the two forms of Li^+ use different cellular mechanisms to mediate LTP. The incubation of brain slices in myo-inositol inhibited the effects LiCl has on LTP, suggesting LiCl has a dose-dependent

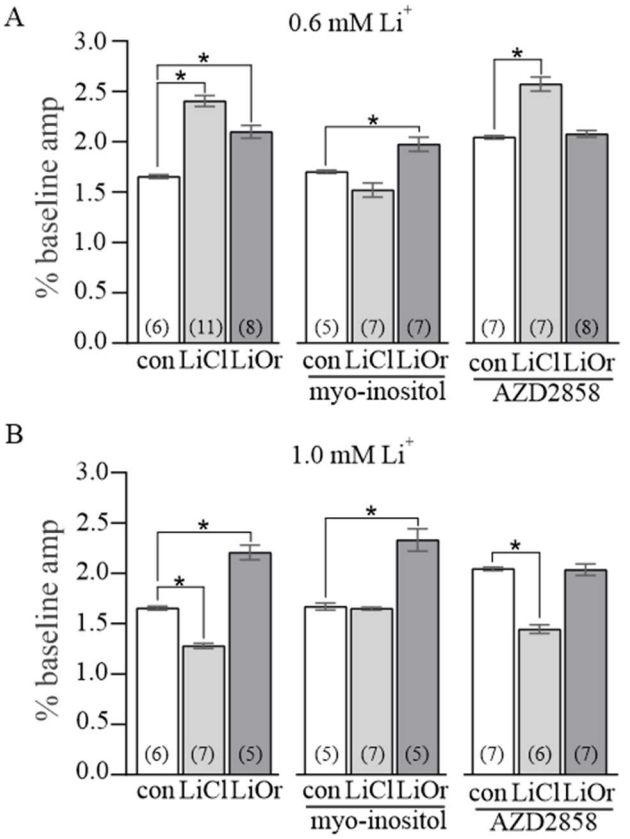

Figure 3.5. LiCl-mediated effects on LTP is inhibited by myo-inositol incubation whereas LiOr-mediated enhancement of LTP is occluded by GSK3β inhibition. A) Summary graph on the effects incubation of 1 mM myo-inositol and acute treatment with 3.5 μM AZD2858 has on 0.6 mM LiOr and LiCl mediated effects on LTP. Myo-inositol incubation decreased the effect 0.6 mM LiCl had on LTP to control levels, but had no effect on LiOr mediated effects on LTP. AZD2858 occluded LiOr-mediated enhancement, but had no effects on LiCl. **B)** Summary graph on the effects incubation of 1 mM myo-inositol had on 1.0 mM LiOr and LiCl mediated effects on LTP. Myo-inositol incubation increased the effect 1.0 mM LiCl had on LTP to control levels, but had no effect on LiOr mediated effects on LTP. AZD2858 occluded LiOr-mediated enhancement, but had no effects on LiCl. Each graph displays average pulse 1 amplitude (mV) during last the 10 minutes after a theta burst stimulation (TBS) (8 bursts at 5 Hz of 4 high-frequency pulses at 100 Hz repeated 3 times, 60 seconds apart) protocol was applied to the Schaffer collateral-CA1 synapse of male C57BL/6 mouse slices at 32˚C. Slices were incubated in 1 mM myo-inositol for 2-3 hours prior to experiments or acutely treated with AZD2858, a GSK3B inhibitor, before acute treatment with LiCl or LiOr. Each recording was normalized to baseline of 1 following LTP stimulation protocol with each bar representing SEM, n = 5-11. * compared to corresponding control <0.05 by two-way ANOVA with Tukey HSD post-hoc.

effect on the phosphatidylinositol pathway (Fig. 3.5). In contrast, LiOr-mediated enhancement is occluded by GSK3β inhibition, indicating LiOr consistently increased LTP through inhibition of GSK3β without affecting phosphoinositol signalling (Fig. 3.5). These experiments indicate LiCl and LiOr mediate synaptic plasticity using different secondary messenger pathways.

3.3.2. Myo-inositol incubation inhibits lithium-mediated attenuation of LTD.

Given that LiCl's effect on LTP can be disrupted with myo-inositol incubation, it would stand to reason that myo-inositol incubation will also reverse the effects LiCl has on LTD. To assess this, we incubated slices in 1 mM myo-inositol to investigate the role LiCl and LiOr have on phosphoinositol signalling during LTD. As expected, there is an interaction between the form of Li^+ used and myo-inositol (interaction $p < 0.0001$), supporting the notion that the two forms of Li^+ use different cellular mechanisms to mediate LTD. Specifically, the inhibitory effect LiCl has on LTD is occluded by incubating brain slices in myo-inositol, whereas the inhibitory effect LiOr has on LTD is not disrupted by myo-inositol incubation (Fig. 3.6). This suggests LiCl affects LTD by disrupting phosphoinositol signalling whereas LiOr does not mediate LTD through phosphoinositol signalling. This further proves that LiOr and LiCl mediate synaptic plasticity using different mechanisms.

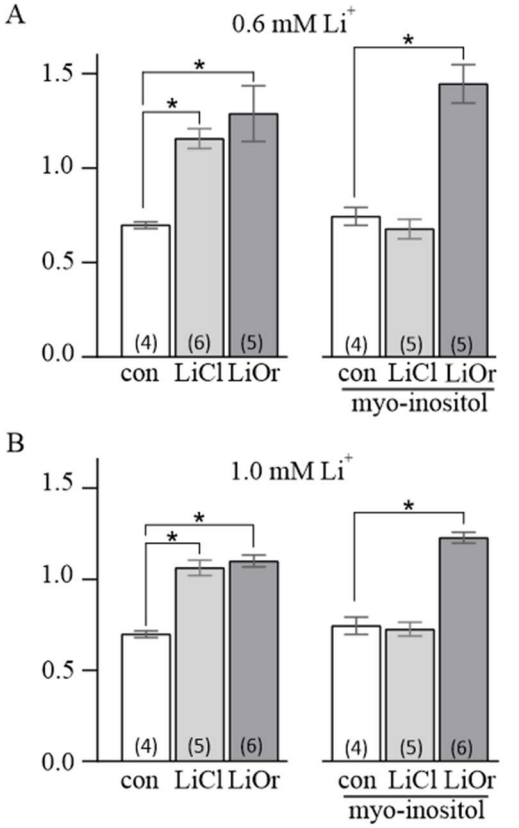

Figure 3.6. LiCl-mediated effects on LTD is inhibited by myo-inositol incubation. A) Summary graph on the effects incubation of 1 mM myo-inositol has on 0.6 mM LiCl and LiOr mediated effects on LTD. Myo-inositol incubation inhibited the effect 0.6 mM LiCl has on LTD, but had no effect on LiOr mediated effects on LTD. **B)** Summary graph on the effects incubation of 1 mM myo-inositol has on 1.0 mM LiCl and LiOr mediated effects on LTD. Myo-inositol incubation inhibited the effect 1.0 mM LiCl has on LTD, but had no effect on LiOr mediated effects on LTD. Each graph displays average pulse 1 amplitude (mV) during last the 10 minutes after a low frequency stimulation (LFS; 1 Hz) protocol was applied to the Schaffer collateral-CA1 synapse of male C57BL/6 mouse slices for 15 minutes at room temperature. Slices were incubated in 1 mM myo-inositol for 2-3 hours prior to experiments before acute treatment with LiCl or LiOr. Each recording was normalized to baseline of 1 following LTD stimulation protocol with each bar representing SEM, n = 4-6. * compared to corresponding control <0.05 by two-way ANOVA with Tukey HSD post-hoc.

3.4. LiCl, but not LiOr, disrupts NMDA-mediated potentials.

3.4.1. An overview of isolated NMDA-mediated potentials

As NMDA receptors mediate the primary forms of synaptic plasticity (see section 1.3.3), we can assume any changes to LTP are, in some way, mediated by NMDA receptors [86,136,137]. Li^+ is observed to influence NMDA receptor expression and signalling (see section 1.2.1.3), suggesting the effect LiOr and LiCl have on LTP are mediated by changes to NMDA receptors [73,75–78]. To quantify the role LiCl and LiOr have on NMDA receptors, we used 5 µM DNQX to isolate NMDA potentials in a 20s - pulse protocol along the Schaffer Collateral in the mouse hippocampus (Fig. 3.7). To ensure we were comparing isolated NMDA mediated potentials, we observed a complete block of synaptic potential following wash in of D-AP5, an NMDA receptor antagonist. We compared the effects 1.0 mM myo-inositol and 3.5 µM AZD2858 in combination with LiCl and LiOr have on isolated NMDA potentials.

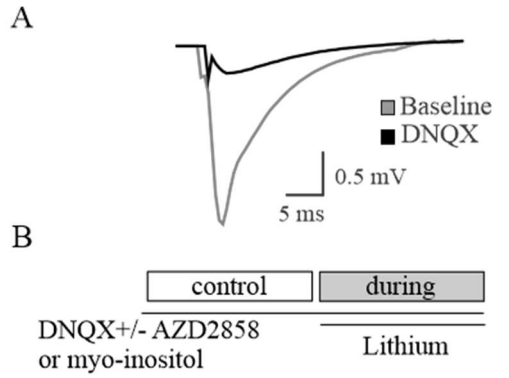

Figure 3.7. DNQX decreases pulse 1 amplitude. A) Example trace of the effect DNQX has on pulse 1 amplitude (mV). **B)** Experimental design. DNQX +/- 1 mM myoinositol or AZD2858 are in solution for the entirety of experiment whereas lithium is washed into solution.

3.4.2. The effects LiCl and LiOr have on LTP are replicated by changes to NMDA potentials.

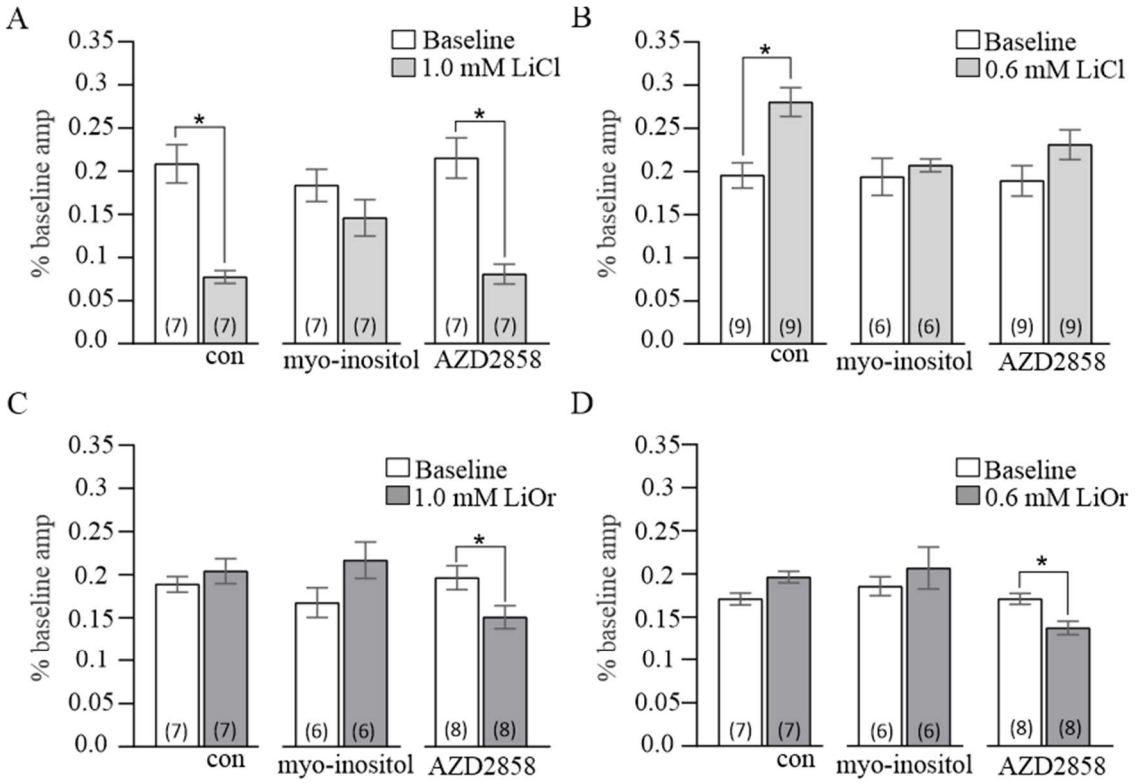

Figure 3.8. LiCl, but not LiOr, inhibits NMDA-mediated potentials. A-D) Average effect 5 μM DNQX 20s paired-pulse stimulation has before (control) and after brain slices were exposed to 1.0 mM and 0.6 mM LiOr and LiCl in the presence of 1 mM myo-inositol and 3.5 uM AZD2858. **A)** Myo-inositol, but not AZD2858, restores the decreased effect 1 mM LiCl has on NMDA mediated potentials. **B)** Neither myo-inositol nor AZD2858 alters the increased effect 0.6 mM LiCl has on NMDA mediated currents. **C)** AZD2858 decreases the effects 1 mM LiOr has on NMDA mediated potentials. **D)** AZD2858 decreases the effects 1 mM LiOr has on NMDA mediated potentials. Each recording was normalized to initial baseline prior to DNQX wash-in with each bar representing SEM, n = 6-10. * compared to control <0.05 by repeated measure two-way ANOVA with Tukey HSD post-hoc.

Similar to the trend we observed with LTP and LTD, LiCl and LiOr differentially effects NMDA-mediated potentials (Fig. 3.8). LiCl increases NMDA potentials at lower concentrations (0.6 mM) and decreases NMDA potentials at higher concentrations (1.0 mM). In contrast, both concentrations of LiOr similarly affect NMDA potential (Li^+ x concentration interaction $p = 0.001$) (Fig. 3.8). Given our observation that LiOr and LiCl have a different effect on NMDA receptors, we next sought to assess the impact LiOr and LiCl have on cellular signalling mechanisms connected to NMDA-receptor dependent synaptic plasticity. We compared the effects incubation of brain slices in myo-inositol and inhibition of GSK3β on the observed effects 0.6 mM and 1.0 mM LiOr and LiCl have on NMDA mediated potentials. The decrease 1.0 mM LiCl has on NMDA receptor potentials was inhibited following incubation with myo-inositol whereas AZD2858 did not have an effect compared to control (1.0 mM LiCl x drug interaction $p = 0.0109$) (Fig. 3.8A).

The increase 0.6 mM LiCl has on NMDA receptor potentials is occluded by the incubation of brain slices in myo-inositol whereas AZD2858 has no effect compared to control (0.6 mM LiCl x drug interaction $p = 0.0302$) (Fig. 3.8B). We did not observe an interaction between 1.0 mM or 0.6 mM LiOr and baseline, indicating LiOr does not directly alter NMDA receptors to mediate synaptic plasticity ($p = 0.9985$, and 0.9546, subsequently) (Fig. 3.8C and Fig. 3.8D). However, there was an interaction between both 1.0 mM and 0.6 mM LiOr, and the drug used, suggesting LiOr indirectly mediates NMDA receptors using secondary messenger pathways (LiOr x drug interaction $p = 0.0005$ and 0.0258 subsequently). Specifically, we observed an interaction between 1.0 mM and 0.6 mM LiOr and AZD2858, indicating LiOr may interact with GSK3β to affect NMDA receptors ($p = 0.05336$ and 0.05878, subsequently). In summary, these experiments suggest that LiCl and LiOr indirectly mediate NMDA receptors using different secondary messenger pathways.

4.0. Discussion

4.1. General discussion:

Despite evidence that LiOr increases brain Li^+ relative to LiCl or LiCO, research into LiOr in psychiatric applications has stopped due to fears of renal impairments if used at concentrations equivalent to LiCO (see section 1.2.3) [6,18,20]. Although concerns about LiOr as a substitute for LiCO to treat psychiatric conditions are valid, it is still important to note that LiOr exists at greater bioavailability in the brain than LiCl at lower concentrations [6,7,16,18]. Considering toxicity and other side effects associated with LiCO therapy, it would be beneficial to decrease the concentration of Li^+ while maintaining suitable Li^+ expression within the brain to decrease BD symptoms [17,68,95,156]. In brief, LiOr may represent an alternative treatment option for BD because it displays lower dosage requirements than LiCO, which may reduce any adverse side effects associated with LiCO[6,7,16,18–20].

This is the first study to assess the role therapeutically relevant concentrations of LiCl and LiOr have on synaptic plasticity using extracellular field recordings. To evaluate the efficiency and potency of LiOr and LiCl, we compared the effects identical concentrations of LiOr have to therapeutic concentrations of LiCl using a hippocampal LTP and LTD model. Although both LiOr and LiCl affect synaptic plasticity, LiOr inhibits LTD and increases LTP at lower concentrations than LiCl. Additionally, we found therapeutic LiCl concentrations demonstrate a dose-dependent response on synaptic plasticity, whereas LiOr consistently affects synaptic plasticity at both high and low concentrations. Given the more efficient cell transport mechanisms associated with orotate compounds (see section 1.2.3)[7,16,18,19], we suspect the potency differences are due to different cellular transport mechanisms between the two Li^+ compounds. The improved potency of LiOr may mitigate adverse effects observed on renal and thyroid health, which improves its safety relative to LiCO.

The potency differences between LiOr and LiCl suggest different pharmacokinetic and pharmacodynamic properties between the two Li^+ compounds. Given these differences, we proposed that LiOr and LiCl mediate synaptic plasticity using different secondary cellular mechanisms (see section 1.3.3). Specifically, we chose to focus on the PP cycle, NMDA receptors and GSK3β because they are inhibited by Li^+ and regulate synaptic plasticity (see sections 1.2.1

and 1.3.4)[68,148,149]. To assess LiCl and LiOr's effect on these secondary cellular mechanisms, we used basic pharmacology to determine the impact LiCl and LiOr had on GSK3β and phosphoinositol signalling. We determined that LiCl mediates LTP and LTD through the phosphatidylinositol pathway and NMDA receptors, whereas LiOr consistently increased LTP through inhibition of GSK3β. We suspect LiCl dissociates extracellularly and diffuses across the membrane through sodium channels and transporters, allowing Li+ ions from LiCl to alter the membrane-bound NMDA receptors and PP cycle. In contrast, LiOr utilizes OATPs for transport and UMPS for intracellular dissociation, leading to Li+ ions from LiOr to directly decreases GSK3β signalling. The differential effects of LiOr and LiCl on the PI cycle, GSK3β and NMDAR support our hypothesis that LiOr and LiCl regulate synaptic plasticity using different mechanisms.

Overall, this research project provides evidence that LiCl and LiOr have different pharmacokinetic and pharmacodynamic properties. Moreover, it confirms LiOr is more potent than LiCl as lower concentrations of LiOr altered LTP and LTD compared to LiCl. These reduced dosing requirements indicate the application of LiOr at lower doses than LiCO, which should dispel renal toxicity concerns raised by Smith *et al*. in 1979. Although further research is needed before LiOr replaces LiCO as a BD treatment, the improvements we have observed in potency displayed by LiOr in our LTP and LTD model suggest that the use of LiOr in BD may dispel compliance-disrupting side effects encountered during Li^+ therapy.

4.2. Contrasting the effects LiOr and LiCl have on synaptic plasticity.

4.2.1. LiOr has an effect on synaptic plasticity at lower doses than LiCl.

As LiOr may be an alternative treatment option for BD, we compared the effects therapeutic doses (ranging from 0.2 mM to 1.0 mM) of LiCl and comparable concentrations of LiOr have on synaptic plasticity. Although inspired by previous studies that found increased potency of LiOr compared to other lithium conjugates, this is the first study to assess the role therapeutically relevant concentrations of LiCl and LiOr have on synaptic plasticity using extracellular field recordings[6]. [6715,157,158]In line with potency differences observed in previous studies, we observed LiOr has a more potent effect on on LTP and LTD than LiCl [6,7,18]. This provides evidence that LiOr and LiCl affect synaptic plasticity using different mechanisms because if these Li^+ compounds had identical pharmacokinetic and pharmacodynamic properties, we would expect a similar effect on synaptic plasticity. These findings support our hypothesis that the

increased CNS penetrations of LiOr would increase its potency relative to LiCl on the effects of LTP and LTD.

Given that BD patients typically take LiCO for an extended period, we decided to determine if there is a change in the effects LiCl and LiOr have on LTP following a long-term incubation of slices in LiCl or LiOr [12,16,159]. Although the differences between acute and long-term incubation of LiCl and LiOr may be the effect of experimental design, it is important to note that LiCl, but not LiOr, has a time-dependent impact on synaptic plasticity. We observed that long-term incubation of 0.6 mM LiCl decreased LTP compared to the acute LiCl counterpart. This indicates that time allows more Li^+ ions to be transported into the cell to affect cellular mechanisms associated with synaptic plasticity. In contrast, we did not observe a difference between acute and long-term incubation of 1.0 mM LiCl, suggesting higher concentrations of LiCl are potent enough to affect cellular mechanisms that mediate synaptic plasticity. Interestingly, long-term incubation of 0.6 mM LiCl had a similar effect on LTP as acute 1.0 mM LiCl, indicating that the increased effect 0.6 mM LiCl has on LTP is a temporary effect on cell signalling. Overall, the comparison between the acute and long-term impact LiOr and LiCl have on LTP supports the notion that LiOr is more potent than LiCl.

In summary, LiOr elicits an effect on synaptic plasticity at a substantially lower dose than LiCl, which would not be the case if the pharmacokinetic and pharmacodynamic properties of the two compounds were identical. As our findings correspond with elevations in brain Li^+ after treatment with LiOr compared to LiCl, as proposed by Kling *et al.,* we suggest LiOr can be used as a substitute for LiCO to reduce dosing requirements[6]. As Smith *et al.* raised concerns regarding the potential renal toxicity of LiOr and cautioned against using the compound to treat BD[20], these reduced dosing requirements of LiOr indicate toxicity concerns of LiOr would be unlikely. We demonstrated that LiOr is more potent than LiCl, making it a better treatment option than LiCO because the decreased dosing requirements negate toxicity concerns connected to high Li^+ concentrations.

4.2.2. The superior potency of LiOr is likely due to differences in cell transport.

There are many possible explanations as to why dosage requirements between LiCl and LiOr are different; however, the most likely explanation is due to the physiological interaction with orotic acid (see section 1.2.3). LiOr is believed to possess differential transport- and dissociation-

related characteristics, enabling the increased cellular uptake of LiOr relative to LiCO[7,16,18,19]. As we found LiOr to be more potent than LiCl on synaptic plasticity, we can assume the pharmacokinetic and pharmacodynamic properties of the two compounds are different. Previous research in our lab characterized the transport- and dissociated-related properties of LiOr that set it apart from LiCO[7]. Pacholko and Bekar (2023) found that, in contrast to LiCO, which dissociates readily in solution, LiOr remains in its non-dissociated state post-administration. These differential dissociative characteristics indicate the existence of different transport pathways. Pacholko and Bekar (2023) provided evidence that LiOr is intercellularly transported as a compound through OATPs, while LiCO readily dissociates into Li^+ and CO_3^{2-}. As LiCO and LiCl readily dissociate, it primarily uses sodium channels and transporters for transport throughout the body (see sections 1.2.2 and 1.2.3)[8–11]. Given sodium channels and transports are abundantly located all over the body, Li^+ ions are dispersed throughout the body and only a small percentage of Li^+ ions actually reach the CNS [8,9,11]. Additionally, Li^+ and sodium are in competing for entry through sodium transporters, suggesting the much greater concentration of extracellular sodium compared to Li^+ results in less Li^+ being transported across biological membranes [8,9,11]. To overcome these restrictions, high concentrations of LiCO are required to overcome the limitations on Li^+ entry to the CNS[12–15]. Unfortunately, these high concentrations of LiCO often increase adverse side effects associated with Li^+ treatments, leading to issues with patient compliance[7,17] .adverse

The different transport mechanisms utilized by LiCl and LiOr explain our observation that LiCl and LiOr differently affected synaptic plasticity. It accounts for the lack of effect lower doses of LiCl had on LTP and LTD as sodium transport mechanisms slow Li^+ entry to the CNS. We speculate that higher doses of LiCl affected synaptic plasticity because these doses were concentrated enough to overcome the competition between sodium and Li^+ for entry into the brain through sodium channels and transporters. If the limiting factor responsible for LiCO's narrow therapeutic window is the use of sodium channels and transporters for entry, a Li^+ compound, such as LiOr, that does not rely on sodium channels and tranporters would overcome dose limitations

in Li^+ treatment. In contrast to LiCl and LiCO, the direct transport of orotates by OATPs enables LiOr to more readily enter cells compared LiCl and LiCO because it is not slowed by sodium transport mechanisms [7]. OATPs are highly concentrated in highly metabolically active tissues, with OATP1A2 localized within neurons, glial cells, and the endothelium of the BBB [7].Pacholko and Bekar (2023) suggest OATP transporters could increase the ease with which LiOr crosses biological barriers to enable the direct delivery of Li^+ to intracellular target sites[7]. The improved transport capabilities LiOr displays explain our results of LiOr being more potent than LiCl as the Li^+ derived from LiCl is slowed by sodium transport, while LiOr is directly and indirectly transported into the CNS through OATPs. The difference in dosing requirements between LiOr and LiCl makes LiOr a better treatment option because the decreased dose needed for therapeutic LiOr treatment may reduce any adverse side effects associated with LiCO due to the high dose requirements of the drug [6,16,18–20].

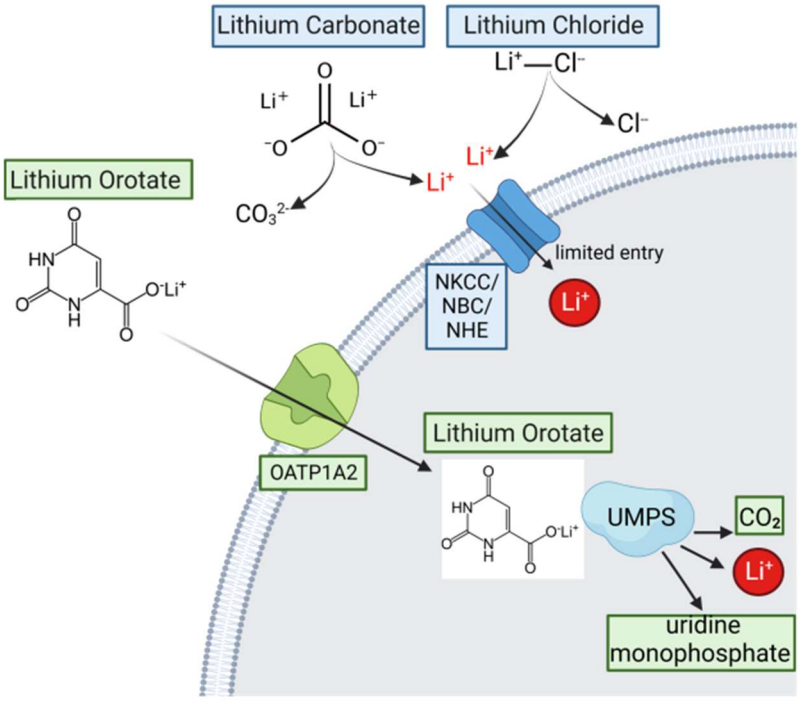

Figure 4.1. Potential relationship between the improved potency of LiOr and transport- and dissociated-related properties. Unlike LiCO and LiCl, which dissociates readily in solution, LiOr remains in its non-dissociated state post-administration. As LiCO and LiCl readily dissociate, it primarily uses sodium channels (sodium potassium chloride cotransporter (NKCC),sodium-bicarbonate cotransporters (NBC), and sodium proton exchanger (NHE) for transport while LiOr utilizes Organic anion-transporting polypeptide (OATPs) for passage across membranes and uridine monophosphate synthase (UMPS) for intracellular dissociation. It is possible the increased potency LiOr displays on synaptic plasticity is due improved transport and dissociation characteristics. Created with BioRender.com.

In summary, we suspect the dose discrepancies we observe LiOr and LiCl have on synaptic plasticity are due to transport pathway differences. We suspect LiOr's utilization of OATPs and LiCl's utilization of sodium transport results in the two compounds having different effects on secondary cellular messengers that regulate LTP and LTD. These findings support our hypothesis that LiOr is more potent than LiCl because of the more efficient cell transport mechanisms associated with orotate compounds[18].

4.3. LiCl and LiOr differentially affect GSK3β and phosphoinositol pathway.

4.3.1. LiCl-mediated inhibition of LTP is inhibited by myo-inositol incubation, whereas LiOr-mediated enhancement of LTP is occluded by GSK3β inhibition.

As our results show LiCl increases LTP at lower therapeutic concentrations and decreases LTP at higher LiCl concentrations compared to the consistent increase observed with LiOr, we hypothesized these two Li compounds have different pharmacokinetic and pharmacodynamic properties. Given these differences, we investigated the effects LiCl and LiOr have on secondary cellular mechanisms associated with synaptic plasticity [68,148,160]. Specifically, we chose to focus on the PI cycle and GSK3β because they are known synaptic plasticity regulators influenced by Li^+ (see section 1.2.1)[35,50,84,152,154,157,161–163]. We observed that the incubation of slices in myo-inositol prior to inducing LTP and LTD did not alter the effect LiOr has on LTP, indicating LiOr does not mediate synaptic plasticity through the phosphoinositol pathway. In contrast, myo-inositol incubation occluded the effect LiCl has on synaptic plasticity, suggesting LiCl affects the phosphoinositol pathway. The occlusion effect myo-inositol has on LiCl mediated changes to LTP and LTD is supported by a study that observed supplementation of 3-4 grams/ daily of myo-inositol decreases side-effects of Li^+ therapy (see section 1.2.1.1) [85]. Interestingly, incubation with myo-inositol eliminated both the increase 0.6 mM LiCl has on LTP and the inhibition 1.0 mM LiCl has on LTP (Fig. 3.5). This suggests both high and low concentrations of LiCl alter intracellular myo-inositol by inhibiting key enzymes in the phosphoinositol pathway. However, the different effects 0.6 mM and 1 mM LiCl have on LTP indicate that LiCl has a concentration-dependent effect on phosphoinositol signalling. We speculate that the increased potentiation observed with 0.6 mM LiCl occurs because this concentration is not potent enough to disrupt the phosphoinositol cycle completely, leading to the accumulation of IP_3 and subsequent increase in LTP [12,50,164]. In contrast, 1.0 mM LiCl is potent enough to have a complete inhibitory effect on phosphoinositol signalling. In summary, these findings suggest LiCl, but not LiOr, affects synaptic plasticity is mediated through the phosphoinositol pathway.

Next, we focused on LiCl and LiOr's effect on GSK3β using AZD2858, a non-specific GSK3β inhibitor, on our LTP model. Given LiCl inhibits GSK3β, we expected changes caused by LiOr and LiCl to LTP to be, in part, mediated by GSK3β [10,15,16,33,86,165]. Interestingly, we found that LiOr, but not LiCl mediates LTP by inhibiting GSK3β. As long-term treatment of LiCl and LiCO

have been observed to alter GSK3β at therapeutically relevant concentrations, we suspect the lack of a GSK3β-dependent effect on synaptic plasticity is due to constraints of our experimental design [145,152,153]. Given LiCl is a more potent inhibitor of IMPase than GSK3β, we speculate acute treatment with 0.6 mM or 1.0 mM LiCl is not a high enough concentration of intracellular Li^+ to cause a GSK3β-dependent increase in LTP whereas it was potent enough to affect the PP cycle[33,91]. We believe these therapeutic concentrations of LiCl would have an effect on GSK3β following chronic administration of the drug. In contrast, GSK3β dependent effect LiOr has on LTP provides further evidence that LiOr is more potent than LiCl.

In summary, LiCl and LiOr mediate synaptic plasticity using different secondary messenger pathways. We determined that LiCl mediates LTP and LTD through the phosphatidylinositol pathway, as the addition of myo-inositol occluded the effect LiCl had on both LTD and LTP. In contrast, we revealed using the GSK3β inhibitor, AZD2858, that LiOr consistently increases LTP through inhibition of GSK3β. While these observations regarding LTP and LTD are unrelated to BD, they nevertheless provide additional context as to why LiOr elicits a different effect than LiCl on synaptic plasticity, which would not be the case if the pharmacokinetic and pharmacodynamic properties of the two compounds were identical.

4.3.2. Due to different transport mechanisms, LiCl acts near the membrane on the phosphatidylinositol pathway whereas LiOr acts more intracellularly on GSK3β.

Given that LiCl and LiOr mediate synaptic plasticity using different secondary messenger pathways, we speculate different transport and dissociation characteristics causes contrasting effects on GSK3β and phosphoinositol signalling. We previously hypothesized LiOr is more potent than LiCl because, unlike LiCl, it is transported intracellularly using alternative transport than sodium transporters and liberated intracellularly by UMPS. In line with this thinking, it is possible that LiOr's utilization of OATPs and LiCl's utilization of sodium transport results in the two

compounds having contrasting effects on secondary cellular messengers that regulate LTP and LTD. The different transport and dissociation mechanisms utilized by LiOr and LiCl create a discrepancy between the amount of brain Li^+ transported into the CNS, which alters the effect LiOr and LiCl have on GSK3β and the PI cycle. We speculate that transport of Li^+ through sodium transporters creates a slow-moving Li^+ gradient across the membrane, allowing LiCl and LiCO to affect more membrane-bound secondary signalling mechanisms compared to LiOr. As a result, LiCl mediates synaptic plasticity through the phosphoinositol pathway, a membrane-bound pathway, because Li^+ ions diffuse across the membrane to inhibit enzymes in the phosphoinositol pathway before Li^+ ions affect more intracellularly located signalling pathways, such as GSK3β [8,11,154]. In contrast, LiOr does not affect the PI cycle because it is transported across cellular membranes as a compound using OATPs and is liberated far from the membrane and the PP cycle by UMPS, suggesting suggests LiOr does not affect the membrane-bound PP cycle because Li^+ is dissociated from orotic acid more intracellularly than the PP cycle is located. Given LiOr is liberated by UMPS intracellularly, we believe Li^+ ions liberated from LiOr have a concentrated effect on intracellularly located GSK3β.

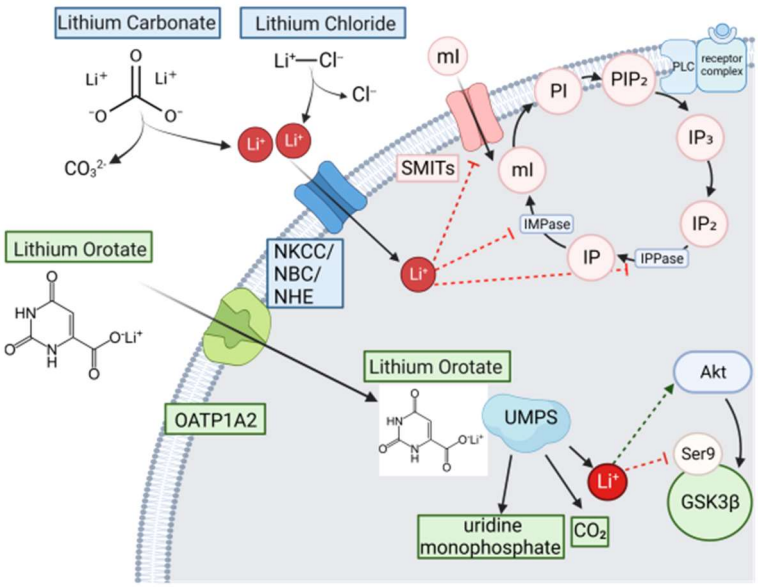

Figure 4.2. LiCl and LiOr mediate synaptic plasticity using different secondary messenger pathways due to transport related differences. A) As LiCl dissociates extracellularly and diffuses across the membrane through sodium channels and transporters, Li^+ ions from LiCl alters membrane-bound phosphoinositol signalling by inhibiting sodium myo-inositol transporters (SMITs), IPPase, and IMPase, which causes a decrease in cellular myo-inositol (mI). **B)** As LiOr utilizes OATPs for transport and UMPS for intracellular dissociation, Li^+ ions from LiOr directly decreases GSK3β signalling by competitive binding of Mg^{2+}, and indirectly inhibits GSK3β by activating Akt to cause an increase in serine phosphoralation. Created with BioRender.com.

Ultimately, LiOr and LiCl mediate synaptic plasticity using different cellular mechanisms because they utilize different transport mechanisms to cross cell membranes. We speculate LiOr specifically mediates synaptic plasticity through GSK3β because it is intracellularly transported using OATPs and liberated by UMPS. In contrast, LiCl mediates synaptic plasticity through the phosphoinositol pathway because, due to sodium transport dynamics, it diffuses across the membrane to inhibit enzymes in the phosphoinositol pathway before Li^+ ions can reach the more

intracellularly located GSK3β. LiOr and LiCl's differential effects on the phosphoinositol pathway and GSK3β provide evidence to support our hypothesis that LiOr and LiCl regulate synaptic plasticity using different mechanisms.

4.4. Contrasting the effects LiCl and LiOr have on NMDA-mediated potentials.

4.4.1. LiCl, but not LiOr, affects NMDA-mediated potentials.

The main forms of synaptic plasticity are mediated by NMDA receptors that trigger downstream synaptic changes [86,136,137]. Synapses undergo a form of bidirectional plasticity where different patterns of synaptic activation result in changes in secondary messenger pathways that cause either LTP or LTD [36,138]. The induction of NMDA receptor-mediated synaptic plasticity requires sufficient depolarization to displace the magnesium block to allow for calcium to flow through the receptor. [143,166,167]. This causes an increase in postsynaptic calcium that activates calcium-dependent kinases and phosphatases that regulate synaptic plasticity [136,140,141]. Previous studies have connected Li^+ to the downregulation of NMDA receptors through inhibition of NR2A tyrosine phosphorylation and its interactions with Src and Fyn mediated by PSD-95; however, the literature on the effects NMDA receptors have on Li^+-mediated changes to synaptic plasticity is nonexistent [73,76,136,155]. Given NMDA receptors mediate synaptic plasticity and Li^+ down-regulates NMDA receptors, we hypothesized LiCl and LiOr interact with NMDA receptors to alter NMDA-dependent synaptic plasticity [73,76,136,155].

Similar to LiCl's differential effect on LTP, we observed 1 mM LiCl decreased NMDA potentials whereas 0.6 mM LiCl increased NMDA potentials. As Li^+ has been shown to induce downregulation of NMDA receptors through inhibition of NR2A tyrosine phosphorylation and its interactions with Src and Fyn mediated by PSD-95, we speculate 1 mM LiCl decreases NMDA mediated potentials through Li^+ induced downregulation of tyrosine mediated phosphorylation of NMDA receptors [73,76,136,155]. We speculate 0.6 mM LiCl is not potent enough to affect tyrosine-mediated phosphorylation of NMDA receptors. In contrast, LiOr did not affect NMDA-mediated potentials, suggesting LiCl, but not LiOr, mediates synaptic plasticity by affecting NMDA-mediated currents. Similar to our explanation as to why LiOr and LiCl have different effects on the PP cycle and GSK3β, we speculate LiCl, but not LiOr, alters NMDA receptors due to the different transport mechanisms between the two compoundsWe speculate sodium transport creates

a slow-moving sodium gradient across the membrane that allows LiCl to affect membrane-bound NMDA receptors. In contrast, we believe LiOr does not affect NMDA receptors because LiOr is transported by OATS and liberated intracellularly by UMPS, indicating LiOr does not affect membrane-bound receptors.

4.4.2. LiCl, but not LiOr, indirectly affects NMDA-mediated potentials.

As our results show LiCl mediates NMDA receptors, we next wanted to determine if the effects LiCl has on NMDA potentials are a direct result of Li^+ or if Li^+ indirectly affects NMDA receptors through secondary cellular messenger pathways. Previously, we have found evidence that LiCl affects the PI cycle whereas LiOr affects GSK3β to mediate synaptic plasticity. Given these differences, we investigated whether LiCl and LiOr indirectly affect NMDA receptors by altering the PI cycle and GSK3β. To assess the effect LiCl and LiOr have on these secondary cellular mechanisms, we used basic pharmacology to determine the effects LiCl and LiOr had on GSK3β and phosphoinositol signalling and used 5 μM DNQX to isolate NMDA currents.

Like the effects on LTP, incubating slices in myo-inositol occluded the effect both 0.6 mM and 1.0 mM LiCl had on NMDA mediated potentials, suggesting LiCl indirectly affects NMDA receptors through mediating the PI cycle. As there is no evidence that Li^+ ions directly affect NMDA receptors, our results are supported by findings that Li^+ is observed to indirectly cause downregulation of NMDA receptor expression and signalling through inhibition of NR2A tyrosine phosphorylation and its interactions with Src and Fyn mediated by PSD-95 [73,75–78].Given the role different concentrations of LiCl have on phosphoinositol signaling, we hypothesize 1 mM LiCl is potent enough to mediate phosphoinositol signaling to cause an inhibition of NMDA receptors whereas lower concentrations (Like 0.6 mM LiCl) affect phosphoinositol signaling but not NMDA receptors. This explains why 0.6 mM LiCl increases LTP by altering the PI cycle whereas 1 mM LiCl decreases LTP by indirectly decreasing NMDA-mediated potentials through altering the PI cycle. In contrast, incubating slices in myo-inositol did not cause LiOr to influence NMDA receptors, confirming our previous results that show LiOr does not mediate synaptic plasticity through phosphoinositol signaling. These results support our previous results that show LiOr and LiCl mediate synaptic plasticity using different cellular mechanisms.

Given GSK3β is a mediator of NMDA receptors [168–170], we assessed the effects LiOr and LiCl have on NMDA currents through regulation with GSK3β. We had previously determined

therapeutic concentrations of LiCl do not mediate LTP through inhibition of GSK3β whereas LiOr was observed to inhibit GSK3β to increase LTP (Fig. 5). In line with our previous findings, we observed AZD2858, a potent GSK3β inhibitor, in conjunction with DNQX does not alter the effect LiOr has on NMDA receptors. In contrast, we did not observe a LiOr-mediated effect on NMDA mediated potentials without AZD2858, suggesting AZD2858 increases the effect LiOr has on GSK3β. There are many possible explanations as to why AZD2858 causes LiOr to decrease NMDA potentials; however, the most likely explanation is that, as AZD2858 is a more potent inhibitor of GSK3β than LiOr, AZD2858 further inhibits GSK3β, which causes GSK3β-dependent inhibition of NMDA-mediated potentials. Given our observation that 0.6 mM and 1.0 mM LiOr mediate LTP through GSK3β, we hypothesize these therapeutically relevant concentrations of LiOr are not potent enough to affect NMDA receptors through GSK3β inhibition. In contrast, AZD2858 is a more potent GSK3β inhibitor than 0.6 mM and 1.0 mM LiOr, suggesting AZD2858 has a greater inhibitory effect on GSK3β compared to 0.6 mM and 1.0 mM LiOr to cause a decrease in NMDA mediated potentials. We speculate higher concentrations of LiOr would affect NMDA receptors like the decrease AZD2858 and LiOr have on NMDA receptors-mediated potential. In any regard, the exact mechanism LiOr has on NMDA receptors is yet to be determined.

Investigating the effects LiOr and LiCl have on NMDA receptors through GSK3β, and the PI cycle has confirmed many of our previous results pertaining to the mechanisms LiCl and LiOr have on secondary messenger pathways. It is apparent LiCl and LiOr have different effects on NMDA receptors, as the pharmacodynamic effects these two Li^+ compounds have on NMDA receptors are different. Overall, it supports our main hypothesis that LiCl and LiOr regulate synaptic plasticity using different mechanisms.

5.0 Conclusion:

1. LiOr affects synaptic plasticity differently than LiCl.
2. LiOr is more potent than LiCl.
3. LiCl and LiOr mediate synaptic plasticity using different cellular mechanisms.
4. LiCl, but not LiOr, indirectly affects NMDA-mediated potentials.

We hypothesized that, as LiOr yields higher brain Li^+ than LiCO due to different transport mechanisms, LiOr will be more potent than LiCl and will selectively inhibit GSK3β to facilitate LTP while LiCl acts closer to the membrane on NMDA receptors and the phosphatidylinositol pathway. After using multiple statistical methods to analyze our data, we can conclude that LiOr is more potent than LiCl and mediates synaptic plasticity differently than LiCl. Specifically, we discovered LiCl mediates synaptic plasticity by altering phosphoinositol signaling and NMDA receptors whereas LiOr mediates LTP by inhibiting GSK3β. Overall, this research project provides evidence that LiCl and LiOr have different pharmacokinetic and pharmacodynamic properties. We speculate these differences occur due to the unique dissociation- and transport-related properties of LiOr. These reduced dosing requirements indicate the application of LiOr at lower doses than LiCO, which should dispel renal toxicity concerns raised by Smith *et al*. in 1979. Although further research is needed before LiOr replaces LiCO as a BD treatment, the improvements we have observed in potency displayed by LiOr in our LTP and LTD model suggest that the use of LiOr in BD may dispel compliance-disrupting side effects encountered during Li^+ therapy.

6.0 Limitations and Future Directions

6.1. Limitations

6.1.1. Absence of a Clinical Model

In this study, we used LTP and LTD as a model to compare and assess the pharmacokinetic and pharmacodynamic properties between LiOr and LiCl. We observed clear differences in dose-responsiveness and mechanistic properties that LiOr and LiCl have on hippocampal LTP and LTD. However, the use of LTP and LTD as a model to study BD limits the clinical translation for LiOr because these plasticity findings have no relation to BD. As we lack a clinical model of BD, we cannot conclude LiOr would be an effective treatment for BD and can only conclude LiOr and LiCl have different effects on synaptic plasticity. In order to understand the effects LiOr has on BD, it must be tested on models that display physiological characteristics of BD, such as mania models similar to the AIH model studied by Pacholko and Bekar (2023). However, BD is difficult to model because there is no unifying theory of BD that currently exists, which indicates further research in BD is necessary to utilize more effective treatment options to treat the disease.

6.1.2. Discrepancies between the acute and chronic effects of lithium.

In general, Li^+ treatment for BD patients is typically taken chronically because therapeutic benefits are only observed until many weeks of treatment, with many therapeutic effects requiring many months of treatment to emerge [15,171]. This means the effects LiOr and LiCl have on LTP and LTD can only be viewed as the initial actions of the drugs and do not translate to the chronic action of the drug, such as the more long-term neuroprotective and plasticity related effects believed to be responsible for the effectiveness of Li^+ therapy. In our experimental design, we compared the short-term and long-term effects of LiCl and LiOr on LTP by incubating slices in LiCl or LiOr prior to experiments in an attempt to display the chronic effects of Li^+ treatment. However, the differences between acute and long-term incubation of LiCl and LiOr are the effect of experimental design, and do not actually model the chronic effects Li^+ has on synaptic plasticity. Thus, it is necessary to study the differences LiCl and LiOr have on synaptic plasticity using a chronic form of treatment to understand therapeutic benefits of LiOr treatment.

6.2. Future directions

6.2.1. Chronic effects LiOr and LiCl have on synaptic plasticity.

Given the primary therapeutic benefits of Li^+ in BD treatment are only observed after long-term use of the drug, we propose the experiments that compare therapeutic concentrations of LiOr and LiCl should be repeated on mice that have been exposed to LiOr and LiCl for several weeks or months. To establish the generalizability of our findings, it is prudent to repeat the differential potency LiOr has on synaptic plasticity compared to LiCl on a chronic model of Li^+ therapy. Moreover, it would be useful to repeat the experiments that study the effects LiCl and LiOr has on the phosphoinositol pathway, GSK3β and NMDA receptors using this chronic model of Li^+ therapy. This study found evidence that LiCl and LiOr acutely affect synaptic plasticity using different mechanisms, which indicates the chronic effects LiCl and LiOr have on synaptic plasticity are necessary to understand the full therapeutic benefits of Li^+ therapy.

6.2.2. Assessment of LiOr and LiCl on synaptic plasticity in a mouse model of BD.

The synaptic plasticity experiments in this study were performed on naïve mice that do not model BD phenotypes, such as the manic and depressive characteristics of BD. We propose the entirety of this study should be repeated on mice brains that display a clinical phenotype of BD. Although this study displays distinct differences between the effects LiCl and LiOr have on synaptic plasticity, the use of a mouse model of BD is necessary to understand the therapeutic effects LiOr potentially has on BD.

6.2.3. Patch-clamp technique to understand the effect LiCl and LiOr have on NMDA receptors.

The results that compare the effects LiOr and LiCl have on NMDA receptors give us many confounding results. For instance, LiOr does not appear to decrease NMDA-mediated potentials, but the application of a GSK3β inhibitor in conjunction with LiOr causes a decrease in NMDA mediate currents. Additionally, LiCl appears to mediate NMDA receptors and synaptic plasticity indirectly by affecting the phosphoinositol pathway. In order to confirm these findings, the use of a patch-clamp technique to specifically look at the effects LiOr and LiCl have on isolated NMDA receptors is necessary to understand the effects on NMDA receptors.

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