EFFECTS OF ERYTHROPOIETIN AND LITHIUM ON NEURAL PRECURSORS AND BLOOD-DERIVED CELLS

by

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A Dissertation submitted to the Graduate School-New Brunswick
Rutgers, The State University of New Jersey
and
The Graduate School of Biomedical Sciences
University of Medicine and Dentistry of New Jersey
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
Graduate Program in Neuroscience
written under the direction of

Dr. Wise Young

and approved by

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New Brunswick, New Jersey
October, 2008
ABSTRACT OF THE DISSERTATION

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by

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Recent studies suggest that two hematopoietic drugs, erythropoietin (EPO) and lithium, can be used to treat various central nervous system (CNS) diseases, and that neural precursor stimulation contributes to their beneficial effects on CNS diseases. We studied the effects of these two drugs on neural precursor cells and N01.1, a neonatal rat blood-derived cell clone generated in our laboratory, exhibiting some features of neural precursors.

My experimental results suggest that EPO promotes neural precursor cell growth in vitro, and may stimulate expression of EPO receptor and neural stem cell marker nestin in rat spinal cords to benefit spinal cord injury. These studies provide important insight into the physiological functions and beneficial effects of EPO in the CNS. In addition, my studies suggest that lithium promotes proliferation but not survival of neural precursors in vitro, and the mechanism does not necessarily involve neurotrophic factors, or inositol depletion, but may involve GSK-3β inhibition and NFAT activation. These
findings not only provide mechanistic insights into the clinical effects of lithium, but also suggest an alternative therapeutic strategy for bipolar disorder and other neural diseases.

EPO activated ERK pathway in N01.1 cells, which may through a c-Raf-independent mechanism. In addition, EPO tended to increase RNA levels of anti-apoptotic gene Bcl-w but not Bcl2 or Bcl-xL in N01.1 cells. These results suggest that EPO may promote N01.1 cell survival or proliferation through a mechanism different from that in erythroid precursor cells.

Lithium significantly promoted cell number increase and stimulated neurotrophic factor RNA expression in N01.1 cell cultures. In addition, lithium treatment of rats that had N01.1 cells transplanted into their injured spinal cords robustly increased N01.1 cell numbers and remarkably increased RNA levels of several neurotrophic factors in the spinal cords. These results suggest a new powerful method for improving survival or proliferation of cells transplanted into the CNS, and a new method for stimulating neurotrophic factor expression in the CNS after injury.
DEDICATION

This thesis is dedicated to my parents,

my husband, Gutian,

my sons, Sean, Ryan, and Ray,

for their unconditional love and support through all my life.
I would like to extend my sincere gratitude and appreciation to all the people who have contributed to the fulfillment of this thesis work. Without their generous help, I would not be able to finish all works I have done in this thesis. First of all, I would like to thank my advisor, Dr. Wise Young for giving me the great opportunity to work with him and learn from him, and for his invaluable guidance, immense support, and wonderful advice throughout my research work. He is an insightful and diligent scientist. His dedication and enthusiasm made him a great paradigm for me to follow in my life. He has generously spent a lot of time to train me, guide me, and help me both in everyday life and in my professional Ph.D. pursuit. Without his patient training and guidance, I will not be able to get to where I am right now. So from bottom of my heart, I really appreciate his mentoring and friendship during my graduate study.

I also would like to thank the members of my thesis committee, Dr. Martin Grumet, Dr. Ronald Hart, Dr. Shu-Chan Hsu and Dr. Arnold Rabson, who spent their precious time in reviewing my thesis and giving me guidance and support to my research work.

I am very grateful to Dr. Dongming Sun, who has given me invaluable advice, enormous support, and helped me in almost every respect of my research. In addition, Jessica Lam gave me the RNA samples extracted from spinal cords of erythropoietin or control vehicle treated rats for me to analyze gene expression changes induced by erythropoietin treatment. Diane Vy generated the neonatal rat blood-derived cell clone
N01.1 and the GFP-expressing N01.1 cells, and together with some students did the study on the effects of lithium on N01.1 cell growth in vitro. Monique Tanna did immunostaining of N01.1 cells. Dr. Masayuki Hashimoto and Dr. Tsutomu Iseda did the in vivo experiments on the effects of lithium on transplanted N01.1 cells in injured rat spinal cords, as well as the effects of lithium and N01.1 cell transplantation on neurotrophic factor RNA expression in injured rat spinal cords. Iman Tadmori helped me in obtaining primary neural precursor cells and provided primary neural precursor cells for my research. I would like to thank all of them. It is their work that greatly benefits my research. Furthermore, I would like to thank Dr. Crista Adamson, Dr. Yi Ren, Dr. Hedong Li, and Dr. Rick Cohen for their wonderful suggestions and great help in my experiments. I also would like to acknowledge all the current and past Keck family members, Joanne Barbiarz, Dr. Sayantani Basak, Dawn Bryant, Dr. Cynthia Camarillo, Dr. Yu-wen Chang, Dr. Jian Chen, Chandrava Chukrabarti, Dr. Max Cristofanilli, Jonathan Davila, Loyal Goff, Sonia N. Guzman-Ramos, Dr. Noriko Kane-Goldsmith, Dr. Masayuki Hashimoto, Dr. Tsutomu Iseda, Ajar Kochar, Jessica Lam, Julie Li, Dr. Kai Liu, Dr. Liping Ma, Dr. Patricia Morton, Dr. Bor Tom Ng, Hock Ng, Dr. Tetsuhito Okuda, Sean O'Leary, Joy Planas, Chris Ricipero, Dr. Mingyu Shao, Judith Stugus, Mavis Swerdel, Iman Tadmori, Monique Tanna, Pui Tom, Evangeline Tzatzalos, Dr. Aswani Valiveti, Dr. Sankar Venkatachalam, Diane Vy, Dr. Junfang Wu, and Dr. Si-Wei You. They are all very nice people, and I appreciate very much their invaluable friendship and great help in my research. I am very lucky to have such a great opportunity to work together with them. I also would like to thank Dr. Martin Grumet for generously providing neural precursor cell clones, RG3.6 and L2.3 for my research.
I cannot begin to thank my family in China enough for their endless support. I cannot give enough thanks to my parents, my brothers and sisters for their unconditional love. I cannot give enough thanks to my parents-in-law for their continuous help and support throughout these years. I am also very grateful to my husband, Gutian Xiao for all of the love and support he has given me.

Last and most importantly, I have been blessed with three adorable sons, Si xiang (Sean) Xiao, Ryan Siyuan Xiao, and Ray Sirui Xiao. They are the source of my great pleasure and pressure. Their smiley faces brighten my life and remind me to work hard everyday. I love them with all of my heart.
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INTRODUCTION

Erythropoietin

Erythropoietin (EPO) was originally identified as a hematopoietic glycoprotein hormone that stimulates red blood cell production. Human native EPO was isolated in 1977 and its gene was cloned in 1985 (Miyake et al., 1977; Jacobs et al., 1985; Lin et al., 1985). Located on the long arm of chromosome 7 (q11-q22) (Law et al., 1986), the human epo gene encodes a protein of 193 amino acids. The N-terminal leader sequence of 27 residues is cleaved before secretion, and the carboxyl terminal arginine is missing in circulating human EPO (Recny et al., 1987). Thus, mature human EPO consists of a protein core of 165 amino acid residues, with three N-linked glycans (linked at Asp24, Asp38, and Asp83), one O-linked glycan (linked at Ser126), and two di-sulfide bonds (Cys7-Cys161 and Cys29-Cys33) (Lai et al., 1986). N-glycosylation is not required for in vitro activity but is necessary for proper biosynthesis and efficient secretion (Wasley et al., 1991; Yamaguchi et al., 1991; Higuchi et al., 1992). The fully sialylated N-glycans are important for stability in circulation, and in vivo activity (Fukuda et al., 1989; Spivak and Hogans, 1989). The rodent EPO lacks O-glycan. The disulfide bond Cys7-Cys161 is crucial for preservation of the molecular structure (Wang et al., 1985; Shimizu et al., 1986). Although the molecular mass deduced from the amino acid backbone is about 18kDa, mature human EPO has an apparent molecular weight of 30 to 40 kDa due to the glycosylations (Jelkmann, 2004).

EPO binds to its cognate receptor, the EPO receptor (EPOR). The epor gene was first cloned in 1989, four years after the identification of the EPO gene (D'Andrea et al.,
The EPO receptor belongs to a subfamily of the type I cytokine receptor superfamily, which is characterized by a single transmembrane domain, an intracellular domain without catalytic activity, and an extracellular domain with a tryptophan-serine-any amino acid-tryptophan-serine (WSXWS) motif near the plasma membrane and 4 cysteine residues that are more distally located (Jelkmann, 2004). EPOR exists as a preformed homodimer with a conformation such that in the absence of EPO the intracellular domains do not interact with each other. Upon EPO binding, the EPOR homodimer undergoes a conformational change, which brings the intracellular domains together, leading to phosphorylation and activation of the associated Janus tyrosine kinase 2 (JAK2) and activation of the downstream signaling cascade (Jelkmann, 2004).

Circulating EPO binds to EPOR expressed on erythroid precursor cells in bone marrow, and activates signal transduction pathways to promote survival, proliferation, and differentiation of erythroid precursor cells, resulting in increased production of red blood cells (Jelkmann, 2004). EPO and EPO receptor are highly conserved across human, rat and mice (Sasaki, 2003). EPO from any of these animals can bind EPO receptor of the other animals.

Made using recombinant DNA technology, human EPO is available as a drug, and used clinically to stimulate erythropoiesis in patients with anemia. It is produced by mammalian cells into which the human *epo* gene has been introduced, and has the identical amino acid sequence of isolated natural EPO, as well as the same biological effects of endogenous EPO (Recny et al., 1987). Its safety profile has been well characterized.
EPO and EPOR are expressed in the central nervous system

The liver produces EPO during fetal development. Shortly after birth, EPO production shifts to the kidney (Li et al., 2004a). In adults, the kidney is the primary site of EPO production and secretion; the liver also produces minor amounts of EPO (Li et al., 2004a). In the kidney, the peritubular interstitial cells, peritubular endothelial cells, tubular epithelial cells, and nephron segments produce and secrete EPO. In the liver, hepatocytes, hepatoma cells, and Kupffer cells produce and secrete EPO.

Stimulation of erythropoiesis was originally believed to be the only physiological action of EPO. The discovery of EPO and EPOR expression outside the erythropoietic system overturned this concept. In addition to liver and kidney, the central nervous system (CNS) and reproductive organs also produce EPO and have receptors to EPO (Yasuda et al., 1998; Chikuma et al., 2000; Sasaki, 2003). Besides erythroid precursor cells, EPO affects EPOR-expressing non-erythroid blood cells (Kimata et al., 1991; Korbel et al., 2004). Many non-hematopoietic cells express EPOR, including vascular smooth muscle cells, cardiomyocytes, myoblasts, capillary endothelial cells, neurons, microglia, and astrocytes (Masuda et al., 1993; Neusser et al., 1993; Anagnostou et al., 1994; Ammarguellat et al., 1996; Wald et al., 1996; Morishita et al., 1997; Ogilvie et al., 2000; Nagai et al., 2001; Wright et al., 2004).

EPO and EPOR expression have been described in specific areas of embryonic, fetal, and adult central nervous system of mice, rats, monkeys, and humans (Digicaylioglu et al., 1995; Li et al., 1996; Marti et al., 1996; Juul et al., 1998; Grasso et al., 2005; Knabe et al., 2005). EPO and EPOR proteins expressed in the CNS are smaller than their counterparts in the periphery, due to differences in sialic acid content (Masuda
et al., 1993; Masuda et al., 1994).

Both EPO and EPOR expression change significantly in the CNS during development. Embryonic mouse brain and other neural tissues express high levels of EPO and EPOR during neurogenesis (Knabe et al., 2004). This expression decreases significantly during brain development and maturation, dropping by up to 100-fold after birth (Liu et al., 1997). The increased expression of EPO and EPOR in the CNS during neural development suggests a role of EPO/EPOR in neurogenesis.

Most regions of the healthy adult CNS express only minimal levels of EPO and EPOR. However, expression of both proteins is markedly higher in ischemic/hypoxic human brain (Siren et al., 2001). Hypoxia can remarkably induce EPO RNA expression in brain as well as in liver and kidney of mice and rats (Tan et al., 1992; Chikuma et al., 2000). EPO RNA level rises transiently in the kidney, and markedly decreases within 8 hours despite continuous hypoxia. In contrast, the brain maintains high levels of EPO mRNA during hypoxia. Likewise, compressed spinal cords show sustained increases of EPO and EPOR proteins (Grasso et al., 2005). In addition, studies in our laboratory indicated that contusive spinal cord injury induced a sustained increase of both EPO and EPOR RNA in injured rat spinal cords (unpublished data of our laboratory). The persistent expression of EPO and EPOR in the adult CNS and up-regulation of their expression after hypoxia or injury suggest a role of EPO in the response of CNS to injury.

**EPO can be beneficial for central nervous system diseases**

Intravenously administered EPO can cross the blood-brain barrier (Brines et al., 2000; Banks et al., 2004; Xenocostas et al., 2005). Many studies have shown that
recombinant human EPO can be beneficial for various CNS diseases. For example, systematically administered EPO reduced brain infarct volume induced by focal ischemia, decreased the extent of concussive brain injury, delayed the onset and reduced the severity of symptoms in experimental autoimmune encephalomyelitis, and ameliorated the latency and severity of seizures induced by the glutamate analog kainic acid in rodents (Brines et al., 2000). Systemic administration of recombinant human erythropoietin after experimental traumatic brain injury in rats reduced blood-brain barrier (BBB) breakdown, brain edema and injury volume, improved recovery from motor dysfunction, and promoted spatial learning or spatial memory restoration (Lu et al., 2005; Grasso et al., 2007; Mahmood et al., 2007). EPO delayed disease onset and progression in a mouse model of amyotrophic lateral sclerosis (Grignaschi et al., 2007; Grunfeld et al., 2007; Koh et al., 2007). EPO also protected dopaminergic neurons and improved neurobehavioral outcome in animal models of Parkinson's disease (Genc et al., 2001; Xue et al., 2007). EPO treatment promoted neurological functional recovery from cerebral ischemia induced by middle cerebral artery occlusion in rats (Wang et al., 2004; Wakida et al., 2007; Wang et al., 2007). EPO administration significantly reduced cortical necrotic neuron count in a rabbit model of subarachnoid hemorrhage-induced acute cerebral ischemia (Grasso et al., 2002). EPO treatment significantly reduced infarct volume as well as number of apoptotic neurons in the brain, and improved long-term spatial memory deficits in a newborn rat hypoxic-ischemic brain injury model (Kumral et al., 2003; Kumral et al., 2004). EPO prevented motor neuron apoptosis and neurologic disability in a rabbit model of spinal cord ischemic injury, and enhanced neurological recovery following rat spinal cord compressive injury or contusion injury (Celik et al.,
EPO treatment has also been reported to improve neurological functional recovery in experimental autoimmune encephalomyelitis mice (Brines et al., 2000; Li et al., 2004b; Zhang et al., 2005). EPO protected CNS neurons from glutamate or hypoxia-induced cell death in vitro (Morishita et al., 1997; Juul et al., 1998). EPO also protected neurons from injury-induced cell death in vivo (Brines et al., 2000; Celik et al., 2002; Grasso et al., 2002; Kumral et al., 2003; Koh et al., 2007; Xue et al., 2007; Liao et al., 2008). Besides the neuroprotective effects of EPO in the CNS, EPO reduced inflammatory infiltrates and demyelination in experimental autoimmune encephalomyelitis (Brines et al., 2000; Li et al., 2004b; Zhang et al., 2005), and promoted angiogenesis after experimental stroke in rats (Wang et al., 2004; Li et al., 2007b). Emerging evidence suggest that EPO may also affect neural precursor cells in the CNS (Studer et al., 2000; Shingo et al., 2001; Yu et al., 2002; Wang et al., 2004; Tsai et al., 2006).

**EPO and EPOR play roles in CNS development and adult neurogenesis**

EPO and EPOR are present in fetal brain and spinal cord during neurogenesis (Juul et al., 1998; Knabe et al., 2004; Knabe et al., 2005). EPOR knockout mice have reduced number of neural precursor cells and increased apoptosis in their brains as early as E10.5, exhibiting neuroepithelial tissue hypoplasia by E11.5 that became prominent by E12.5 (Yu et al., 2002). EPO and EPOR knockout mice have similar phenotypes (Tsai et al., 2006), suggesting that EPOR is necessary for EPO action in early embryonic development. Although no major structures are absent in EPO and EPOR knockout
mice, the mutant brains are consistently smaller and less developed than their littermate controls, particularly in the subventricular zone of the developing forebrain (Tsai et al., 2006). EPO or EPOR knockout mice die early in utero (Yu et al., 2002; Tsai et al., 2006). Tsai et al generated mice with EPOR expression selectively knocked down in the brain (Tsai et al., 2006). These mice exhibited normal hematopoiesis and erythrocyte production, survived to adulthood and were fertile. However, these mice displayed reduced cell proliferation in the subventricular zone, and impaired post-stroke neurogenesis. These results suggest that EPO and EPOR play roles in CNS development and adult neurogenesis.

**EPO affects neural precursor cells**

EPO receptor is expressed in the embryonic germinal zone during neurogenesis and in adult brain subventricular zone, where neural precursors reside and continue to generate neurons throughout adulthood (Shingo et al., 2001), suggesting a role of EPO/EPOR in neurogenesis. Supporting this hypothesis, EPO increases generation of neurons by promoting the production of neuronal progenitor cells at the expense of multipotent progenitors (Shingo et al., 2001). In addition, EPO increases dopaminergic differentiation of cultured primary neural precursor cells (Studer et al., 2000). Studies in our laboratory showed that neural precursor cells isolated from various sources express EPO receptor. Since EPO promotes survival or proliferation of various precursor cells, such as erythroid precursor cells, endothelial progenitor cells, myoblasts, etc. (Umemura et al., 1989; Koury and Bondurant, 1990; Ogilvie et al., 2000; Bahlmann et al., 2004; George et al., 2005), and EPOR knockout mice have fewer neural precursor cells in their
brain (Yu et al., 2002), it is plausible that EPO may promote neural precursor cell survival or proliferation. So I investigated the effects of EPO on neural precursor cell survival and proliferation. In addition, I examined EPO activation of intracellular signaling pathways in neural precursor cells. These studies will be described in chapter I.

**Lithium is a hematopoietic reagent.**

Another drug, lithium, has been used clinically to stimulate leucocytosis and thrombopoiesis, i.e., leukocyte and platelet production, respectively (Lyman et al., 1980; Scanni et al., 1980; Steinherz et al., 1980; Chang et al., 1989; Hager et al., 2001). Lithium is a monovalent cation that belongs to the group of alkali metals together with sodium, potassium and other elements. Lithium increases white blood cell count, reducing leucopenia (white blood cell deficiency) and associated infection complications caused by chemotherapy and radiotherapy in cancer patients (Lyman et al., 1980; Scanni et al., 1980; Steinherz et al., 1980; Chang et al., 1989). Lithium increases platelet count in cancer patients with persistent thrombocytopenia (platelet deficiency) following chemotherapy or radiotherapy (Hager et al., 2001). In addition, several recent clinical studies indicate that lithium reduces neutropenia or granuloctopenia in schizophrenic patients treated with clozapine (Sporn et al., 2003; Papetti et al., 2004; Esposito et al., 2005).

Consistent with the clinical effects of lithium on leukocytosis and thrombopoiesis induction, lithium also stimulates the growth of bone marrow stromal cells, granulocyte-macrophage precursor cells and other hematopoietic precursor cells in vitro (Gamba-Vitalo et al., 1983; Doukas et al., 1986; Korycka and Robak, 1991; Huang et al., 1999).
These effects of lithium may account for the leukocytosis observed in patients that take lithium.

**Lithium has long been used clinically to treat bipolar disorder.**

Bipolar disorder, also called manic depressive illness, is a chronic mood disorder with symptoms of alternating mania (overly "high" or irritable) and depression (sad and hopeless), often with periods of normal mood in between. Severe changes in energy and behavior go along with these changes in mood. Bipolar disorder is a common disease, with an overall lifetime incidence of about 1% in the general population (Quiroz et al., 2004).

Over one hundred years ago in the nineteenth century, lithium was used as a sedative and a putative anticonvulsant. However, its use as therapeutics was withdrawn later due to the severe intoxication as a result of the erroneous employment of lithium as a salt substitute for cardiac and other chronically ill patients (Wada et al., 2005). In 1949, the Australian physician-scientist John Cade rediscovered lithium’s anti-manic effects (Manji et al., 1999). Subsequent clinical studies showed that it was not only efficacious in the control of acute mania, but was also prophylactic against recurrent manic and depressive episodes, and enhances the effects of classical antidepressants in some depressive patients (Chuang, 2005). Lithium has been the reference standard medication for acute and prophylactic treatment of bipolar disorder.

**Lithium can be used for other central nervous system diseases**
Lithium may have beneficial effects on several other central nervous system (CNS) diseases aside from bipolar disorder. For example, lithium may be beneficial for ischemic brain injury. Chronic lithium pretreatment before left middle cerebral artery occlusion in rats significantly improved neurological deficits and reduced brain infarct volume (Nonaka and Chuang, 1998). Lithium treatment after the ischemic insult also significantly reduced brain damage and improved neurological deficits (Ren et al., 2003). Lithium improved behavioral disorder induced by transient global cerebral ischemia in Sprague-Dawley rats (Yan et al., 2007).

Lithium may also be beneficial for Huntington’s disease (HD). Dr. Chuang’s group (2001) assessed the effect of lithium on striatal lesion formation in a rat model of Huntington's disease (HD) in which quinolinic acid was unilaterally infused into the striatum. They demonstrated that subcutaneous injections of lithium chloride for 16 days before quinolinic acid infusion considerably reduced the striatal lesion (Wei et al., 2001). Wood and Morton (2003) assessed the effects of lithium on R6/2 mice that carry Huntington's disease mutation and exhibit a progressive neurological phenotype including deterioration of motor function resembling that seen in HD. They found that post-symptomatic chronic treatment with lithium caused a significant improvement in rotarod performance (Wood and Morton, 2003).

Lithium may benefit spinal cord injury. Dr. Wu’s group (2004) reported that lithium together with chondroitinase ABC, a bacterial enzyme that breaks down an extracellular matrix protein known to inhibit axonal growth, improved functional recovery after C7 spinal cord hemi-section in rats (Yick et al., 2004).
Lithium may be useful to treat amyotrophic lateral sclerosis (ALS). Lithium administration alone or combined with concurrent administration of 2-hydroxy-5-(2,3,5,6-tetrafluoro-4-trifluoromethyl-benzylamino)-benzoic acid (Neu2000), a potent antioxidant, markedly promoted motor neuron survival, improved motor function, and decreased mortality in a mouse ALS model, the G93A transgenic mice carrying the G93A human copper/zinc superoxide dismutase 1(SOD1) gene mutation (Shin et al., 2007). Combined lithium and valproate treatment delayed disease symptom onset, reduced neurological deficits, and prolonged survival in the same mouse ALS model (Feng et al., 2008). Lithium administration delayed disease progression and augmented life span in both the mouse ALS model and human ALS patients (Fornai et al., 2008).

Lithium may be beneficial for glaucoma. Lithium protected retinal ganglion cells (RGC) and their axons against delayed degeneration triggered by optic nerve crash, a model of both brain axonal injury and certain aspects of the glaucomatous degeneration of RGC (Schuettauf et al., 2006). Simultaneous application of lithium with astrotoxin (a-aminoadipate), a selective killer of scar forming astrocytes, induced robust optic nerve regeneration in adult mice after optic nerve crash (Cho and Chen, 2008).

**Lithium affects neural precursor cells**

Lithium may benefit CNS diseases by protecting neurons as well as by stimulating neural precursor cells. The neuroprotective effect of lithium has been well demonstrated in various in vitro and in vivo models (Chuang et al., 2002). Converging evidence indicate that lithium can also affect neural precursor cells and induce neurogenesis in vitro and in vivo (Chen et al., 2000; Hashimoto et al., 2003; Kim et al.,
Lithium increased neurogenesis in the dentate gyrus of rodent hippocampus and selectively increased neuronal differentiation of cultured rat hippocampal neural progenitor cells (Chen et al., 2000; Kim et al., 2004). Lithium also stimulated neural precursor cell proliferation in embryonic rat cerebral cortical cultures as well as postnatal rat cerebellar granule cell cultures (Hashimoto et al., 2003). In agreement with the other reports, my studies showed that lithium promoted neural precursor cell proliferation. Despite the studies demonstrating that lithium promotes neural precursor cell proliferation, there is still a dearth of knowledge on how lithium promotes neural precursor cell proliferation. I therefore focused on the mechanisms of lithium effects on neural precursor cells. Chapter II will describe these studies.

**Effects of EPO and lithium on blood-derived cell clone N01.1**

Our laboratory developed a mononuclear cell clone called N01.1 from neonatal rat blood. Interestingly, these cells exhibit some features of neural precursors. They are positive for nestin, an intermediate filament protein expressed by neural stem cells and progenitors (Lendahl et al., 1990). Serum withdrawal promoted formation of spherical aggregates, similar to the neurospheres formed by neural precursor cells in culture. Treatment with retinoic acid failed to induce these cells into βIII-tubulin positive neurons, GFAP-positive astrocytes, or GALC-positive oligodendrocytes, suggesting that they are not really neural precursor cells. These cells present another interesting feature: they express EPO receptor. To better characterize these cells, I studied the effects of EPO on these cells, which will be described in chapter I.
We were very interested to know whether transplantation of these blood-derived cells with some neural precursor features could benefit spinal cord injury or other CNS diseases. When the N01.1 cells were transplanted into normal rat spinal cords, they respected the host tissue boundary and did not form tumors, suggesting that they are safe for transplantation. However, when they were transplanted into injured rat spinal cords, they suffered enormous cell loss. We therefore would like to find ways to promote N01.1 cell survival or proliferation. Since lithium stimulates survival or proliferation of various types of cells (Gallicchio and Chen, 1981; Doukas et al., 1986; Kucharz et al., 1988; Hashimoto et al., 2003; Huang et al., 2003; Rao et al., 2005), we hypothesize that lithium may also promote N01.1 cell survival or proliferation. To test this hypothesis, we studied the effects of EPO and lithium on N01.1 cells in vitro. Lithium remarkably induced cell number increase in N01.1 cell cultures. In addition, lithium significantly increased RNA levels of two neurotrophic factors in cultured N01.1 cells. Excited by these in vitro results, Dr. Masayuki Hashimoto and Dr. Tsutomu Iseda in our laboratory started to investigate whether lithium can promote N01.1 cell survival or proliferation after they are transplanted into injured rat spinal cords, and whether lithium could stimulate neurotrophic factors in the injured rat spinal cords transplanted with these N01.1 cells. Chapters II will describe these studies.
CHAPTER I

EFFECTS OF ERYTHROPOIETIN ON BLOOD-DERIVED
CELLS AND NEURAL PRECURSOR CELLS

Abstract

Previous studies in our laboratory have shown that EPO receptor is expressed in neural precursor cells, as well as in N01.1 cells, a cell clone grown from neonatal rat blood and exhibiting some features of neural precursors. I therefore investigated the effects of EPO on neural precursor cells and N01.1 cells. In N01.1 cells, EPO activated ERK pathway, which may through a c-Raf independent mechanism. In addition, EPO tended to increase RNA levels of anti-apoptotic gene Bcl-w, but not Bcl2 or Bcl-xL. These results suggest that EPO may promote N01.1 cell survival or proliferation through a mechanism different from that in erythroid precursor cells. EPO stimulated cell number increase in cultures of neural precursor cell clone RG3.6 cells, suggesting that EPO directly affects neural precursor cell survival, proliferation, or both. Furthermore, EPO treatment of rats with contusive spinal cord injury significantly increased RNA levels of nestin and EPOR in the spinal cords, suggesting that EPO may stimulate EPO and nestin expression in the spinal cord to benefit spinal cord injury. These studies provide important insight into the physiological functions of EPO in the CNS and the beneficial effects of EPO on CNS disorders.
Introduction

Erythropoietin (EPO) is a glycoprotein well known for erythropoiesis stimulation, and is used clinically to treat anemia. It exerts this function through binding to EPO receptor (EPOR) expressed on erythroid precursor cells in bone marrow. Recent studies in our laboratory showed that neural precursors also express EPOR, suggesting that EPO may also affect neural precursor cells.

As I stated before, our laboratory generated the N01.1 cell clone from neonatal rat blood. Interestingly, these cells exhibit some features of neural precursors and express EPO receptor. To better characterize these blood-derived cells with some neural precursor cell properties, I studied the effects of EPO on these cells.

EPO activates one of the three mitogen-activated protein kinase (MAPK) pathways, the extracellular signal-regulated kinase (ERK) pathway in erythroid precursor cells (Carroll et al., 1991; Devemy et al., 1997). In the MAPK pathway, MAPKKK enzymes are the first members of the pathway to be phosphorylated and activated by upstream signals. The activated MAPKKK then phosphorylates and activates MAPKK, and the activated MAPKK then phosphorylates and activates MAPK. In erythroid precursor cells, EPO activates c-Raf (Raf-1, a MAPKKK), MEK1/2 (mitogen-activated protein kinase/ERK kinase 1/2, MAPKKs), and ERK1/2 (Carroll et al., 1991). I therefore checked whether EPO could also activate c-Raf, MEK1/2, and ERK1/2 in N01.1 cells. Section 1 will describe these studies.

EPO up-regulates expression of the anti-apoptotic gene Bcl-xL or Bcl2 to promote cell survival in several types of cells. For example, in erythroid precursor cell line HCD-57 cells, which require EPO for proliferation and survival, EPO withdrawal
down-regulates anti-apoptotic genes Bcl-2 and Bcl-xL, and the cells underwent apoptotic cell death (Silva et al., 1996). Ectopic expression of Bcl-2 or Bcl-xL rescued these cells from apoptosis in the absence of EPO (Silva et al., 1996). EPO also up-regulates Bcl-xL in the EPO-dependent leukemia cell line UT-7/EPO, normal erythroid precursor cells, endothelial cells, and neurons (Wen et al., 2002; Mori et al., 2003; Zhande and Karsan, 2007). In addition, EPO up-regulates Bcl2 in cardiac myocytes after traumatic brain injury (Emir et al., 2004).

Recently, a relatively new Bcl2 family member with anti-apoptotic property, Bcl-w, was found to be neuroprotective and protect hippocampus in a mouse model of status epilepticus evoked by intra-amygdala kainic acid (Yan et al., 2000; Zhu et al., 2004; Murphy et al., 2007). I therefore examined whether EPO could up-regulate RNA levels of Bcl-w, Bcl2 and Bcl-xL, in N01.1 cells. These studies will be described in Section 1.

The central nervous system (CNS) also expresses EPO and EPOR, and their expression levels are up-regulated after hypoxia or CNS injury (Chikuma et al., 2000; Siren et al., 2001; Grasso et al., 2005). However, the physiological functions of EPO and EPOR in the CNS are not clear. Recent animal studies suggest that EPO can be beneficial for several CNS conditions, including spinal cord injury (Celik et al., 2002; Gorio et al., 2002; Boran et al., 2005), ischemic stroke (Wang et al., 2004; Wakida et al., 2007; Wang et al., 2007), traumatic brain injury (Lu et al., 2005; Grasso et al., 2007; Mahmood et al., 2007), amyotrophic lateral sclerosis (Grignaschi et al., 2007; Grunfeld et al., 2007; Koh et al., 2007), Parkinson's disease (Genc et al., 2001; Xue et al., 2007), and experimental autoimmune encephalomyelitis (Brines et al., 2000; Li et al., 2004b; Zhang et al., 2005). However, the mechanisms of EPO's beneficial effects in the CNS are not
well understood.

Several studies suggest that neural precursor stimulation may be involved in the physiological function and beneficial effects of EPO in the CNS (Wang et al., 2004; Lu et al., 2005; Tsai et al., 2006). Previous studies in our laboratory showed that neural precursor cells express EPO receptor, suggesting that EPO may play a direct role in neural precursor cells. I therefore studied the effects of EPO on neural precursor cell clone RG3.6 (courtesy from Dr. Martin Grumet). RG3.6 cell clone was established and well characterized by Dr. Martin Grumet’s group. It was derived from embryonic forebrain of green fluorescent protein (GFP)-transgenic rat, and exhibits features of both radial glial cells and neural precursor cells. To check whether EPO could stimulate endogenous neural precursors after spinal cord injury, I examined whether EPO could up-regulate RNA levels of two genes expressed in neural precursors, the EPO receptor and the neural stem cell marker nestin, in injured rat spinal cords. Section 2 will describe these studies.
Materials and Methods

Antibodies and reagents

Table 1 lists the antibodies used in the experiments and their sources. Table 2 lists the reagents used in the experiments and their sources.

Cell culture

Dr. Martin Grumet’s group kindly provided us the cell clone RG3.6 (Hasegawa et al., 2005). These cells were maintained in DMEM/F12 (Invitrogen) supplemented with 25 mM glucose (Sigma-Aldrich), 100 µg/ml streptomycin (Invitrogen), 100 units/ml penicillin (Invitrogen), 10 ng/ml FGF2 (BD biosciences), 2 µg/ml heparin (Sigma-Aldrich), and 1X B27 (Invitrogen). The cultures were passaged when the neurospheres that had formed grew into balls of 50 to 100 cells, usually in about 4-5 days. The following passage procedure was used. Remove the supernatant after spinning down the cells. Add one-tenth culture medium volume of 0.05% trypsin-EDTA (Invitrogen), and incubate at room temperature for about 2-3 minutes, tap the cells occasionally. Add an equal amount of trypsin inhibitor (Sigma-Aldrich) to stop the trypsin digestion, and pipette up and down to dissociate the cells. Spin down the cells and wash the cells for 2 times with culture medium. Incubate the cells in culture medium at 37 °C in a humidified atmosphere of 95% air + 5% CO2.

Diane Vy in our laboratory generated the neonatal rat blood derived cell clone N01.1 by the following procedure. P0 Sprague-Dawley rats were decapitated to obtain blood. The blood was diluted in HBSS. The samples were layered on Lymphocyte
Separation Medium (Fisher) and centrifuged at 1,200 g for 20 minutes at room temperature to separate mono-nucleated cells from other cells types. Mono-nucleated cells were retrieved from the buffy coat layer and washed with HBSS. The cells were counted and suspended at a concentration of $1 \times 10^6$ cells/ml in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 10 ng/ml EGF (BD Biosciences), 20 ng/ml FGF2 (BD Biosciences), 100 µg/ml streptomycin (Invitrogen), and 100 units/ml penicillin (Invitrogen). Cells were incubated at 37 °C in a humidified atmosphere of 95% air + 5% CO₂. Cells started multiplying after approximately 4 weeks, and continued multiplying afterwards. The cell clone N01.1 was obtained by seeding the cells in a 96-well plate after passage with the density of close to one cell per well, and growing the cells from a single cell.

**Immunocytochemistry**

For immunocytochemistry, the cells were plated on coverslips in culture medium. When the cells reach 70%-90% confluence, the cultures were stopped by fixing the cells with 4% para-formaldehyde at room temperature for 15 minutes. The fixed cells were washed three times with 0.01 M phosphate buffered saline (PBS, pH 7.4). The coverslips were treated for 30 minutes with 5% normal goat serum (NGS, Invitrogen) in PBS (plus 0.3% triton for intracellular antigen detection) to block non-specific binding of antibodies. Then primary antibodies were applied for one hour at room temperature. After removal of the primary antibodies, the coverslips were washed with PBS for 3 times at 5 minutes per wash. Fluorescent dye-conjugated secondary antibodies (Molecular Probes) were subsequently applied for about 30 minutes followed by Hoechst
(Molecular Probes) nuclear counterstaining for 10 minutes. The coverslips were washed again with PBS for 3 times at 5 minutes per wash, and mounted onto slides in mounting media (Invitrogen). The fluorescent staining was visualized and photographed using fluorescent microscope.

**Western blotting**

Western Blotting was carried out according to the described procedure (Qu et al., 2004). Cells were lysed in radioimmunoprecipitation assay buffer (RIPA buffer) [50 mM Tris-HCl pH7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)] supplemented with a protease inhibitor cocktail (Roche). Aliquots of cell lysates were subjected to SDS-PAGE, transferred to PVDF membrane by electrophoresis. The membrane was incubated in blocking buffer, i.e. TBS/T (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween-20) with 5% (w/v) non-fat dry milk, for one hour at room temperature to block non-specific binding of antibodies. After washed 3 times for 5 minutes each with TBS/T, the membrane was incubated in primary antibody solution with gentle agitation overnight at 4 °C (for phospho-antibody) or at room temperature for one hour (for regular antibody). The membrane was washed 3 times for 5 minutes each with TBS/T, followed by incubation in secondary antibody solution with gentle agitation for one hour at room temperature. After washed 3 times for 5 minutes each with TBS/T, the membrane was incubated with the ECL plus western blotting detection reagents (Amersham Biosciences) for 5 minutes at room temperature. The chemiluminescent signal was detected using a Storm Imager.
Spinal cord injury model and treatment

Marlin Matthews in our laboratory did the spinal cord injury and treatment. The procedure is as follows. Adult Sprague-Dawley rats (77±5 days old) were anesthetized with intraperitoneal injection of pentobarbital (45±5 mg/kg for female and 65±5 mg/kg for male). Laminectomies were performed to expose the thoracic segments T9–10. 60±5 minutes after anesthesia induction, spinal cords were contused by dropping a 10-gram rod from a height of 25 mm using the Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor (formerly known as the NYU impactor). Injury at this level causes hind limb motor and sensory paralysis and lack of bladder control. Respiratory and front limb motor and sensory function above the injury site remain intact.

Shortly after injury, 40,000 U/kg body weight of EPO (Johnson and Johnson) or vehicle control was injected intravenously through the external jugular vein. The vehicle control is the solution in which EPO was dissolved, i.e. 4.38 mg/ml sodium chloride, 1.1 mg/ml sodium dihydrogen phosphate monobasic, 1.6 mg/ml sodium phosphate dibasic, 5.0 mg/ml glycine, 0.3 mg/ml Tween-80, pH 6.9 (pH is adjusted with phosphoric acid or sodium hydroxide if necessary). The exposed muscle and skin were closed in layers. After the surgery, the rats were kept on warming pads until they wake, and then returned to their home cage.

RNA extraction from spinal cord tissue

Dr. Bor Tom Ng and other colleagues in our laboratory did the animal euthanasia, spinal cord removal and RNA extraction from the spinal cord pieces, which is according
to the following procedure. Six hours after spinal cord injury, the animals were
anesthetized with intraperitoneal injection of pentobarbital (45±5 mg/kg for female and
65±5 mg/kg for male) and decapitated. Spinal cords were quickly removed, chilled on
dry ice, and cut into 5 mm segments centered at the impact site. Five consecutive
segments of each spinal cord were collected, from the proximal to the distal of the spinal
cord: P2, P1, I, D1, D2, with segment I having the impact site at its center. Each spinal
cord segment was placed in 0.5 ml trizol reagent (Invitrogen) and homogenized using a
tissue homogenizer with a micro-grinding tip. 100 µl of chloroform was added into each
tube. The samples were then mixed by vortex or pipetting for 5 seconds or until cloudy,
placed on ice for 5 minutes, and centrifuged at 13,000 rpm for 15 minutes at 4°C. The
RNA containing upper aqueous phase was transferred to a new tube, and an equal volume
of 70% ethanol was added into the new tubes. Samples were mixed by pipetting and
applied to RNeasy mini spin columns (Qiagen). RNA was subsequently purified using
RNeasy Mini kit (Qiagen) according to the manufacturer recommended RNeasy spin
protocol.

**RNA extraction from cell culture**

RNA from cultured cells was extracted using the RNeasy Mini kit (Qiagen)
according the manufacture recommended RNeasy spin protocol.

**Quantitative real time PCR**

After RNA concentration and purity were measured using a spectrophotometer,
the same amount of RNA from each sample was reverse transcribed into cDNA in equal
conditions using SuperScript™ first-strand synthesis system for RT-PCR (Invitrogen). The cDNAs were diluted 1:10. Equal volume of each diluted cDNA, together with equal amounts of primer pair and 2X SYBR Green PCR master mix (Applied Biosystems) was loaded into each well of a 384-well plate. The plate was sealed with optical adhesive cover. Quantitative real time PCR was performed using ABI 7900HT Fast real time PCR system. The primers used in the experiments are listed in Table 3.

**Cell count analysis**

Cells were grown in 96-well plate. Quantification of cell number was carried out using the Cyquant cell proliferation assay kit (Invitrogen) according to the manufacturer’s protocol.
Table 1. Antibodies used in the experiments and their sources.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-EPO receptor polyclonal antibody</td>
<td>Upstate</td>
</tr>
<tr>
<td>Mouse anti- nestin monoclonal antibody</td>
<td>Chemicon</td>
</tr>
<tr>
<td>Mouse anti- BrdU monoclonal antibody</td>
<td>DakoCytomation</td>
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<tr>
<td>Rabbit anti-glial fibrillary acidic protein (GFAP) polyclonal antibody</td>
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<td>Mouse anti-betaIII-tubulin monoclonal antibody</td>
<td>Covance</td>
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<td>Mouse anti-galactocerebroside monoclonal antibody</td>
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<td>Fluorescent dye-conjugated secondary antibodies</td>
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<td>Rabbit phospho-ERK1/2 antibody</td>
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<tr>
<td>Rabbit phospho-MEK1/2 antibody</td>
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Table 2. Reagents used in the experiments and their sources.

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<td>Fetal bovine serum</td>
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<td>B27</td>
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<td>Fibroblast Growth Factor 2 (FGF2)</td>
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<td>Lymphocyte separation medium</td>
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<td>Hoechst 33342</td>
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<tr>
<td>Erythropoietin (EPO)</td>
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<td>SuperScript™ first-strand synthesis system for RT-PCR</td>
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Table 3. Primers used for quantitative real time PCR experiments.

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Section 1

Effects of Erythropoietin on Neural Precursor Cells

Results

*N01.1 cells express EPO receptor*

Monique Tanna in our laboratory examined EPO receptor expression in N01.1 cells through immunocytochemistry. The results showed that most of the cells express low levels of EPO receptor; few of them express EPO receptor at high level (Figure 1). The expression of EPO receptor in N01.1 cells suggests that EPO may directly affect these cells.

*EPO activates the ERK pathway in N01.1 cells*

In erythroid precursor cells, EPO activates c-Raf, MEK1/2, and ERK1/2 in the ERK pathway (Carroll et al., 1991). To determine whether EPO activates the ERK pathway in N01.1 cells, I first examined EPO activation of ERK1/2, using various doses of EPO, i.e. 0, 5, 10, 20 U/ml. My preliminary results showed that EPO at dose of 20 U/ml activated ERK1/2 in N01.1 cells, while at lower doses had no obvious effect on ERK1/2 activation (Figure 3).

I next performed a time course study on EPO activation of the ERK pathway in N01.1 cells. I examined activation of c-Raf, MEK1/2, and ERK1/2 at various time points after adding EPO (20 U/ml) to the culture medium. The western blotting results showed that EPO induced an early activation of MEK1/2 and ERK1/2, with peak activation
within 20 minutes after EPO addition. However, no activation of c-Raf was detected at all the time points examined (Figure 4), suggesting that c-Raf is not the upstream MAPKKK and that some other enzyme may be involved. Since activation of the ERK pathway contributes to EPO-mediated cell survival or proliferation in various types of cells (Carroll et al., 1991; Devery et al., 1997; Sui et al., 1998; Kilic et al., 2005), ERK pathway activation by EPO in N01.1 cells suggests that EPO may promote N01.1 cell survival or proliferation.

*EPO does not significantly increase Bcl2 or Bcl-xL RNA levels but may increase Bcl-w RNA levels in N01.1 cells*

EPO up-regulates expression of the anti-apoptotic gene Bcl-xL and Bcl2 to promote cell survival in several types of cells, such as erythroid precursor cells, endothelial cells, cardiac myocytes, and neurons (Silva et al., 1996; Wen et al., 2002; Mori et al., 2003; Emir et al., 2004; Zhande and Karsan, 2007). Using quantitative real-time PCR analysis, I examined whether EPO increases RNA levels of Bcl2 family of anti-apoptotic genes Bcl2, Bcl-xL and Bcl-w in N01.1 cells. To our surprise, it did not significantly increase Bcl2 and Bcl-xL RNA levels during 4 hours of treatment (Figure 6). Interestingly, EPO showed a trend of increasing RNA levels of anti-apoptotic gene Bcl-w. These results suggest that EPO may up-regulate Bcl-w in N01.1 cells to promote cell survival.
Discussion

*N01.1 cells express EPO receptor*

The immunocytochemistry results from our laboratory showed that N01.1 cells express EPO receptor (Figure 1). The expression level is low in most cells; only few cells express high levels of EPO receptor. When I stained neural precursor cell clone RG3.6 cells for EPO receptor, the RG3.6 cells also displayed heterogeneous levels of EPO receptor expression (Figure 5). This likely reflects different stages of the cells in the cell cycle. N01.1 cells expressing high levels of EPO receptor also expressed high levels of nestin, and vice versa (data not shown). Similarly, RG3.6 cells expressing higher levels of EPO receptor also expressed higher levels of GFP, and vice versa (data not shown). These suggest that the cells expressing higher levels of EPO receptor and nestin or GFP were at more active stage of protein production. Co-examination of EPO receptor expression together with cell cycle stage markers will help define the cell cycle stage of the cells expressing higher and lower levels of EPO receptor. Another possible reason for the heterogeneous levels of EPO receptor expression is that the cells may contain a heterogeneous population. Although both N01.1 and RG3.6 were derived form a single cell, it is possible that during passage, some of the cells may drift in some characteristics, resulting in heterogeneous population.

The cells were permeabilized with 0.3% triton (Page 20) to also stain intracellular nestin. As a result, immunostaining revealed intracellular EPO receptor that had been synthesized but had not been delivered to the cell surface yet. In N01.1 cells that express high levels of EPO receptor, some EPO receptors seem to be present at the perinuclear region. However, these immunostainings were viewed and photographed with regular
fluorescence microscope, which was not able to reveal accurate localization of the fluorescent signals. To reveal cell surface localization of EPO receptor, it would be better to stain EPO receptor with another known cell surface protein without permeabilizing the cells and view the staining using confocal microscopy. In addition, it would be better to verify the specificity of the EPO receptor staining by adding a negative control using excessive amount of the EPO receptor epitope that was used to generate the EPO receptor antibody to compete out the EPO receptor on the cell surface when adding the EPO receptor antibody. Another specific EPO receptor negative control could be N01.1 cells with EPO receptor knocked out or knocked down via EPO receptor specific SiRNA.

EPO activates the ERK pathway in N01.1 cells

Activation of the ERK pathway contributes to EPO-mediated cell survival or proliferation in various types of cells, including erythroid precursor cells (Carroll et al., 1991; Devemy et al., 1997; Sui et al., 1998; Kilic et al., 2005). My experimental results showed that EPO activated MEK1/2 and ERK1/2 in the ERK pathway in neonatal rat blood-derived N01.1 cells (Figures 2-3). My preliminary results showed that EPO at 20 U/ml activated ERK1/2, while at 5, 10, or 50 U/ml did not induce obvious activation of ERK1/2 in N01.1 cells (Figure 2). Although my later experiments confirmed that EPO at 20 U/ml did activate ERK1/2 in N01.1 cells, more experiments need to be done to check whether this pattern of ERK1/2 activation is repeatable.

The activation of MEK1/2 and ERK1/2 in N01.1 cells was indicated by increased phosphorylation of MEK1/2 and ERK1/2, respectively, in the lanes loaded with the same
amount of cell lysate protein (Figures 2-3). A better quantification of MEK1/2 and ERK1/2 activation could be obtained by examining P-MEK1/2 and P-ERK1/2 together with MEK1/2 and ERK1/2 and using the ratio of P-MEK1/2 to MEK1/2 and P-ERK1/2 to ERK1/2 as an indication of their activation. EPO activated MEK1/2 and ERK1/2 at the same time points in N01.1 cells (Figure 3), suggesting that MEK1/2 activation is responsible for ERK1/2 activation in N01.1 cells. This could be verified by checking whether specific MEK1/2 inhibitor, such as U0126, could inhibit EPO-induced ERK1/2 activation.

In N01.1 cells, EPO induced ERK pathway activation in a way different from PMA (phorbol myristate acetate), a potent ERK pathway activator (Frost et al., 1994; Lee et al., 2002). As shown in Figure 3, PMA activated c-Raf in N01.1 cells, which is indicated by the phosphorylation-caused band shift in lane 6. While no band shift of c-Raf was observed in EPO treated samples (lanes 2-5), suggesting that EPO did not activate c-Raf in N01.1 cells. These results suggest that in N01.1 cells EPO induces ERK pathway activation through a c-Raf-independent mechanism, which is different from the mechanism by which EPO activates ERK pathway in HCD-57 cells, a murine erythroid precursor cell line that requires EPO for survival and proliferation. In HCD-57 cells, c-Raf activation by EPO is essential for EPO-mediated proliferation (Carroll et al., 1991). Thus, EPO may activate the ERK pathway in N01.1 cells through a mechanism different from that in erythroid precursor cells. To verify that c-Raf activation is not responsible for EPO-induced MEK1/2 and ERK1/2 in N01.1 cells, we could check whether specific Raf inhibitor, such as BAY43-9006, could inhibit EPO-induced MEK1/2 and ERK1/2 activation in N01.1 cells.
EPO may induce Bcl-w expression in N01.1 cells

EPO can up-regulate Bcl2 family of anti-apoptotic gene, such as Bcl2 or Bcl-xL, to promote cell survival in several types of cells, including EPO-dependent leukemia cell line UT-7/EPO, normal erythroid precursor cells, endothelial cells, and neurons (Wen et al., 2002; Mori et al., 2003; Zhande and Karsan, 2007). I therefore examined EPO-induced RNA level changes of Bcl2 family of anti-apoptotic genes, including Bcl-w, Bcl2 and Bcl-xL, in N01.1 cells. Surprisingly, EPO did not significantly change Bcl2 or Bcl-xL RNA levels during 4 hours of treatment (Figure 4). However, interestingly, EPO showed a trend of increasing anti-apoptotic gene Bcl-w RNA level (Figure 4). Treatment of N01.1 cells with EPO for longer period of time will help to determine the time course and extent of EPO induction of Bcl-w RNA.

Bcl-w is a relatively new Bcl2 family member with anti-apoptotic property (Gibson et al., 1996). It is neuroprotective and protects hippocampus in a mouse model of status epilepticus evoked by intra-amygdala kainic acid (Yan et al., 2000; Zhu et al., 2004; Murphy et al., 2007). To our knowledge, other investigators have not reported that EPO up-regulates Bcl-w expression. Thus, EPO induction of Bcl-w in N01.1 cells would be an interesting novel finding. Further investigation of EPO-induced Bcl-w expression changes at the protein level will be needed to substantiate this finding.

EPO did not induce significant Bcl2 or Bcl-xL RNA level changes in N01.1 cells within 4 hours of treatment. However, I cannot rule out the possibility that EPO may still up-regulate expression of these genes. Examination of their expression after longer periods of EPO treatment and at the protein level will help clarify this issue.
In summary, EPO activates MEK1/2 and ERK1/2 in the ERK pathway in N01.1 cells. EPO may activate ERK pathway through a c-Raf-independent mechanism, a mechanism different from the EPO-induced c-Raf dependent ERK pathway activation in erythroid precursor cells. In addition, EPO may stimulate anti-apoptotic gene Bcl-w expression. Taken together, these results suggest that EPO may promote N01.1 cell survival or proliferation.
N01.1 cells were cultured in DMEM supplemented with 10% FBS, 10 ng/ml FGF2 and 10 ng/ml EGF. They were fixed with 4% para-formaldehyde at room temperature for 15 minutes and stained with rabbit anti-EPOR (1:200), followed by Texas Red goat anti-rabbit IgG (1:200) secondary antibody staining, and then nuclear counterstaining with Hoechst (1:1000). The calibration bars in the figure represent 20 µm. Red represents EPOR staining, and blue represents nuclei staining. A few cells express high levels of EPOR, while most of the cells express low levels of EPOR.
Cells were starved overnight in DMEM with 0.5% FBS to reduce basal level of ERK1/2 activation, and then treated with increasing doses of EPO or phorbol myristate acetate (PMA, a potent MAPK pathway activator, 20 ng/ml) for 10 minutes, and then lysed in RIPA buffer. Protein concentrations were measured and equal amounts of protein were loaded to SDS-PAGE gel. Activated ERK1/2 was detected by immunoblotting with anti-phospho-ERK1/2 antibody.
Figure 3. EPO activated MEK1/2 and ERK1/2 in N01.1 cells in the absence of Raf activation.

Cells were starved overnight in DMEM with 0.5% FBS to reduce basal level activation of these kinases, then treated with EPO (20 U/ml) or phorbol myristate acetate (PMA, 20 ng/ml) for the indicated time, and then lysed in RIPA buffer. Aliquots were subjected to SDS-PAGE. Activated ERK1/2 and MEK1/2 were detected by immunoblotting with anti-phospho-ERK1/2 antibody and anti-phospho-MEK1/2 antibody, respectively. The activation of Raf is indicated by the band shift in lane 6 of PMA treated sample.
Figure 4. EPO tended to increase RNA levels of anti-apoptotic gene Bcl-w in N01.1 cells.

RNAs were extracted from N01.1 cells treated with EPO (20 U/ml) for the indicated time (legend on the right, n = 3 for each treatment group). Quantitative real time PCR was carried out using the cDNAs reverse transcribed from these purified RNAs and primers for the indicated genes (Table 3). Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) level. The data points represent mean ± standard error. EPO did not significantly change RNA levels of anti-apoptotic genes Bcl2 and Bcl-xL. There was a trend of increasing the anti-apoptotic gene Bcl-w RNA level.
Section 2

Effects of Erythropoietin on Neural Precursor Cells

Results

*Neural precursor cell clone RG3.6 expresses EPO receptor*

Previous studies in our laboratory showed that EPO receptor is expressed in neural precursors isolated from various sources, such embryonic rat cerebral cortex, neonatal rat subventricular zone, and neonatal rat olfactory bulb. I examined EPO receptor expression in RG3.6 cells, a radial glial (neural precursor) cell clone established and well characterized by Dr. Martin Grumet’s group. The immunocytochemistry result showed that RG3.6 cells express EPO receptor, suggesting that EPO may directly affect these cells (Figure 5).

*EPO increases cell numbers in cultures of neural precursor cell clone RG3.6 cells*

I examined whether EPO could increase cell number in RG3.6 cell cultures. EPO administered daily at doses of 50-100 U/ml significantly increased cell numbers in neural precursor cell clone RG3.6 cell cultures, while at doses of 20 U/ml and below had no significant effect on RG3.6 cell numbers (Figure 6). These results suggest that EPO may promote neural precursor cell survival, proliferation, or both.
My experimental results showed that EPO stimulates neural precursor cells in vitro. Our laboratory has RNA samples extracted from spinal cords of rats treated with EPO (40,000 U/kg body weight, intravenous injection) or vehicle solution shortly after spinal cord injury. These rats were euthanized six hours after the spinal cord injury. To check the possibility that EPO may stimulate neural precursors in vivo in injured rat spinal cord, I examined the RNA levels of two genes expressed in neural precursor cells, EPO receptor and the neural stem cell marker nestin, in these spinal cord RNA samples. I also examined the RNA levels of the EPO gene itself in these samples. Through quantitative real-time PCR analysis, I found that EPO significantly increased RNA levels of EPOR and the neural stem cell marker nestin, but it had no significant effect on EPO RNA levels in injured rat spinal cords (Figure 7). These results suggest that EPO may up-regulate EPO receptor and nestin expression in spinal cord cells, or stimulates survival or proliferation of EPO receptor-expressing cells and nestin-expressing cells in the spinal cord after injury.
Discussion

*EPO promotes neural precursor cell growth*

My immunocytochemistry result showed that neural precursor cell clone RG3.6 cells express EPO receptor (Figure 5). These cells were permeabilized to also stain intracellular GFP (data not shown), thus the detected EPO receptor represents both cell surface and intracellular EPO receptor. To locate EPO receptor on the cell surface, it would be more desirable to stain the cells with EPO receptor together with a known cell surface protein without cell permeabilization and use confocal microscopy to locate the staining more accurately. In addition, as discussed in section 1, it would be better to verify the specificity of the EPO receptor staining.

The presence of EPO receptor in RG3.6 cells suggests that EPO may directly affect these cells. Indeed, EPO at doses of 50-100 U/ml significantly increased cell numbers in RG3.6 cell cultures, while at doses of 20 U/ml and below had no significant effect on cell counts in RG3.6 cultures (Figure 6), suggesting that EPO may promote RG3.6 cell survival, proliferation, or both. We could determine whether lithium promotes RG3.6 cell survival by measuring proportion of viable cells, dead cells, or apoptotic cells in RG3.6 cell cultures. In addition, we could determine whether lithium could stimulate RG3.6 cell proliferation by measuring proportion of cells in active proliferation in RG3.6 cell cultures. Although there are numerous commercial cell viability assay kits, we could simply use trypan blue or propidium iodide to stain dead cells, or even more simply, use GFP as a marker for RG3.6 cell survival. I will explain why GFP can be used as a marker for RG3.6 cell survival in chapter II. To measure
apoptotic cells, we could use terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. As for measuring cell proliferation, we could use Ki-67 immunostaining or BrdU incorporation assay. Ki-67 is a protein strictly associated with cell proliferation (Scholzen and Gerdes, 2000). It is present during all active phases of the cell cycle (G1, S, G2 and mitosis), but absent from resting cells (G0). Thus Ki-67 is an excellent marker for cells undergoing active proliferation. BrdU incorporation has been used frequently as an indication of cell proliferation both in vitro and in vivo, since BrdU only gets incorporated into chromosome DNA in significant amount during DNA synthesis of cell proliferation. One drawback of this method is that it cannot rule out the possibility that the difference in BrdU incorporation may be due to the difference in the survival of some cells instead of the difference in cell proliferation rate. Thus other measurement(s) such as cell survival examination is needed to draw a final conclusion.

My results suggest that EPO may promote neural precursor cell survival, proliferation, or both. This is consistent with the in vivo findings that EPO receptor knock out mice have fewer neural stem cells in their brain (Yu et al., 2002), and that mice with EPO receptor selectively knocked down in the brain have reduced cell proliferation in the subventricular zone and impaired post-stroke neurogenesis (Tsai et al., 2006). In a more recent report, Chen et al generated another mice with EPO receptor expression driven by the endogenous EPO receptor promoter in hematopoietic tissue but not in brain (Chen et al., 2007). Consistent with my results and the other in vivo findings, these mice exhibit reduced neural cell proliferation in adult brain regions associated with neurogenesis, the hippocampus and the subventricular zone.
The level of tissue oxygenation normally regulates endogenous production of EPO. In normal human subjects, plasma EPO levels range from 0.01 to 0.03 U/ml, and increase up to 100- to 1000-fold during hypoxia or anemia (Graber and Krantz, 1978). Thus EPO level could reach up to 30 U/ml in plasma during hypoxia or anemia. EPO expression in the CNS also increases markedly but for a much longer period of time during hypoxia, anemia, or after CNS injury (Tan et al., 1992; Chikuma et al., 2000; Grasso et al., 2005). However, EPO expression in these studies was measured at the RNA level or at the protein level using immunocytochemistry. Thus we don’t know how high the EPO concentration could reach in the CNS after CNS injury. In normal human subjects, EPO level in the cerebrospinal fluid is about one 10th of that in plasma (Widl et al., 2007). To reach 50 U/ml, the concentration that significantly promoted RG3.6 cell growth (Figure 6), the EPO level in the cerebrospinal fluid has to increase at least 10,000 times, which is unlikely, although may not be absolutely impossible. However, EPO may not distribute evenly in the CNS, thus it remains a question whether the microenvironment around neural precursor cells could accumulate enough EPO to trigger neural precursor cell proliferation while the cerebrospinal fluid EPO does not reach that high level. Systemic administration of EPO at up to 5,000 U/kg improves neurological function in various animal models of CNS injury or disease, such as spinal cord injury, traumatic brain injury, cerebral ischemia, amyotrophic lateral sclerosis, and Parkinson’s disease (Brines et al., 2000; Celik et al., 2002; Gorio et al., 2002; Wang et al., 2004; Lu et al., 2005; Grasso et al., 2007; Grignaschi et al., 2007; Wang et al., 2007; Xue et al., 2007). This dose of EPO is similar to the in vitro dose of 5 U/ml, which is a dose that did not promote RG3.6 cell growth (Figure 6). Thus my experimental results suggest that
EPO does not promote neural precursor cell growth at physiological doses that improves neurological functions in vivo, but does promote neural precursor cell growth at higher doses.

Different from my results, Chen et al reported that EPO at 10 U/ml optimally induced neural precursor cell number increase in cultures of primary neural precursor cells isolated from mouse embryonic hippocampus (Chen et al., 2007). One potential cause for this dose difference is that the primary neural precursor cell cultures contain a mixture of cells. As shown by their data, in the primary neural precursor cell cultures, all nestin-positive cells are also EPO receptor-positive, but a majority of cells are not nestin-positive, yet they are EPO receptor-positive. Thus, EPO may affect not only neural precursor cells but also the other EPO receptor-expressing cells in these cultures. The changes induced by EPO in the other EPO receptor-expressing cells may indirectly contribute to EPO’s stimulation of neural precursor cells. Or, the presence of other types of cells may facilitate EPO’s stimulation of neural precursor cells in these cultures. In support of this idea, a recent report showed that EPO at 10 U/ml did not promote neuronal differentiation of neuronal precursor cells, however, it promoted neuronal differentiation when the neuronal precursor cells were co-cultured with astrocytes or incubated with medium from EPO-treated astrocytes (Park et al., 2006). Thus, my results suggest that promotion of neural precursor cell growth by endogenous EPO or exogenous EPO at physiological dose is more likely due to concerted effects of EPO on the body cell mixture rather than due to the sole interaction between EPO and the neural precursor cells. Another potential but less likely cause for this dose difference in EPO effect is that RG3.6 is immortalized via v-myc retroviral infection, some changes due to this process
may affect the ability of RG3.6 to respond to EPO. To rule out this possibility and to test the idea that the ability of primary neural precursors to respond to lower doses of EPO was due to the mixture of cells in the culture, we will need to enrich nestin-positive primary neural precursors and check this EPO effect on pure nestin-positive primary neural precursor cell cultures.

**EPO stimulates EPO receptor and nestin expression in injured spinal cords**

EPO administered intravenously at 40,000 U/kg body weight significantly increased RNA levels of EPO receptor and the neural stem cell marker nestin in spinal cord at 6 hours after contusion but did not significantly alter EPO RNA level (Figure 7). These results bring up the possibility that EPO may promote neural precursor cell survival or proliferation after spinal cord injury, as neural precursor cells express both nestin and EPO receptor. However, other possibilities exist. In rat spinal cords, EPO receptor is present in neurons, glial cells, endothelial cells, besides the presumptive neural precursor ependymal cells, and up-regulated in these cells after spinal cord injury (Grasso et al., 2005). EPO may further up-regulate EPO receptor expression in these cells or a subset of them. Alternatively, EPO-induced EPO receptor up-regulation may be due to increased number of these cells or a subset of them in the injured spinal cords. In regard to nestin, its expression is hardly detectable in normal rat spinal cord. After spinal cord injury, nestin expression is induced, and cell proliferation is concomitantly increased, in the ependyma adjacent to the lesion site, suggesting that spinal cord injury activates latent neural precursor cells (the ependymal cells) and stimulates their proliferation (Namiki and Tator, 1999). Spinal cord injury also induces lots of nestin-
expressing reactive astrocytes close to and at the lesion site (Frisen et al., 1995). Many of these cells show active proliferation. Thus EPO-induced nestin up-regulation in the injured spinal cords could be due to up-regulation of nestin in neural precursor cells or reactive astrocytes, or due to increased number of neural precursor cells or reactive astrocytes. Immunohistochemical analysis of EPO receptor, nestin and other cell type markers in the injured spinal cords will help to clarify whether some cells up-regulate nestin and EPO receptor, and to identify these cells if so, and to determine whether EPO increases numbers of neural precursors, reactive astrocytes, or both, in the injured spinal cords.

In summary, my experimental results suggest that EPO does not promote neural precursor cell growth at physiological doses that improves functional recovery in vivo, but does promote their growth at higher doses. In addition, EPO may up-regulate EPO receptor expression in spinal cord cells, or stimulate survival or proliferation of some EPO receptor-expressing cells after spinal cord injury. Furthermore, EPO may stimulate nestin expression in spinal cord cells, or promote survival or proliferation of neural precursor cells or reactive astrocytes after spinal cord injury. These effects may contribute to EPO’s beneficial effects on spinal cord injury.
Figure 5. Radial glial cells RG3.6 expressed erythropoietin receptor.

RG3.6 cells were plated on laminin-coated coverslips, and cultured overnight in DMEM/F12 supplemented with 25 mM glucose, 100 µg/ml streptomycin, 100 units/ml penicillin, 10 ng/ml FGF2, 2 µg/ml heparin, and 1X B27, and then fixed with 4% paraformaldehyde at room temperature for 15 minutes. Cells were stained with rabbit anti-erythropoietin receptor (1:200) primary antibody, Texas Red goat anti-rabbit IgG (1:200) secondary antibody, and counterstained with Hoechst nuclear dye. EPO receptor is present in RG3.6 cell cytoplasm, and it is hard to tell its cell surface localization (red).
RG3.6 cells were seeded in equal number into wells of a 96-well plate (n=6 for each condition). The indicated doses of EPO were administered daily to the cultures. The cultures were stopped 5 days after plating. The cell number in each well was determined using cyquant cell proliferation assay kit (Invitrogen) according to the manufacturer’s protocol. EPO at doses 50-100 U/ml significantly increased cell numbers in RG3.6 cell cultures (*: Bonferroni/Dunn, P < 0.0083 compared to cultures treated with 0 amount of EPO).
Figure 7. EPO significantly increased RNA levels of EPOR and nestin but not EPO in injured rat spinal cords.

Adult Sprague–Dawley rats (n=8) received contusive spinal cord injury (Page 22). Shortly after injury, the rats received EPO (40,000 U/kg, Johnson and Johnson, n=3) or vehicle solution (n=5). The rats were euthanized six hours after injury. RNAs were purified from the impact site spinal cord pieces (segment I), and reversed transcribed into cDNA (Page 23). Quantitative real-time PCR was carried out using these cDNAs and primers for the indicated genes and peptidylprolyl isomerase A (Ppia, Table 3, Page 28). Expression levels of nestin, EPO and EPOR were normalized to Ppia expression. Results were analyzed using analysis of variance (ANOVA) in StatView software. The data represent mean ± standard error. EPO administration significantly increased nestin and EPO receptor RNA levels without much affection on EPO RNA levels (*: Bonferroni/Dunn, P < 0.05).
CHAPTER II

EFFECTS OF LITHIUM ON NEURAL PRECURSOR CELLS AND BLOOD-DERIVED CELLS

Abstract

Lithium has long been used clinically to treat manic depression. Recent studies indicate that lithium may be beneficial for other central nervous system (CNS) diseases. Lithium stimulates neural precursor cell proliferation, which may be involved in its beneficial effects on CNS diseases. However, the mechanism by which lithium promotes neural precursor cell proliferation is not clear. My studies suggest that lithium stimulates neural precursor proliferation without necessarily increasing neurotrophic factors, nor via inositol depletion, but through glycogen synthase kinase 3β (GSK-3β) inhibition and subsequent activation of nuclear factor of activated T cells (NFAT).

Lithium also remarkably increased numbers of N01.1 cells, a cell clone derived from neonatal rat blood, both in vitro cell culture and in injured rat spinal cord transplanted with these cells. In addition, lithium markedly increased RNA levels of neurotrophic factors both in vitro N01.1 cell culture and in vivo injured N01.1 cell-transplanted spinal cords. These studies suggest a new powerful method of promoting
transplanted cell survival or proliferation in the CNS, and a new method of stimulating neurotrophic factors in the CNS after injury.
Introduction

Lithium is a monovalent cation that belongs to the family of alkali metals including sodium and potassium. It has long been used clinically to treat manic depression (bipolar disorder), a chronic mood disorder characterized by alternating mania and depression, often with normal mood in between. It has also been used clinically to stimulate leukocyte and platelet production in cancer patients with chemotherapy or radiotherapy-induced leucopenia or thrombocytopenia. Lithium promotes proliferation or survival of many types of cells, including neurons, neural precursor cells and various hematopoietic cells (Gamba-Vitalo et al., 1983; Doukas et al., 1986; Kucharz et al., 1988; Korycka and Robak, 1991; Levine et al., 2000; Hashimoto et al., 2002; Hashimoto et al., 2003).

As I stated before, our laboratory generated an interesting blood-derived cell clone, N01.1, which exhibit some features of neural precursors. We were eager to find out whether transplantation of these cells could benefit spinal cord injury or other CNS diseases. To track the cells after they are transplanted, Mrs. Diane Vy in our laboratory introduced green fluorescent protein (GFP) gene into N01.1 cells and established N01.1 cells stably expressing GFP. These GFP-expressing N01.1 cells did not form tumors after they were transplanted into normal rat spinal cords, suggesting that they are safe for transplantation. However, when they were transplanted into injured rat spinal cords, these cells suffered enormous cell loss. Preliminary result of our laboratory showed that lithium considerably increased N01.1 cell number in vitro. Dr. Masayuki Hashimoto and Dr. Tsutomu Iseda in our laboratory therefore tested whether lithium could increase
numbers of GFP-expressing N01.1 cells after they were transplanted into injured rat spinal cords. Section 1 will describe these studies.

Neurotrophic factors are growth factors that support nerve cell survival and growth. Four neurotrophin family members have been identified in the mammalian brain: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurophin-3 (NT-3), and NT-4 (Ip and Yancopoulos, 1996). Besides neurotrophin family proteins, ciliary neurotrophic factor (CNTF), glial cell line-derived neurotrophic factor (GDNF), as well as leukemia inhibitory factor (LIF), are also recognized as neurotrophic factors (Ip and Yancopoulos, 1996; Murphy et al., 1997; Sariola and Saarma, 2003).

Lithium treatment stimulates neurotrophic factor expression in specific brain regions of normal rats and rat models of mood disorders. For example, chronic treatment with lithium, as well as another well prescribed mood stabilizer valproate, increases BDNF content in the hippocampus and frontal cortex of normal rat brain (Fukumoto et al., 2001). In addition, subchronic lithium treatment increases NGF content in normal adult rat hippocampus, frontal cortex, amygdala and limbic forebrain (Hellweg et al., 2002). In a rat model of mania induced by application of D-amphetamine, lithium treatment increased BDNF, NGF, and NT-3 in the hippocampus (Frey et al., 2006a; Frey et al., 2006b; Walz et al., 2008). In an animal model of depression, Flinders Sensitive Line (FSL) rats, lithium increased NGF level in the hippocampus (Angelucci et al., 2003). Compared to the FSL rats, the control normal Flinders Resistant Line rats did not show significant lithium-induced alteration in NGF level in the hippocampus.
Lithium stimulation of neurotrophic factor expression in rodent brain regions suggests that neurotrophic factor stimulation may contribute to the therapeutic effects of lithium in manic depression. We were interested to know whether lithium could stimulate neurotrophic factor expression in the spinal cord to benefit spinal cord injury. We also would like to find out whether N01.1 cell transplantation could benefit spinal cord injury by stimulating neurotrophic factor expression in the spinal cord. Lithium stimulates neurotrophic factor BDNF to protect neurons from glutamate-induced excitotoxicity (Hashimoto et al., 2002). We wonder whether lithium could stimulate neurotrophic factor expression in cultured blood-derived N01.1 cells. Interestingly, my experimental results showed that lithium significantly increased RNA levels of neurotrophic factor LIF and NGFβ in cultured N01.1 cells. We thus get very interested to find out whether lithium could stimulate neurotrophic factor expression in injured rat spinal cords with GFP-expressing N01.1 cell transplantation. Dr. Masayuki Hashimoto and Dr. Tsutomu Iseda in our laboratory therefore performed a study to check neurotrophic factor expression in injured spinal cords of four groups of rats (n=3 per group): Injury, Injury/LiCl, Injury/N01.1, and Injury/N01.1/LiCl. These studies will also be described in section 1.

Recent animal studies suggest that lithium can also be used to treat other central nervous system (CNS) diseases, such as brain ischemia, spinal cord injury, Alzheimer’s disease, Huntington’s disease, etc (Nonaka and Chuang, 1998; Wei et al., 2001; Alvarez et al., 2002; Wood and Morton, 2003; Xu et al., 2003; Senatorov et al., 2004; Terao et al., 2006; Yan et al., 2007). The mechanisms of lithium’s beneficial effects on CNS diseases may involve stimulation of neural precursor cells. Lithium increases neurogenesis in the
dentate gyrus of rodent hippocampus, and selectively increases neuronal differentiation of hippocampal neural progenitor cells in vitro (Chen et al., 2000; Kim et al., 2004). In addition, lithium promotes neural precursor cell proliferation in embryonic rat cerebral cortical cultures as well as postnatal rat cerebellar granule cell cultures (Hashimoto et al., 2003). My studies showed that lithium considerably increased cell numbers in both cultures of primary neural precursor cells derived from neonatal rat subventricular zone and olfactory bulb, and cultures of radial glial (neural precursor) cell clone RG3.6 derived from embryonic rat cerebral cortex, mainly due to increased cell proliferation rather than increased cell survival.

The molecular mechanism by which lithium stimulates neural precursor proliferation is not well understood. Lithium inhibits many enzymes that participate in a wide arrange of cellular activities (Quiroz et al., 2004). Many investigators have proposed theories concerning the molecular mechanisms of lithium’s beneficial effects on CNS diseases including bipolar disorder. Currently three major theories dominate the field: stimulation of neurotrophic factors, inositol depletion, and GSK-3 inhibition.

**Neurotrophic factor stimulation hypothesis:**

Neurotrophic factors support nerve cell survival and growth and other functions in the CNS (Ip and Yancopoulos, 1996). As I stated before, lithium stimulates neurotrophic factor expression in specific brain regions of normal rats and rat models of mood disorders, suggesting that neurotrophic factor stimulation may contribute to lithium’s therapeutic effects in CNS diseases including bipolar disorder. Lithium stimulates neurotrophic factor BDNF expression in rat cerebral cortical neurons, and BDNF plays
an essential role in lithium-mediated neuroprotection against glutamate-induced excitotoxicity in vitro (Hashimoto et al., 2002).

*Inositol depletion hypothesis*

Lithium inhibits the activity of inositol polyphosphate 1-phosphatase (IPPase) and inositol monophosphate phosphatase (IMPase) (Hallcher and Sherman, 1980; Agam and Livne, 1989). IPPase and IMPase are two critical enzymes involved in the recycling and de novo synthesis of inositol. IPPase catalyzes the formation of inositol monophosphate from inositol (1, 4) bis-phosphate. IMPase catalyzes the formation of inositol from inositol 1 (or 4) monophosphate.

Inositol is the substrate for synthesis of the membrane lipid phosphatidylinositol (PI), which is then phosphorylated on the inositol carbon ring to form mono- bis- tris-phosphatidylinositol phosphate forms. Phosphatidylinositol (4, 5) bis-phosphate (PIP\(_2\)) is hydrolyzed by the enzyme phospholipase C (PLC), whose activity is regulated by a wide range of extracellular stimuli, to form diacyl glycerol (DAG) and inositol (1, 4, 5) tris-phosphate (IP\(_3\)), two important intracellular signaling molecules that modulate the activity of a multitude of intracellular events. Inositol is recycled through sequential dephosphorylation of IP\(_3\), which includes dephosphorylation of IP\(_3\) to form inositol (1, 4) bis-phosphate (IP\(_2\)), dephosphorylation of IP\(_2\) to form inositol monophosphate (IMP) by IPPase, and dephosphorylation of IMP to form inositol by IMPase. De novo synthesis of inositol involves formation of IMP by isomerization of glucose-6-phosphate, and subsequent dephosphorylation of IMP by IMPase.
Suppression of IPPase and IMPase by lithium inhibits inositol recycling and de novo synthesis, which can lead to depletion of inositol. Inositol depletion has been proposed to be the mechanism of lithium action in the central nervous system (Quiroz et al., 2004). The inositol depletion theory suggests that inositol depletion leads to reduced synthesis of the membrane lipid phosphatidylinositol from inositol, and subsequent synthesis of phosphatidylinositol phosphate forms, thus decreasing the amount of phosphatidylinositol polyphosphate available for intracellular signaling.

Much evidence supports the inositol depletion hypothesis. For example, lithium reduced inositol or myo-inositol (another form of inositol) levels in rat or mouse brain and in cholinergically stimulated rat parotid gland slices or cerebral cortex slices (Allison and Stewart, 1971; Downes and Stone, 1986; Kennedy et al., 1990; Hirvonen, 1991; Huang et al., 2000; O'Donnell et al., 2003; McGrath et al., 2006). Lithium administration reduced myo-inositol level in the brain of human subjects with manic depressive illness (Moore et al., 1999; Davanzo et al., 2001). Lithium prevented the accumulation of inositol polyphosphate second messengers following cholinergic stimulation of cerebral cortex slices, and pre-incubation with myo-inositol reversed this effect of lithium (Kennedy et al., 1989).

**GSK-3 inhibition hypothesis**

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase that has diverse functions in many cellular activities, including regulation of cell survival, control of cell division, transport of intracellular organelles, regulation of neuronal growth cones, glycogen synthesis, amyloid metabolism, phosphorylation of the microtubule associated
proteins tau and MAP1B, etc (Gould and Manji, 2005). First identified in 1980 as a protein that phosphorylates and deactivates glycogen synthase, and hence its name (Embi et al., 1980), GSK-3 is highly conserved throughout eukaryotes. It is found in vertebrates as two isoforms, GSK-3α and GSK-3β, which have 97% sequence homology in their catalytic domains, and have similar, but not identical biological functions (Gould and Manji, 2005). It is generally considered constitutively active inside the cell and is inactivated by various signaling events (Quiroz et al., 2004).

Several investigators reported in 1996 that lithium directly inhibits GSK-3β (Klein and Melton, 1996; Stambolic et al., 1996). Since then, lithium has been shown to inhibit GSK-3β activity in many types of cells and in the frontal cortex of rat brain (Stambolic et al., 1996; Choi and Sung, 2000; Hongisto et al., 2003; Beurel et al., 2004; Gould et al., 2004; Jonathan Ryves et al., 2005; Li et al., 2007a). Loss of GSK-3 function in Xenopus and Dictyostelium results in developmental abnormalities that are phenocopied by lithium treatment (Klein and Melton, 1996; Stambolic et al., 1996). Mice with heterozygous loss of GSK-3β genotype exhibit behavioral and molecular changes similar to those induced by lithium treatment (O'Brien et al., 2004). On the other hand, transgenic mice overexpressing GSK-3β show hyperactivity resembling that observed in the manic phase of bipolar disorders (Prickaerts et al., 2006). These data suggest that GSK-3β may be involved in the pathophysiology of bipolar disorder and the therapeutic action of lithium.

To determine the mechanism by which lithium stimulates neural precursor cell proliferation, I tested these three hypotheses of lithium action (Figure 10). My results
suggest that lithium might stimulate neural precursor cell proliferation without necessarily stimulating neurotrophic factors, nor through inositol depletion, but through GSK-3β inhibition, and subsequent NFAT activation. These studies will be described in section 2.
My experiments showed that lithium significantly increased cell numbers in rat primary neural precursor cells, mouse primary neural precursor cells, and neural precursor cell clone RG3.6 cells. Whether lithium-induced increase in cell numbers was due to increased cell proliferation or increased cell survival was examined. In addition, I tried to decipher the mechanism by which lithium promotes neural precursor cell growth. Specifically, whether lithium stimulates neural precursor cell growth through neurotrophic factor stimulation, inositol depletion, or GSK-3β inhibition and subsequent NFAT activation was investigated.

**Figure 8. Study of lithium effects on neural precursor cells.**
Materials and Methods

Antibodies and Reagents

Table 1 (Page 25) lists the antibodies used in the experiments and their sources. Table 2 (Page 26) lists the reagents used in the experiments and their sources.

Cell Culture

Neural precursor cell clone RG3.6, and blood-derived N01.1 cells were obtained and cultured as described in the materials and methods section of chapter I.

Mrs. Iman Tadmori and I isolated rat primary neural precursor cells according to the following procedure. Mrs. Iman Tadmori isolated primary neural precursor cells from mice using the same procedure. Neonatal rats or mice were dissected to obtain neural precursor cells from the olfactory bulbs and subventricular zones. For each animal, the skull and the dura mater were dissected away from the cortex after removing the head skin. Two olfactory bulbs were extracted from the front of the cortex above the eyes, and the cortical hemispheres were then separated. Tissue samples from the subventricular zone of each hemisphere were dissected out. All the tissue samples were mixed together. After washing with cold Hanks Balanced Salt Solution (HBSS, Invitrogen), the samples were suspended in 0.05% trypsin (Sigma-Aldrich), minced by sequential passage through an 18-gauge needle 5 times, a 21-gauge needle 5 times, and a 25-gauge needle 5 times, and then incubated at 37 °C for 5 minutes to dissociate cells. After adding an equal volume of trypsin inhibitor (0.25 mg/ml, Sigma-Aldrich), cells
were further dissociated by pipetting up and down (about 10 times). Samples were spun down at \(~200\) g for 5 minutes. The supernatant was removed. The samples were washed twice with DMEM/F12 (Invitrogen), and resuspended in culture medium, i.e. DMEM/F12 (Invitrogen) supplemented with 25 mM glucose (Sigma-Aldrich), 100 µg/ml streptomycin (Invitrogen), 100 units/ml penicillin (Invitrogen), 10 ng/ml FGF2 (BD Biosciences), 10 ng/ml EGF (BD Biosciences), and 1X B27 (Invitrogen). Finally, after filtered through a 70 µm nylon mesh, the cell samples were incubated at 37 °C in a humidified atmosphere of 95% air + 5% CO₂.

Cells were further dissociated 24 hours later using the following procedure. The samples were washed three times with DMEM/F12, pipetted up and down for about 25 times at each wash. Samples were then re-suspended in new culture medium, filtered through a 40 µm nylon mesh and incubated at 37 °C in a humidified atmosphere of 95% air + 5% CO₂. One-tenth of the culture medium was replenished every other day with 10X FGF medium, i.e. DMEM/F12 (Invitrogen) supplemented with 25 mM glucose (Sigma-Aldrich), 100 µg/ml streptomycin (Invitrogen), 100 units/ml penicillin (Invitrogen), 100 ng/ml FGF2 (BD Biosciences) and 1X B27 (Invitrogen).

The cultures were passaged when the neurospheres that had formed grew into balls of 50 to 100 cells, usually in about one week. The following passage procedure was used. Remove the supernatant after spinning down the cells. Add one-tenth culture medium volume of 0.05% trypsin-EDTA (Invitrogen), and incubate at room temperature for about 2-3 minutes, tap the cells occasionally. Add an equal amount of trypsin inhibitor (Sigma-Aldrich) to stop the trypsin digestion, and pipette up and down to dissociate the cells. Spin down the cells and wash the cells for 2 times with culture
medium. Incubate the cells in culture medium at 37 °C in a humidified atmosphere of 95% air + 5% CO2.

**Cell Count Analysis**

Cells count analysis was done as described in the materials and methods section of chapter I.

**BrdU Labeling and Immunocytochemistry**

The procedure is modified from the Millipore online protocol (http://www.millipore.com/cellbiology/cb3/brdulabeling). RG3.6 cells were plated on coverslips coated with laminin (20 µg/ml, Invitrogen) in culture medium. After 3 days of culturing, Bromodeoxyuridine (BrdU, Sigma-Aldrich) was added to the medium to reach a final concentration of 10 µM to label proliferating cells. Four hours after BrdU addition, the cultures were stopped by fixing the cells with 4% para-formaldehyde at room temperature for 15 minutes. The fixed cells were washed three times with 0.01 M phosphate buffered saline (PBS, pH 7.4). The coverslips were treated for 30 minutes with 2 N HCl in water at room temperature. After the acid was removed, the coverslips were neutralized by washing 3 times in borate buffer (0.1 M, pH 8.5). The coverslips were treated with 2% normal goat serum (NGS, Invitrogen) in PBS-T (PBS with 0.05% Tween 20) for 30 minutes to block non-specific binding of antibodies. Then mouse monoclonal anti-BrdU antibody (DakoCytomation, 1:100 in PBS-T/2% NGS) was applied for 30 minutes at room temperature. After removal of the primary antibodies, the coverslips were washed with PBS-T for 3 times, 5 minutes per wash. Goat-anti-mouse
AlexaFluor 546 (Molecular Probes, 1:500 in PBS-T/2% NGS) was subsequently applied for about 30 minutes followed by DAPI (4',6-Diamidino-2-phenylindole, Molecular Probes, 1:1000 in PBS-T/2% NGS) nuclear counterstaining for 10 minutes at room temperature. The coverslips were washed again with PBS-T for 3 times, 5 minutes per wash. Coverslips were mounted onto slides in mounting media (Invitrogen). The fluorescent staining was visualized and photographed using fluorescent microscope.

**Flow Cytometry**

RG3.6 neurospheres were treated with trypsin-EDTA (0.25% trypsin, 1 mM EDTA•4Na, Invitrogen) for 3 to 5 minutes at room temperature. Afterwards, add an equal volume of 0.25 mg/ml trypsin inhibitor (Sigma-Aldrich) and pipette up and down to dissociate the neurospheres. Wash the cells twice with 1 X PBS (pH 7.4). Flow cytometry was performed to analyze the GFP signals in the samples using a flow cytometer.

**Western Blotting**

Western Blotting was carried out as described in the materials and methods section of chapter I.

**GFP Transfection and Selection**

N01.1 cells were transfected with a plasmid (pCAGGS) containing enhanced green fluorescent protein (EGFP) gene and neomycin-resistance gene (Niwa et al., 1991) using Amaxa nucleofector. The transfected cells were selected by treating the culture
with G418 (neomycin, 200 µg/ml, Invitrogen) and verified by viewing the GFP signal under fluorescent microscope.

**Spinal Cord Injury Model and Treatment**

Rats were housed under 12 h light-dark cycles and were given free access to food and water. Adult Sprague-Dawley rats (77±5 days old) were anesthetized with intraperitoneal injection of pentobarbital (45±5 mg/kg for female and 65±5 mg/kg for male). Laminectomies were performed to expose thoracic segments T9–10. 60±5 minutes after anesthesia induction, spinal cords were contused by dropping a 10-gram rod from a height of 25 mm using the Multicenter Animal Spinal Cord Injury Study (MASCIS) impactor (formerly known as the NYU impactor).

Immediately after injury, GFP-positive N01.1 cells were injected into the spinal cord at 2 points, 2 mm proximal and 2 mm distal to the impact center, at 100,000 cells/µl/point. The exposed muscle and skin were closed in layers. After the surgery, the rats were kept on warming pads until they wake, and then returned to their home cage. LiCl (80 mg/ml in saline, 100 mg/kg body weight) or equal volume of control saline was injected subcutaneously once daily for 2 weeks before the animals were euthanized. Saline was provided ad libitum to reduce potential toxicity. The therapeutic serum concentrations of lithium in humans is 0.6 – 1.2 mEq/L (Hopkins and Gelenberg, 2000), and this lithium treatment regimen should produce therapeutic serum levels of lithium (McQuade et al., 2004; Tsaltas et al., 2007). No significant differences in the body weights of lithium-treated and control saline-treated rats were observed. Rats that
received lithium treatment developed polyuria, a side effect also reported by other investigators (Smith, 1976; Pies, 1993).

**RNA and DNA Extraction from Spinal Cord Tissue**

Animals were anesthetized with intraperitoneal injection of pentobarbital (45±5 mg/kg for female and 65±5 mg/kg for male) and decapitated. Spinal cords were quickly removed, chilled on dry ice. Each 25 mm-long spinal cord segment centered at the impact site was cut out, placed in 2.5 ml trizol reagent (Invitrogen), and homogenized using a tissue homogenizer with a micro-grinding tip. For each 0.5 ml homogenized sample, 100 µl of chloroform was added. Samples were mixed by vortex or pipetting for 5 seconds or until cloudy. After placed on ice for 5 minutes, samples were centrifuged at 13,000 rpm for 15 minutes at 4 °C. The RNA containing upper aqueous phase was transferred to a new tube, and an equal volume of 70% ethanol was added into each new tube. Samples were mixed by pipetting and applied to RNeasy mini-spin column (Qiagen). RNA was subsequently purified using RNeasy Mini kit (Qiagen) according to the manufacturer’s RNeasy spin protocol. The interphase and the lower phenol-chloroform phase of the initial homogenates contain DNA. After complete removal of the RNA-containing upper aqueous phase, DNA was extracted from the interphase and the lower organic phase of the initial homogenates according to the Trizol reagent (Invitrogen) manufacturer’s protocol.

**RNA Extraction from Cell Culture**
RNA from cultured cells was extracted using the RNeasy Mini kit (Qiagen) according the manufacturer’s RNeasy spin protocol.

**Quantitative Real Time PCR**

Quantitative real time PCR was performed as described in the materials and methods section of Chapter I.

**Affymetrix Microarray Analysis**

Total RNA was extracted from RG3.6 cells grown for 3 days in culture medium (DMEM/F12 (Invitrogen) supplemented with 25 mM glucose (Sigma-Aldrich), 100 µg/ml streptomycin (Invitrogen), 100 units/ml penicillin (Invitrogen), 0.2 ng/ml FGF2 (BD Biosciences), 2 µg/ml heparin (Sigma-Aldrich), and 1X B27 (Invitrogen)) containing 3 mM lithium chloride or 3 mM control sodium chloride (3 samples per condition). The integrity of the RNAs was examined using the Agilent platform (Agilent 2100 Bioanalyzer). GeneChip® Rat Genome 230 2.0 Array (Affymetrix) was used. The Transcriptional Facility Shared Resource of the Cancer Institute of New Jersey performed biotin-labeling of cDNA and subsequent hybridization. GeneSpring GX 9 software (Agilent) was used to screen genes whose RNA expression was significantly altered by lithium treatment.
Section 1

Effects of Lithium on Blood-Derived Cells

Results

*Lithium increases numbers of N01.1 cells in culture*

Lithium promotes proliferation or survival of many types of cells, including neurons, neural precursor cells and various hematopoietic cells (Gamba-Vitalo et al., 1983; Doukas et al., 1986; Kucharz et al., 1988; Korycka and Robak, 1991; Levine et al., 2000; Hashimoto et al., 2002; Hashimoto et al., 2003). Mrs. Diane Vy in our laboratory together with some undergraduate students checked whether lithium could stimulate N01.1 cell growth. Their preliminary result showed that one week treatment with 3 mM lithium chloride, a dose that optimally promoted neural precursor cell clone RG3.6 proliferation (Figures 15-16, 19-21), induced almost 4-fold cell number increase in N01.1 cell cultures (Figure 9). This result suggests that lithium may promote in vitro N01.1 cell survival, proliferation, or both.

*Lithium increases numbers of N01.1 cells transplanted into injured rat spinal cords*

Excited by the result that lithium remarkably increased numbers of N01.1 cells in vitro (Figure 9), Dr. Masayuki Hashimoto and Dr. Tsutomu Iseda in our laboratory checked whether lithium could increase numbers of GFP-expressing N01.1 cells after they were transplanted into injured rat spinal cords. These N01.1 cells were transplanted
into rat spinal cords right after contusive spinal cord injury, produced by dropping a 10-gram rod from 25 mm height using the MASCIS impactor (n=12). Lithium chloride (100 mg/kg body weight) or control saline was injected subcutaneously every day (n=6 for each treatment group). At two weeks after spinal cord injury and cell transplantation, the rats were euthanized, the spinal cords were taken out and examined with an epifluorescent dissecting microscope. Spinal cords from lithium chloride treated animals had much more green fluorescent signals than control saline treated animals (Figure 10), indicating much more transplanted N01.1 cells in spinal cords of lithium-treated rats. To quantify the GFP in the spinal cords, DNA and RNA were extracted from spinal cord samples (25 mm long centered at the impact center) containing the impact and cell injection site. Quantitative real time PCR for GFP RNA expression showed that spinal cords from lithium chloride treated animals contain about 1000 times more GFP RNA than spinal cords from control saline treated animals (Figure 11). Genomic DNA PCR also showed that spinal cords from lithium-treated animals contain much more GFP DNA than spinal cords from control saline treated animals (Figure 12). These results clearly demonstrated that lithium promoted N01.1 cell survival, proliferation, or both, in injured rat spinal cords.

**Lithium increases RNA levels of LIF and NGFβ in cultured N01.1 cells**

Lithium stimulates neurotrophic factors in specific regions of rodent brain and in cultured cortical neurons (Fukumoto et al., 2001; Hashimoto et al., 2002; Hellweg et al., 2002; Angelucci et al., 2003; Frey et al., 2006a; Frey et al., 2006b; Walz et al., 2008). I checked whether lithium could increase RNA levels of neurotrophic factors BDNF,
GDNF, LIF, NGFβ, and NGFγ in N01.1 cells, using quantitative real time PCR. Treatment with 3 mM lithium for one week significantly increased RNA levels of LIF and NGFβ, and had no significant effect on RNA levels of BDNF, GDNF, and NGFγ in cultured N01.1 cells (Figure 13). These results suggest that lithium may stimulate LIF and NGFβ expression in N01.1 cells.

*Lithium combined with N01.1 transplantation increases neurotrophic factor RNA levels in injured rat spinal cords*

Lithium stimulates neurotrophic factor expression in specific regions of rodent brain (Fukumoto et al., 2001; Angelucci et al., 2003; Frey et al., 2006b). Lithium also increased LIF and NGFβ RNA levels in cultured N01.1 cells. Dr. Masayuki Hashimoto and Dr. Tsutomu Iseda in our laboratory therefore performed a study to check neurotrophic factor expression in injured spinal cords of four groups of rats (n=3 per group): Injury, Injury/LiCl, Injury/N01.1, and Injury/N01.1/LiCl. Spinal cords of rats in all four groups were injured at T10 level by dropping a 10-gram rod from 25 mm height using the MASCIS impactor. The Injury group animals received daily saline administration. The Injury/LiCl animals received daily lithium chloride (LiCl, 100 mg/kg) administration. The Injury/N01.1 group animals received N01.1 cell transplantation right after spinal cord injury and daily saline administration. The Injury/N01.1/LiCl group animals received N01.1 cell transplantation right after spinal cord injury and daily lithium chloride (100 mg/kg) administration. At two weeks after spinal cord injury, the rats were euthanized by decapitation, and the spinal cords were quickly removed. RNAs were extracted from the spinal cord samples (25 mm long
centered at the impact center) containing the injury and cell injection sites. They assessed RNA levels of neurotrophic factors BDNF, GDNF, LIF, NT-3, NGFβ and NGFγ in these RNAs using quantitative real time PCR. Lithium administration alone did not significantly alter neurotrophic factor RNA levels in injured rat spinal cords (Figure 14). N01.1 cell transplantation alone tended to increase RNA levels of BDNF, GDNF, LIF, NT-3, and NGFγ but NGFβ, although the increase was not statistically significant. Lithium treatment combined with N01.1 cell transplantation significantly increased RNA levels of GDNF, LIF, NT-3, NGFβ and NGFγ, and tended to increase BDNF RNA levels in the injured spinal cords. These results suggest that lithium treatment does not stimulate neurotrophic factor expression in the spinal cords after injury, and while transplantation of N01.1 cells into injured spinal cords may stimulate neurotrophic factor expression there, lithium treatment may further enhance neurotrophic factor expression in the injured spinal cords.
Discussion

*Lithium promotes N01.1 cell growth in vitro*

Preliminary result of our laboratory showed that lithium markedly induced almost 4-fold increase of N01.1 cell numbers in vitro after one week of treatment (Figure 9). This experiment needs to be repeated to demonstrate statistical significance of this lithium effect. The result suggests that lithium may promote N01.1 cell survival, proliferation, or both. Since the survival rate of N01.1 cells in this culture condition is very high, lithium may not have much influence on N01.1 cell survival. I observed that after one week of culturing, when N01.1 cell cultures reached 100% confluency, in N01.1 cell cultures treated with 3 mM lithium chloride, there were many three-dimensional clusters of cells, which was absent in control cultures. This suggests that lithium may overcome contact inhibition, promoting cell proliferation in N01.1 cell cultures. Similar to what I discussed in section 1 of chapter I, we could determine whether lithium promotes N01.1 cell survival by measuring proportion of viable cells, dead cells, or apoptotic cells in N01.1 cell cultures. In addition, we could determine whether lithium could stimulate N01.1 cell proliferation by measuring proportion of cells in active proliferation in N01.1 cell cultures.

*Lithium promotes N01.1 cell survival, proliferation, or both, in injured rat spinal cords*

Lithium also robustly increased N01.1 cells two weeks after they were transplanted into injured rat spinal cords (Figures 10-12). This could have been due to stimulation of proliferation, promotion of survival, or both. To determine whether
lithium stimulates N01.1 cell proliferation, we could check whether lithium increases the ratio of GFP-expressing Ki-67-positive cells to GFP-expressing cells in the injured spinal cords at various times after spinal cord injury and cell transplantation, using immunohistochemistry.

The robust increase of transplanted N01.1 cells by lithium in the injured rat spinal cords suggests a powerful method for promoting survival or proliferation of transplanted cells in the CNS after injury. Lithium stimulates growth of bone marrow stromal cells, granulocyte-macrophage progenitor cells, and other hematopoietic cells in vitro (Gallicchio and Chen, 1981; Gamba-Vitalo et al., 1983; Doukas et al., 1986; Korycka and Robak, 1991; Huang et al., 1999). Lithium also stimulates proliferation of neural precursor cells in vitro and after transplanted into normal rat spinal cords (Figures 15-21) (Hashimoto et al., 2003; Kim et al., 2004; Su et al., 2007). Lithium may be useful as a treatment to promote survival of transplanted cells of many types, including neural precursor cells, bone marrow stromal cells, and other cells.

**Lithium may stimulate neurotrophic factor expression in blood-derived N01.1 cells**

Lithium significantly increased RNA levels of two neurotrophic factors, LIF and NGFβ, in cultured blood-derived N01.1 cells (Figure 13). These results suggest that lithium may stimulate LIF and NGFβ expression in N01.1 cells. We will need to confirm LIF and NGFβ expression at the protein level to substantiate this finding. This could be achieved by measuring LIF and NGFβ protein concentration in N01.1 culture medium using enzyme-linked immunosorbent assay (ELISA). To check whether LIF stimulation contributes to lithium’s promotion of N01.1 cell growth, we could use LIF antibody that
block LIF interaction with its receptor or LIF receptor neutralizing antibody to block LIF receptor activation, and check whether this can counteract lithium’s promotion of N01.1 cell growth. Similarly, we could check whether NGFβ stimulation contributes to lithium’s promotion of N01.1 cell growth.

Lithium stimulates neurotrophic factors in specific regions of rodent brain (Fukumoto et al., 2001; Hellweg et al., 2002; Angelucci et al., 2003; Frey et al., 2006a; Frey et al., 2006b; Walz et al., 2008). The source cells for the neurotrophic factors are not clear. Lithium stimulated BDNF in cultured cortical neurons (Hashimoto et al., 2002), suggesting that neurons are one potential source for BDNF. Lithium stimulation of LIF and NGFβ RNA expression in N01.1 cells suggests that blood or blood-derived cells could be another source for neurotrophic factors stimulated by lithium.

*Lithium may stimulate neurotrophic factor expression in injured rat spinal cords transplanted with N01.1 cells*

Lithium treatment alone did not affect neurotrophic factor BDNF, GDNF, LIF, NGFβ, NGFγ and NT-3 RNA levels in the spinal cords two weeks after spinal cord injury (Figure 14). This suggests that lithium does not stimulate neurotrophic factor expression in spinal cord after injury. This could be one reason why lithium treatment alone does not promote regeneration of rubrospinal tract axons, and does not produce significant functional improvement after spinal cord injury (Yick et al., 2004).

N01.1 cell transplantation tended to increase RNA levels of BDNF, GDNF, LIF, NT-3, and NGFγ, in the spinal cords two weeks after injury, although the increase was not statistically significant (Figure 14). This is a very interesting finding that deserves
further investigation. Two weeks after spinal cord injury and N01.1 cell transplantation, most of the transplanted cells had already died. Yet there was a tendency of increased neurotrophic factor expression in the spinal cords, suggesting that N01.1 cell transplantation may improve the microenvironment of the spinal cord and the improved microenvironment may stimulate endogenous spinal cord cells to produce more neurotrophic factors. Next we should check whether N01.1 cell transplantation promotes neurotrophic factor expression more profoundly and more significantly at earlier times when there are more surviving N01.1 cells. We should also check neurotrophic factor expression at the protein level. However, we may not be able to detect an increase in neurotrophic factor protein production confined to a small region in the spinal cord since the neurotrophic factors are diffusible.

Lithium treatment combined with N01.1 cell transplantation significantly increased RNA levels of GDNF, LIF, NT-3, NGFβ and NGFγ, and tended to increase BDNF RNA levels in rat spinal cords two weeks after spinal cord injury (Figure 14). The increased expression of neurotrophic factors is not likely the driving force for the robust increase of transplanted N01.1 in the injured spinal cords of rats treated with lithium. Neurotrophic factor RNA levels tended to be higher in the injured rat spinal cords transplanted with N01.1 cells than in the spinal cords without N01.1 cell transplantation, yet most of the transplanted cells died. Further increase in neurotrophic factors should not make such a big difference in transplanted N01.1 cell survival. On the other hand, the increased N01.1 cell number may contribute to the increased neurotrophic factor RNA levels in the injured rat spinal cords, since more N01.1 cells may better improve the microenvironment in the spinal cord, leading to further increased neurotrophic factor
expression by endogenous spinal cord cells. In addition, the increase of neurotrophic factor LIF and NGFβ RNA in lithium-treated rat spinal cords may be partially from the N01.1 cells, since lithium robustly increased N01.1 cells in the spinal cords, and lithium stimulates LIF and NGFβ RNA expression in cultured N01.1 cells.

In summary, our experimental results suggest that lithium promotes N01.1 cells growth in vitro, and substantially promotes N01.1 cell survival, proliferation, or both, after they are transplanted into injured rat spinal cords. In addition, lithium may stimulate neurotrophic factor expression in cultured N01.1 cells, suggesting that blood or blood-derived cells could be one source for increased neurotrophic factor expression in the CNS of lithium-treated animals. Furthermore, N01.1 cell transplantation may stimulate neurotrophic factor expression in spinal cord after injury, and N01.1 cell transplantation combined with lithium treatment may further enhance neurotrophic factor expression in injured rat spinal cords. These data suggest that lithium treatment may provide a powerful method of improving survival or proliferation of cells transplanted into the CNS after injury, and that blood-derived cell transplantation combined with lithium treatment may benefit CNS injury.
Figure 9. Lithium increased cell number in N01.1 cell cultures.

N01.1 cells were cultured in DMEM medium supplemented with 5% fetal bovine serum (FBS), in the absence (control) or presence of 3 mM LiCl for 7 days. Then the cells were detached by trypsinization, and mechanically dissociated after adding the 10% FBS-containing medium to stop the trypsin activity. Cell number in each culture condition was calculated after counting the cells using a hemacytometer. The result is preliminary. Lithium treatment induced almost 4-fold cell number increase in N01.1 cell cultures.
Figure 10. Much stronger green fluorescent signal from transplanted GFP expressing N01.1 cells was detected in injured rat spinal cords treated with lithium.

Spinal cords of Sprague-Dawley rats (77 ± 1 days) were contused by dropping a 10-gram rod from 25 mm height (n=12). Immediately after injury, GFP-positive N01.1 cells were injected into the injured spinal cords. Lithium chloride (100 mg/kg) (n=6) or control saline (n=6) was administered daily via subcutaneous injection. Two weeks after spinal cord injury and cell transplantation, the animals were euthanized and the spinal cords were taken out for examination under epifluorescent dissecting microscope. Much stronger green fluorescent signal was detected in all the spinal cords of animals treated with lithium, indicating the presence of much more N01.1 cells. Left: samples of 2 spinal cords from animals treated with saline. Right: samples of 2 spinal cords from animals treated with lithium chloride. Space between bars: 1 mm.
Figure 11. Lithium markedly increased GFP RNA levels in injured rat spinal cords transplanted with GFP-expressing N01.1 cells.

Sprague-Dawley rats received contusive spinal cord injury followed by GFP-positive N01.1 cell transplantation and lithium chloride (LiCl) (n=6) or saline treatment (n=6) for 2 weeks, as indicated in the legends of Figure 32. RNA was extracted from the spinal cords of these rats for quantitative real-time PCR analysis. The primers used are listed in Table 3 (Page 28). GFP expression was normalized to that of GAPDH. The data represent mean ± standard deviation. Note the scale is logarithmic. *: Bonferroni/Dunn, P < 0.0001. GFP RNA level in spinal cords of lithium treated rats is about 1000 times higher than that in spinal cords of control rats.
Figure 12. Lithium markedly increased GFP DNA in injured rat spinal cords transplanted with GFP-expressing N01.1 cells.

Rats received contusive spinal cord injury followed by GFP-positive N01.1 cell transplantation and lithium or control saline treatment for 2 weeks. PCR for GFP DNA was carried out using equal amounts of DNAs extracted from spinal cord samples of these rats. The following primers were used. GFP-forward: 5'-TGA ACC GCA TCG AGC TGA AGG G-3'; and GFP-reverse: 5'-TCC AGC AGG ACC ATG TGA TCG C-3'. Much more GFP DNA was present in spinal cords of rats (n=5) treated with lithium chloride than in spinal cords of rats (n=3) treated with control saline.
Figure 13. Lithium increased LIF and NGFβ RNA levels in cultured N01.1 cells.

N01.1 cells were grown in culture medium (DMEM supplemented with 5% fetal bovine serum) containing 3 mM LiCl or 3 mM NaCl (n=3 for each condition). After one week of culturing, the cells were lysed for RNA extraction and subsequent quantitative real time PCR analysis. RNA levels of neurotrophic factors were normalized to that of GAPDH. The data represent mean ± standard error. *: Bonferroni/Dunn, P < 0.05 compared to NaCl control condition.
Figure 14. Lithium treatment combined with N01.1 cell transplantation greatly increased RNA levels of neurotrophic factors in injured rat spinal cords.

Four groups of Sprague-Dawley rats (n=12) received spinal cord injury. The Injury group animals (blue, n=3) received daily saline administration. The Injury/LiCl group animals (red, n=3) received daily lithium chloride (100 mg/kg) administration. The Injury/N01.1 group animals (green, n=3) received N01.1 cell transplantation right after spinal cord injury and daily saline administration. The Injury/N01.1/LiCl group animals (purple, n=3) received N01.1 cell transplantation right after spinal cord injury
and daily lithium chloride (100 mg/kg) administration. At two weeks after spinal cord injury, the animals were euthanized. RNAs were extracted from the spinal cord samples of these rats. Quantitative real time PCR was carried out to assess RNA levels of neurotrophic factors in the spinal cords. The data represent mean ± standard deviation. Compared to the injury group without treatment, N01.1 cell transplantation tended to increase RNA levels of BDNF, GDNF, LIF, NT-3, and NGFγ. However, the increase was not statistically significant. Compared to the other three groups, lithium treatment combined with N01.1 cell transplantation significantly increased RNA levels of GDNF, LIF, NT-3, NGFβ and NGFγ in the spinal cord two weeks after injury. *: Bonferroni/Dunn, P < 0.01 compared to the other three groups.
Section 2

Effects of Lithium on Neural Precursor Cells

Results

*Lithium significantly increases cell numbers in neural precursor cell cultures*

I first tested whether lithium could stimulate cell number increase in cultures of neural precursor cell clone RG3.6 cells. To determine the dose response of RG3.6 cells to lithium, RG3.6 cells were grown in culture media with 0, 0.5, 1.0, 3.0, and 5.0 mM of lithium chloride (LiCl) for one week, and cell numbers were determined using cell count analysis. Lithium chloride at 3 mM optimally increased RG3.6 cell numbers (Figure 15), and this dose was thus used in later in vitro experiments. To rule out possible effects of chloride or osmolarity, I also treated RG3.6 cells with the same concentration (3 mM) of sodium chloride (NaCl) as a control. In contrast to LiCl treated cultures, NaCl-treated cultures contained similar cell number as control medium-treated cultures (Figure 16), suggesting that the cell number increase induced by LiCl treatment was through the lithium ion but not the chloride ion. To confirm that this is not a special property of the neural precursor clone RG3.6 cells but a general effect of lithium on neural precursor cells, I checked whether lithium could also induce cell number increase in primary neural precursor cell cultures. My results showed that lithium also significantly increased cell numbers in cultures of primary neural precursor cells isolated from neonatal rat subventricular zone and olfactory bulb (Figure 17), and in cultures of primary neural precursor cells isolated from neonatal mouse subventricular zone and olfactory bulb
Figure 18. These results suggest that lithium promotes neural precursor cell survival, proliferation, or both.

**Lithium has no significant effect on percentage of surviving cells in RG3.6 cell cultures**

We next would like to figure out whether lithium promotes neural precursor cell survival, proliferation, or both. We used RG3.6 cells as a model system to determine this, since RG3.6 cells are easier to manipulate than primary neural precursor cells. RG3.6 cells are derived from GFP transgenic rat and express GFP. I wonder whether GFP can be used as a marker for RG3.6 cell survival. I stained RG3.6 cells with propidium iodide, a red fluorescent dye that stains only dead cells. I found that propidium iodide-positive red dead cells do not express GFP, and vice versa, GFP-expressing cells are negative for propidium iodide (Figure 19). This suggests that GFP can be used as a marker for RG3.6 cell survival. I therefore did flow cytometry to determine whether lithium increases the proportion of GFP-expressing live cells in RG3.6 cell cultures. RG3.6 cells grown in 3 mM LiCl or 3 mM control NaCl-containing medium had similar GFP histograms, and there was no significant difference in the percentage of GFP-expressing cells in these two culture conditions (Figure 20). These results suggest that lithium does not affect RG3.6 cell survival. In each GFP histogram of the LiCl or control NaCl-treated cultures, there were two curves. I found that the lower curve on the left corresponds to a smaller population of smaller cells that express lower levels of GFP, and the higher curve on the right corresponds to a larger population of larger cells that express higher levels of GFP. Those smaller cells expressing lower levels of GFP might be quiescent cells or cells
undergoing cell differentiation under these culture conditions. Immunostaining with cell cycle markers and cell type markers will help to identify these cells.

$Lithium significantly increases percentage of BrdU$\(^+\) $cells in RG3.6 cell cultures$

Lithium does not affect RG3.6 cell survival, thus lithium-induced cell number increase in neural precursor cell cultures must be due to lithium-promoted cell proliferation. To confirm this, I used Bromodeoxyuridine (BrdU) incorporation assay to label proliferating cells in RG3.6 cell cultures, since BrdU only gets incorporated into cell chromosome in significant amount during DNA synthesis of cell proliferation. My results showed that lithium significantly increased the percentage of BrdU-positive cells (Figure 21), suggesting that lithium indeed promotes RG3.6 cell proliferation.

$Lithium stimulates RNA expression of proliferation-related genes in RG3.6 cells$

To assess lithium-induced gene expression changes in neural precursor cells, I did gene array analyses of RG3.6 cells grown in culture medium (DMEM/F12 supplemented with 25 mM glucose, 100 $\mu$g/ml streptomycin, 100 units/ml penicillin, 0.2 ng/ml FGF2, 2 $\mu$g/ml heparin, and 1X B27) containing 3 mM LiCl (n=3) or control 3 mM NaCl (n=3) for 3 days. The microarray data files were analyzed using GeneSpring GX 9 software (Agilent). The gene entities were filtered by expression based on all six samples having signal intensity between 20% and 100%. Statistical analysis using paired t test, asymptotic P-value computation, and Benjamini-Hochberg multiple testing correction resulted 0 entry for P < 0.05. Thus I used unpaired t test and more stringent P value (P < 0.02) in the statistical analysis. After statistical analysis using unpaired t test, asymptotic
P-value computation, and Benjamini-Hochberg multiple testing correction, and then eliminating the entities that are transcribed loci without gene symbols, the analysis revealed a list of 19 genes whose RNA levels were significantly altered more than two fold by lithium (P < 0.02, compared to control 3 mM NaCl-treated samples). Among these genes, 17 genes were up-regulated and 2 genes were down-regulated by lithium. Through GO (gene ontology) analysis, Pubmed gene search using the Entrez gene number, and literature research (Tatsumoto et al., 1999; Saito et al., 2003; Miki et al., 2005; Oceguera-Yanez et al., 2005), 14 genes (Table 4) were found to be involved in proliferation-related biological processes, such as cell division, cell cycle, regulation of cell cycle, cytokinesis, meiosis, or mitosis. Thus, of the 17 genes that were significantly up-regulated by lithium, a large majority (82%) are proliferation related. The gene array analysis did not reveal any known cell survival-related genes or neurotrophic factors that were significantly up-regulated by lithium. These data add another support that lithium promotes proliferation but not survival of RG3.6 cells, and suggest that lithium does not stimulate neurotrophic factor expression in RG3.6 cells.

Lithium has diverse effects on neurotrophic factor RNA expression in neural precursors

Lithium stimulates brain-derived neurotrophic factor (BDNF) expression in cultured cortical neurons and BDNF plays an important role in lithium-induced neuroprotection against glutamate excitotoxicity (Hashimoto et al., 2002). Lithium stimulates neurotrophic factor LIF and NGFβ RNA expression in cultured blood-derived N01.1 cells (Figure 13). We thus expected that lithium might also stimulate some neurotrophic factor RNA expression in neural precursor clone RG3.6 cells. It was a
surprise that the gene array analysis did not reveal any known neurotrophic factors that were significantly up-regulated by lithium. We tried to confirm this using quantitative real time PCR. Even more surprisingly, lithium not only did not increase, but significantly reduced RNA levels of BDNF, CNTF, GDNF, LIF, NGFβ, NGFγ, and NT-3, in RG3.6 cells (Figure 22). I repeated the whole experiment, and still found the same pattern of lithium-induced neurotrophic factor RNA reduction in RG3.6 cells. The failure for the gene array analysis to detect these gene expression changes is possibly due to the lower sensitivity of the gene array assay and the smaller sample size (n=3) in the gene array experiments. We then checked what effects lithium exerts on neurotrophic factor RNA expression in primary neural precursor cells isolated from neonatal rat subventricular zone and olfactory bulb. In contrast to RG3.6 cells, lithium had no significant effect on RNA levels of CNTF, GDNF, LIF, NGFβ, NGFγ, and NT-3, but significantly increased RNA levels of BDNF in rat primary neural precursor cells (Figure 23). Since lithium promotes the growth of both RG3.6 cells and rat primary neural precursor cells, but has very different effects on neurotrophic factor expression, it is unlikely that stimulation of neurotrophic factor expression accounts for a common mechanism by which lithium stimulates neural precursor cell growth.

Adding myo-inositol does not affect lithium-induced RG3.6 cell number increase

Lithium can inhibit the activity of IPPase (inositol polyphosphate 1-phosphatase), and IMPase (inositol monophosphate phosphatase), two critical enzymes involved in the recycling and de novo synthesis of inositol (Hallcher and Sherman, 1980; Agam and Livne, 1989). Suppression of these two enzymes by lithium can result in inositol
depletion. Lithium-induced inositol depletion has been shown in rodent brain and in vitro culture (Allison and Stewart, 1971; Downes and Stone, 1986; Kennedy et al., 1990; Hirvonen, 1991; Huang et al., 2000; O'Donnell et al., 2003; McGrath et al., 2006), and has been proposed to be a mechanism for lithium’s action in bipolar disorder.

To find out whether inositol depletion is the mechanism by which lithium promotes neural precursor cell proliferation, I added myo-inositol (1 mM and 10 mM) to RG3.6 cultures treated with or without 3 mM LiCl. Adding myo-inositol did not significantly change RG3.6 cell numbers and did not alter the lithium-induced cell number increase (Figure 24). These results suggest that inositol depletion does not contribute to lithium’s promotion of neural precursor cell proliferation.

Lithium may increase inhibitory phosphorylation of GSK-3β in RG3.6 cells

Lithium inhibits GSK-3β activity in many types of cells (Stambolic et al., 1996; Choi and Sung, 2000; Hongisto et al., 2003; Beurel et al., 2004; Jonathan Ryves et al., 2005; Li et al., 2007a). Lithium can inhibit GSK-3β directly by competing with the metal co-factor magnesium of GSK-3β. Lithium can also inhibit GSK-3β indirectly by increasing inhibitory phosphorylation at position Serine 9 (Jope, 2003; Zhang et al., 2003). I examined the inhibitory phosphorylation (Serine 9) of GSK-3β in RG3.6 cells cultured in the presence of 3 mM LiCl or 3 mM control NaCl for 3 days. My preliminary western blotting results showed that lithium increased inhibitory phosphorylation of GSK-3β (Figure 25). This suggests that lithium inhibits GSK-3β in RG3.6 cells.
Other GSK-3β inhibitors increases cell numbers in RG3.6 cell cultures

Since lithium induced inhibitory phosphorylation of GSK-3β in RG3.6 cells, I hypothesize that inhibition of GSK-3β mediates lithium stimulation of RG3.6 cell proliferation. If so, then other GSK-3β inhibitors should have similar effects on RG3.6 cell growth. I tested the effects of two potent and specific GSK-3 inhibitors, SB216763 and SB415286, on RG3.6 cell cultures, using concentrations that were previously shown to inhibit GSK-3β activity (Coghlan et al., 2000; Cross et al., 2001; Bachelder et al., 2005; Yoshimura et al., 2005). Consistent with our hypothesis, SB216763 (5 µM) and SB415286 (25 µM) both significantly increased cell numbers in RG3.6 cultures (Figure 26).

Lithium reduces RNA levels of Dscr1l1 and Sez6 in RG3.6 cells

As described above, I did gene array analysis to examine gene expression changes induced by lithium in RG3.6 cells. Besides the 17 genes that were significantly up-regulated more than two fold by lithium, I also identified 2 genes that were significantly down-regulated more than two fold by lithium (Table 5). These are Dscr1l1 (Down syndrome critical region gene 1-like 1) and Sez6 (Seizure related 6 homolog). Quantitative real time PCR analysis confirmed that lithium reduced Dscr1l1 and Sez6 RNA levels in RG3.6 cells (Figure 27).

Sez6 is a seizure related gene specifically expressed in the brain (Shimizu-Nishikawa et al., 1995; Yucel et al., 2007). It encodes a membrane protein with unknown function. Dscr1l1 belongs to Down’s syndrome critical region gene 1 (Dscr1) family. DSCR1 encodes a regulatory protein in the calcineurin/NFAT signal transduction
pathway (Fuentes et al., 2000). The DSCR1 protein contains a conserved calcipressin domain that binds to calcineurin, and suppresses the phosphatase activity of calcineurin. The rat DSCR1L1 protein also contains a calcipressin domain (Figure 28). DSCR1L1 thus may also bind to calcineurin, inhibit calcineurin phosphatase activity, and subsequent NFAT activation. Thus, lithium down-regulation of Dscr1l1 gene expression may dis-inhibit calcineurin, leading to increased NFAT activity in RG3.6 cells.

Cyclosporin A antagonizes lithium-induced cell number increase in RG3.6 cell cultures

Lithium reduced Dscr1l1 RNA levels in RG3.6 cells (Table 5 and Figure 27). As described above, Dscr1l1 may inhibit the major NFAT activator calcineurin. Thus down-regulation of Dscr1l1 may dis-inhibit calcineurin, leading to NFAT activation. My experimental results suggest that lithium inhibits GSK-3β in RG3.6 cells (Figure 25). Since GSK-3β inhibits the activity of NFAT (Beals et al., 1997; Haq et al., 2000; Antos et al., 2002), lithium inhibition of GSK-3β may also lead to NFAT activation in RG3.6 cells. To investigate the involvement of NFAT activation in lithium-induced proliferation stimulation of RG3.6 cells, I assessed the effects of cyclosporin A (CsA), a specific inhibitor of the major NFAT activator calcineurin, on lithium-induced cell number increase in RG3.6 cell cultures. To make sure that the dose of CsA I use has no effect on RG3.6 cell growth by itself, I tested the effect of various doses of CsA (0.01 µM, 0.1 µM, 1 µM, 10 µM and 50 µM) on RG3.6 cell growth. CsA at doses of 0.01 µM up to 1 µM did not significantly change cell numbers, but CsA at doses of 10 µM and above significantly reduced cell numbers in RG3.6 cell cultures (Figure 29). Interestingly, although 1 µM CsA (the highest dose I used) did not significantly change
cell numbers in RG3.6 cell cultures, it completely abolished lithium (3 mM)-induced cell number increase in RG3.6 cell cultures (Figure 30). These results suggest that NFAT activation is involved in lithium’s promotion of RG3.6 cell proliferation.

CsA antagonizes lithium’s effect on Dscr1l1 and Sez6 RNA expression in RG3.6 cells

NFAT regulates Dscr1 gene expression (Lange et al., 2004). I postulate that NFAT may also regulate the Dscr1 family member Dscr1l1 gene expression. The genomic sequence of rat Dscr1l1 contains a consensus NFAT binding motif A/TGGAAAA/T/CN (Rao et al., 1997) at position -327 (upstream of the start of the first exon, Figure 31). Examination of the Sez6 genomic sequence revealed that it also contains a consensus NFAT binding motif at a similar location, position -325 (upstream of the start of the first exon, Figure 32). To determine whether NFAT mediates lithium-induced down-regulation of Dscr1l1 and Sez6 RNA expression, I added cyclosporin A (CsA), a specific inhibitor of the major NFAT activator calcineurin, to lithium-treated RG3.6 cell cultures, and did quantitative real time PCR analysis on RNAs extracted from these cultures. CsA significantly increased Dscr1l1 and Sez6 RNA levels in lithium (3 mM LiCl) treated RG3.6 cell cultures (Figure 33), which is a reverse of the effect of lithium on RNA levels of these two genes in RG3.6 cells (Table 5 and Figure 27). These results suggest that NFAT activation is involved in lithium’s reduction of these two genes’ RNA expression in RG3.6 cells.
**Discussion**

*Lithium stimulates neural precursor cell proliferation*

Lithium significantly increased cell numbers in cultures of neural precursor cells, including rat and mouse primary neural precursor cells and radial glial (neural precursor) cell clone RG3.6 cells (Figures 15-18). Lithium also significantly increased the percentage of BrdU positive cells when BrdU was added to RG3.6 cell cultures to label proliferating cells (Figure 21), whereas lithium did not significantly change the percentage of surviving cells in RG3.6 cell cultures (Figure 19-20). Gene array analysis revealed that a majority of genes significantly up-regulated more than two fold by lithium are proliferation-related genes (Table 4), and did not reveal any known cell survival-related genes that were significantly altered by lithium. These data altogether suggest that lithium promotes cell number increase in neural precursor cell cultures by stimulating cell proliferation but not cell survival.

*Lithium promotion of neural precursor cell proliferation does not involve neurotrophic factors*

Lithium has been reported to increase expression of neurotrophic factors such as BDNF, NGF, and NT-3, in specific regions of rodent brain (Fukumoto et al., 2001; Hellweg et al., 2002; Angelucci et al., 2003; Frey et al., 2006a; Frey et al., 2006b; Walz et al., 2008). Stimulation of neurotrophic factors especially BDNF has been proposed to be a mechanism of the anti-depressant drugs including lithium (Post, 2007), although the source cells for the neurotrophic factors are not clear. Lithium stimulates BDNF
expression and BDNF is essential for lithium-induced neuroprotection against glutamate excitotoxicity in cultured cortical neurons (Hashimoto et al., 2002). This suggests that neurons are one potential resource cells for lithium-induced BDNF in the brain. My experiments showed that lithium increased RNA levels of BDNF in rat primary neural precursor cells (Figure 23), suggesting that neural precursor cells might be another potential resource cells. However, in neural precursor cell clone RG3.6 cells, lithium significantly reduced RNA levels of all the neurotrophic factors I tested, including BDNF, CNTF, GDNF, LIF, NGFβ, NGFγ and NT-3 (Figure 22). Since lithium has very different effects on neurotrophic factor RNA expression in primary neural precursors and RG3.6 cells, it is unlikely that neurotrophic factor stimulation is a common mechanism for lithium’s promotion of neural precursor cell proliferation.

**Inositol depletion does not contribute to lithium’s promotion of RG3.6 cell proliferation**

Lithium’s inhibition of IMPase and IPPase, two critical enzymes involved in de novo synthesis and recycling of inositol, leads to inositol depletion hypothesis, which suggests that inositol depletion caused by suppression of these two enzymes mediates lithium action in bipolar disorder (Harwood, 2005). In support of this hypothesis, lithium reduced inositol or myo-inositol levels in rodent brains and in the brains of human subjects with bipolar disorder (Allison and Stewart, 1971; Hirvonen, 1991; Huang et al., 2000; O'Donnell et al., 2003; McGrath et al., 2006). In addition, sodium-myoinositol transporter 1 (smit1) knock out mice, which have huge reduction in myo-inositol level in their brains, behave similarly to lithium-treated animals (Bersudsky et al., 2008). However, much higher inositol depletion is required for achievement of the behavioral
effects in mice than that achieved by lithium administration, since smit1+/- mice show lower levels of myo-inositol deficiency in the brain but no significant difference in lithium-sensitive behavior (Shaldubina et al., 2006). This suggests that the inositol depletion role of lithium is not responsible for all of its actions. My experimental results showed that addition of myo-inositol (1 mM and 10 mM) to RG3.6 cell cultures did not cause significant change in RG3.6 cell number, and addition of myo-inositol (1 mM and 10 mM) had no significant effect on lithium-induced cell number increase (Figure 24). These results suggest that inositol depletion does not contribute to lithium stimulation of neural precursor cell proliferation. In both the RG3.6 cell cultures with and without lithium treatment, as the concentration of myo-inositol increased, there was a very slight but insignificant decrease in average cell number, suggesting that the myo-inositol used might be bioactive. However, it would be better if we could verify the bioactivity of myo-inositol clearly to rule out the possibility that the myo-inositol used was inactive and this very slight and insignificant effect of myo-inositol was just by chance.

*Lithium may promote neural precursor cell growth through GSK-3β inhibition and NFAT activation*

My preliminary results showed that lithium induced the inhibitory phosphorylation (Ser9) of GSK-3β in neural precursor cell clone RG3.6 cells (Figure 25), suggesting that lithium inhibits GSK-3β in RG3.6 cells. However, since lithium can also directly inhibit GSK-3β by competing with its metal cofactor magnesium (Quiroz et al., 2004), the increased inhibitory phosphorylation may not represent the full extent of GSK-3β inhibition by lithium. Other specific GSK-3β inhibitors, SB216763, and SB415286,
similarly increased cell numbers in RG3.6 cell cultures (Figure 26). Altogether, these data suggest that lithium stimulates neural precursor cell proliferation through inhibition of GSK-3β. Interestingly, cyclosporin A (CsA), a specific inhibitor of the major NFAT activator calcineurin, completely blocked lithium–induced cell number increase (Figure 30), suggesting that NFAT activation contributes to lithium’s promotion of neural precursor cell proliferation. In addition, while lithium significantly reduced Dscr1l1 and Sez6 RNA levels (Table 5 and Figure 27), CsA reversed lithium’s effects on RNA levels of these two genes in RG3.6 cells (Figure 33). These results suggest that NFAT activation might also contribute to lithium-induced down-regulation of Dscr1l1 and Sez6 RNA expression in RG3.6 cells. Both Dscr1l1 and Sez6 gene contain a conserved NFAT binding motif close to their transcription start site (Figures 31 and 32). This brings up the possibility that NFAT may directly bind to the promoter region of these two genes and inhibit their gene expression. Further experiments such as electrophoretic mobility shift assay, chromatin immunoprecipitation assay, and reporter assay are needed to verify the direct interaction between NFAT and the promoter regions of these two genes.

Based on my data, I propose a mechanism by which lithium promotes neural precursor cell proliferation (Figure 34). NFAT is activated via dephosphorylation by calcineurin, and inactivated via phosphorylation by GSK-3β (Rao et al., 1997). Lithium inhibits GSK-3β, which in turn dis-inhibits NFAT. Activated NFAT suppresses the expression of calcineurin inhibitor Dscr1l1, which leads to further enhancement of NFAT activity. Activated NFAT together with other factors causes gene expression changes, including down-regulation of Sez6 and Dscr1l1, leading to further changes inside the cell, and eventually enhanced cell proliferation. CsA antagonizes lithium’s effects by
inhibiting calcineurin phosphatase activity, which in turn leads to NFAT inactivation, and thus cell growth inhibition.

CsA is usually administered at 10 mg/kg as an immunosuppressive drug in rats receiving cell transplantation. The molecular weight of CsA is 1202.63. This dose is thus similar to a dose of 8 µM in vitro. My experiments showed that CsA at doses of up to 1 µM had no significant effect on RG3.6 cell growth, but at doses of 10 µM and above was detrimental to RG3.6 cells. The dosage used in rats therefore is likely to be detrimental to RG3.6 cells and more generally to neural precursor cells. However, in vivo, despite the high lipid solubility of CsA, the transportation of CsA across the blood-brain barrier is very restricted (Cefalu and Pardridge, 1985). Thus the CsA concentration in the CNS could be much lower. In the cases of CNS injury or some other diseased condition, the blood-brain barrier permeability to CsA may increase. Thus CsA concentration in the CNS may reach levels high enough to suppress neural precursor cells and possibly some other cells. This may contribute to some adverse effects of CsA in the CNS, such as tremor, headache, seizures, altered mental functioning, impaired consciousness, visual disturbances (including cortical blindness), loss of motor function, and movement disorders (Gijtenbeek et al., 1999).

Since there is no clinical need for using CsA together with lithium, the drug interaction between CsA and lithium has not been studied yet. Now the experimental results of our laboratory showed that lithium treatment robustly increased numbers of N01.1 cells transplanted into injured rat spinal cords (Figures 10-12). On the other hand, my experiments showed that CsA at low dose (1 µM) antagonized lithium’s promotion of RG3.6 cell growth (Figure 30). It is possible that CsA may also antagonize lithium’s
effects on cells transplanted into the CNS. Since CsA is also used to promote transplanted cell survival. It would be interesting to check whether administering lithium together with CsA at the same time will attenuate or boost lithium’s effect on transplanted cells. If CsA attenuate lithium’s effect on transplanted cells, then we should avoid using them together at the same time.

In summary, my experimental results suggest that lithium promotes proliferation but not survival of neural precursor cells by induction of proliferation-related genes. Lithium stimulation of neural precursor cell proliferation may not involve neurotrophic factor stimulation, or inositol depletion, but may involve GSK-3β inhibition and subsequent NFAT activation. These results not only provide mechanistic insight into the beneficial effects of lithium on manic depression and other CNS diseases, but also suggest an alternative strategy for these diseases by targeting the GSK-3β/NFAT signaling pathway.
Figure 15. Lithium chloride induced cell number increase in RG3.6 cultures.

RG3.6 cells were seeded in equal number into wells of a 96-well plate in various concentrations of lithium chloride (LiCl) added to the culture medium (DMEM/F12 supplemented with 25 mM glucose, 100 µg/ml streptomycin, 100 units/ml penicillin, 0.2 ng/ml FGF2, 2 µg/ml heparin, and 1X B27). Cells were incubated at 37 °C in a humidified atmosphere of 90% air/10% CO₂ for 7 days. Cell number in each well (n=6 for each condition) was determined using cyquant cell proliferation assay kit (Invitrogen) according to the manufacturer’s protocol. The data represent mean ± standard error. The 3 mM LiCl group had the greatest RG3.6 cell numbers (*: Bonferroni/Dunn, P < 0.002 compared to no LiCl control).
Figure 16. Lithium chloride but not sodium chloride induced cell number increase in RG3.6 cell cultures.

To rule out an osmotic effect, this experiment compared numbers of RG3.6 cells grown in 96-well plate in culture medium (Page 77) alone, medium + 3 mM NaCl, and medium + 3 mM LiCl (n=6 for each condition), incubated at 37 °C in a humidified atmosphere of 90% air/10% CO₂ for 5 days. Cell number in each well was determined using cyquant cell proliferation assay kit (Invitrogen) according to the manufacturer’s protocol. The data represent mean ± standard error. The 3 mM LiCl group had significantly greater cell numbers (*: paired t test, P < 0.01 compared to 3 mM NaCl or control medium treatment), whereas 3 mM NaCl had no significant effect on cell numbers in RG3.6 cell cultures.
Figure 17. Lithium increased cell numbers in rat primary neural precursor cell cultures.

Primary neural precursor cells isolated from both neonatal rat subventricular zone and olfactory bulb was seeded in equal number into wells of a 96-well plate. LiCl or control NaCl was added to the culture medium (DMEM/F12 supplemented with 25 mM glucose, 100 µg/ml streptomycin, 100 units/ml penicillin, 0.2 ng/ml FGF2, 0.2 ng/ml EGF, and 1X B27) to reach a final concentration of 3 mM (n=6 for each condition). Cells were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂ for 5 days. Cell number in each well was determined using cyquant cell proliferation assay kit (Invitrogen) according to the manufacturer’s protocol. The data represent mean ± standard error. *: Bonferroni/Dunn, P < 0.002.
Figure 18. Lithium increased cell numbers in mouse primary neural precursor cell cultures.

Primary neural precursor cells isolated from both neonatal mouse subventricular zone and olfactory bulb was seeded in equal number into wells of a 96-well plate. LiCl or control NaCl was added to the culture medium (DMEM/F12 supplemented with 25 mM glucose, 100 µg/ml streptomycin, 100 units/ml penicillin, 0.2 ng/ml FGF2, 0.2 ng/ml EGF, and 1X B27) to reach a final concentration of 3 mM. Cells were incubated at 37 °C in a humidified atmosphere of 95% air/5% CO₂ for 5 days. Cell number in each well was determined using cyquant cell proliferation assay kit (Invitrogen) according to the manufacturer’s protocol. The data represent mean ± standard error. *: Bonferroni/Dunn, P < 0.002.
RG3.6 neurospheres were dissociated by trypsinization. The dissociated cells were stained with propidium iodide (PI), a red fluorescent nuclear dye that specifically stains dead cells. The green GFP signal and the red PI staining were visualized and photographed using fluorescent microscope. The green GFP signal and the red propidium iodide signal are mutually exclusive. The GFP expressing cells are live cells that are negative for PI, while the non-GFP expressing cells are dead cells that are positive for PI. The relative high proportion of PI-positive, non-GFP expressing dead cells was possibly due to the relative long trypsinization time.

Figure 19. GFP signal can be used as an indication of RG3.6 cell survival.
Figure 20. Lithium had no significant effect on percentage of GFP-expressing live cells in RG3.6 cell cultures.

RG3.6 neurospheres were grown in culture medium (Page 77) containing 3 mM LiCl or 3 mM NaCl for 3 days, and then dissociated by trypsinization. Flow cytometry was carried out to determine the percentage of GFP-expressing live cells. Cells grown in non-FGF2 containing medium for 6 days were used as a negative control to gate GFP signal, since most cells grown in this condition were dead. The bottom panel shows quantification of the results. RG3.6 cells grown in 3 mM LiCl or 3 mM control NaCl-containing medium had similar GFP histograms, and there were no significant difference in the percentage of GFP expressing cells in these two culture conditions. In each GFP histogram of the LiCl or control NaCl-treated cultures, there were two curves, a lower curve on the left corresponding to a smaller population of cells that express lower levels of GFP, and a higher curve on the right corresponding to a larger population of cells that express higher levels of GFP.
Figure 21. Lithium significantly increased percentage of BrdU+ cells in RG3.6 cell cultures.

RG3.6 cells were plated on laminin-coated coverslips, grown in culture medium (Page 77) containing 3 mM LiCl or 3 mM NaCl. After 3 days in culture, the cells were
labeled with BrdU (10 µM) for 4 hours, and then fixed for BrdU immunostaining. The data represent mean ± standard error. Lithium significantly increased percentage of BrdU positive cells (*: Bonferroni/Dunn, P < 0.0001, n=8 for each condition).
Table 4. Proliferation-related genes significantly up-regulated more than two fold by lithium in RG3.6 cells.

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Figure 22. Lithium significantly reduced neurotrophic factor RNA levels in RG3.6 cells.

RG3.6 cells were grown in culture medium (Page 77) containing 3 mM LiCl or 3 mM NaCl for 3 days. Then the cells were lysed for RNA extraction and subsequent quantitative real time PCR analysis. The primers used are listed in Table 3 (Page 28). RNA levels of neurotrophic factors were normalized to β-actin. The data represent mean ± standard error. *: Bonferroni/Dunn, P < 0.05; **: Bonferroni/Dunn, P < 0.005; n = 5 for each condition.
Figure 23. Lithium significantly increased BDNF RNA level and had no significant effect on CNTF, GDNF, LIF, NGFβ, NGFγ and NT-3 RNA in rat primary neural precursor cells.

Rat primary neural precursor cells were isolated from neonatal rat subventricular zone and olfactory bulb. The cells were grown in culture medium (Page 79) containing 3 mM LiCl or 3 mM NaCl for 3 days. Then the cells were lysed for RNA extraction and subsequent quantitative real time PCR analysis. The primers used are listed in Table 3 (Page 28). RNA levels of neurotrophic factors were normalized to that of peptidylprolyl isomerase A (Ppia). The data represent mean ± standard error. *: Bonferroni/Dunn, P < 0.05, n=3 for each condition.
Figure 24. Inositol did not significantly change cell numbers in RG3.6 cell cultures.

RG3.6 cells were grown in culture medium (Page 77) with or without 3 mM LiCl, and with or without 1 mM or 10 mM myo-inositol (n=6 for each condition) for 5 days, and then cell count analysis was carried out. The data represent mean ± standard error, and significance was determined with the Fisher’s PLSD post hoc test following analysis of variance (ANOVA). LiCl (3 mM) significantly increased cell numbers, while myo-inositol (1 mM or 10 mM) did not significantly change cell numbers in RG3.6 cell cultures either with or without LiCl (3 mM) treatment.
Figure 25. Lithium may increase inhibitory phosphorylation of GSK-3β in RG3.6 cells.

RG3.6 cells were grown in culture medium (Page 77) containing 3 mM LiCl or 3 mM control NaCl for 3 days. Then the cells were lysed for western blotting analysis. The blotting results were analyzed using the ImageJ software (Abramoff, 2004), and the ratio of P-GSK-3β/GSK-3β was plotted in the bottom panel.
Figure 26. Other GSK-3β inhibitors, like lithium, also increased cell numbers in RG3.6 cell cultures.

RG3.6 cells were grown in culture medium (Page 77) without or with 3 mM LiCl, 0.1% DMSO, 5 µM SB216763, or 25 µM SB415286 (n=6 for each condition) for 6 days, and then cell count analysis was carried out. DMSO was used as vehicle control for SB216763 and SB415286, since these two drugs were dissolved in DMSO. The data represent mean ± standard error, and significance was determined with Bonferroni/Dunn post hoc test following analysis of variance (ANOVA).
Table 5. Genes significantly down-regulated more than two fold by lithium in RG3.6 cells.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Entrez Gene</th>
<th>Summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sez6</td>
<td>192247</td>
<td>Membrane protein; specific expression in the brain</td>
</tr>
<tr>
<td>Dscr111</td>
<td>140666</td>
<td>Potential calcineurin inhibitor; may have a role in brain development</td>
</tr>
</tbody>
</table>
Figure 27. Lithium reduced RNA levels of Sez6 and Dscr111 in RG3.6 cells.

RG3.6 cells were grown in culture medium (Page 77) containing either 3 mM LiCl or 3 mM NaCl for 3 days. Then the cells were lysed for RNA extraction and subsequent quantitative real time PCR analysis. The primers used are listed in Table 3 (Page 28). The expression level of Sez6 and Dscr111 was normalized to that of β-actin. The data represent mean ± standard error. *: Bonferroni/Dunn, P < 0.001, n=5 for each condition.
**Figure 28.** Rat Dscr1l1 gene contains a conserved calcipressin domain that binds to calcineurin and suppresses calcineurin phosphatase activity.

Rat DSCR1L1 protein sequence (gi_28212228) is 243 amino acid (aa) long. Research online for conserved domains revealed that it contains a calcipressin domain at its C-terminus, starting from aa80 to aa237, with 92% alignment, and 68% identity. The alignment picture was copied from the following web site:

Figure 29. Effect of cyclosporin A on RG3.6 cell numbers in culture.

RG3.6 cells were grown for 5 days in culture medium (Page 77) containing various doses of cyclosporin A (CsA): 0 (Control), 0.01 µM, 0.1 µM, 1 µM, 10 µM, 50 µM, n=6 for each dose. Then cell count analysis was carried out. Treatment with 0.01 µM up to 1 µM CsA did not significantly change cell numbers, while 10 µM CsA and above significantly reduced cell numbers in RG3.6 cell cultures (*: paired t test, P < 0.005 compared to control, 0.01 µM, 0.1 µM, or 1 µM CsA group).
Figure 30. Cyclosporin A antagonized lithium-induced cell number increase in RG3.6 cell cultures.

RG3.6 cells were grown for 5 days in culture medium (Page 77) with or without 3 mM LiCl, and with or without 1 µM cyclosporin A (CsA) (n=6 for each condition). Then cell count analysis was carried out. The data represent mean ± standard error. *: paired t test, P < 0.02 compared to No CsA + No LiCl, or CsA + No LiCl condition. Lithium (3 mM) alone but not CsA (1 µM) alone significantly increased cell numbers in RG3.6 cell cultures. However, when the cells were treated with both CsA and lithium, CsA completely blocked the lithium-induced cell number increase.
Figure 31. Rat Dscr1ll1 gene contains a conserved NFAT binding motif close to the transcription start site.

Top: Schematic representation of the rat Dscr1ll1 gene. Rat Dscr1ll1 gene contains 5 exons (blue blocks). The sequence of 500 nucleotides upstream of the transcription start site is shown at the bottom. The conserved NFAT binding motif (Rao et al., 1997) at position -327 is highlighted in red and underlined.
Figure 32. Rat Sez6 gene contains a conserved NFAT binding motif close to the transcription start site.

Top: Schematic representation of rat Sez6 gene. Exons are shown as blue blocks. The sequence of 500 nucleotides upstream of the transcription start site is shown at the bottom. The conserved NFAT binding motif (Rao et al., 1997) at position -325 is highlighted in red and underlined.
Figure 33. Cyclosporin A increased Dscr1l1 and Sez6 RNA levels in RG3.6 cells treated with lithium.

RG3.6 cells were grown in culture medium (Page 77) containing 3 mM LiCl, and 0 or 1 µM of cyclosporin A (CsA). After 3 days of culture, the cells were lysed for RNA extraction and subsequent quantitative real time PCR analysis. The primers used are listed in Table 3 (Page 28). The data represent mean ± standard error. *: Bonferroni/Dunn, P < 0.05, n=3 for each condition.
Figure 34. Model for one mechanism by which lithium promotes neural precursor cell proliferation.

NFAT activity is regulated through phosphorylation. Calcineurin dephosphorylates NFAT. Dephosphorylated NFAT enters the nucleus, and participates in gene expression regulation. GSK-3β phosphorylates NFAT, promoting its nuclear export, and thus inhibits NFAT activity. Lithium inhibits GSK-3β, which in turn disinhibits NFAT. Activated NFAT suppresses the expression of calcineurin inhibitor Dscr1III, which leads to further enhancement of NFAT activity. Activated NFAT and possibly other factors, cause gene expression changes, which in turn lead to further changes inside the cells, promoting cell proliferation. CsA antagonizes lithium’s effects by inhibiting calcineurin phosphatase activity, which in turn leads to NFAT inactivation and cell growth inhibition.
CHAPTER III

CONCLUSION AND FUTURE DIRECTIONS

1. EPO Promotes Neural Precursor Cell Growth

The CNS expresses EPO and EPO receptor, and up-regulates their expression after hypoxia or CNS injury (Siren et al., 2001; Grasso et al., 2005). Animal studies have shown that application of exogenous EPO is beneficial for various CNS diseases and injuries (Brines et al., 2000; Celik et al., 2002; Gorio et al., 2002; Boran et al., 2005; Lu et al., 2005; Grasso et al., 2007; Grunfeld et al., 2007; Koh et al., 2007; Xue et al., 2007). However, the physiological functions of EPO and the mechanisms of the beneficial effects of EPO in the CNS are not clear. Recent studies suggest that EPO and EPOR may be involved neurogenesis during development and in adult after CNS injury. My experiments showed that EPO does not promote neural precursor clone RG3.6 cell growth at doses (up to 5 U/ml) that improves neurological function in animals. However, it does so at higher doses (50-100 U/ml) that is unlikely, although may not be absolutely impossible to reach in the microenvironment around endogenous neural precursor cells in the CNS after injury (Figure 6). Different from my results, a recent report showed that EPO at 10 U/ml optimally promotes neural precursor cell growth in cultures of primary neural precursor cells (Chen et al., 2007). However, their primary neural precursor cell cultures contain a majority of non-nestin expressing other types of cells, many of which express EPO receptor. My results together with their data suggest that the presence of other types of cells may facilitate EPO’s promotion of neural precursor cell growth,
which is highly likely the case in vivo. To find out what types of cells may facilitate this EPO effect, We could co-culture neural precursor clone RG3.6 cells or pure nestin-expressing primary neural precursor cells with various types of cells, such as astrocytes, microglia, neurons, etc, and check the effect of EPO on neural precursor cell growth in this co-culture system. If we find out one cell type that facilitates EPO’s promotion of neural precursor cell growth, we could then use the culture medium or EPO-treated culture medium from that cell type to check whether cell contact is necessary for this facilitation, and whether EPO stimulation of this cell type plays a role in this facilitation.

2. EPO May Stimulate EPO Receptor Expression in the Spinal Cord to Benefit Spinal Cord Injury

Animal studies have shown that EPO benefits spinal cord injury (Celik et al., 2002; Gorio et al., 2002; Boran et al., 2005). However, the mechanisms are not well understood. My experiments showed for the first time that EPO significantly increased RNA levels of EPO receptor in rat spinal cords six hours after spinal cord injury (Figure 7), suggesting that EPO may stimulate EPO receptor expression in the spinal cord to benefit spinal cord injury. In rat spinal cords, EPO receptor is present in neurons, glial cells, endothelial cells, besides the presumptive neural precursor ependymal cells, and up-regulated in these cells after spinal cord injury (Grasso et al., 2005). By immuno-costaining with EPO receptor and other cell type-specific proteins in the injured spinal cords of EPO and control vehicle-treated rats, we could find out whether EPO further up-regulates EPO receptor expression in these or some of these cells, or increases number of these or some of these cells, or both.
As I described in the previous section, EPO promotes neural precursor cell growth. EPO may also stimulate neuronal differentiation of neural precursor cells (Shingo et al., 2001). Moreover, EPO protects neurons against hypoxia and neurotoxicity induced by various chemicals in vitro (Morishita et al., 1997; Lewczuk et al., 2000; Viviani et al., 2005; Shang et al., 2007; Won et al., 2007; Zhong et al., 2007), and is neuroprotective in various animal models of CNS injury and diseases (Sakanaka et al., 1998; Celik et al., 2002; Junk et al., 2002; Demers et al., 2005; Grimm et al., 2006; Kanaan et al., 2006; Zhang et al., 2006; Koh et al., 2007; Liao et al., 2007; Xue et al., 2007). Furthermore, EPO is cytoprotective for astroglial cells and endothelial cells (Diaz et al., 2005; Zhande and Karsan, 2007). Thus injury-induced EPO receptor up-regulation in these cells may represent an endogenous protective mechanism of the animals after spinal cord injury, and EPO-induced EPO receptor up-regulation in the spinal cord may further enhance these protective effects.

3. EPO May Stimulate Nestin Expression in the Spinal Cord to Benefit Spinal Cord Injury

My experiments also showed for the first time that EPO significantly increased RNA levels of nestin in rat spinal cords six hours after spinal cord injury (Figure 7), suggesting that EPO may stimulate nestin expression in the spinal cord to benefit spinal cord injury. Nestin expression is hardly detectable in normal rat spinal cords. After spinal cord injury, nestin expression is induced in the ependyma adjacent to the lesion site (Namiki and Tator, 1999), and in reactive astrocytes close to and at the lesion site (Frisen et al., 1995). Cell proliferation is concomitantly induced in these nestin-
expressing cells. Since EPO promotes neural precursor cell growth in vitro (Figure 6 and Chen et al., 2007), EPO may promote neural precursor cell growth in the spinal cord after injury, which could be one cause for the EPO-induced nestin RNA level increase in the injured rat spinal cords. However, reactive astrocytes also express nestin, EPO-induced nestin RNA level increase in the injured rat spinal cords might as well be due to up-regulation of nestin in reactive astrocytes, or due to increased number of reactive astrocytes. Immunohistochemical analysis of nestin and astrocyte marker GFAP or other reactive astrocyte markers in the injured spinal cords will help to clarify whether EPO up-regulates nestin in neural precursor cells or reactive astrocytes, and whether EPO promotes growth of neural precursor cells or reactive astrocytes in the spinal cords after injury. Reactive astrocytes were originally considered detrimental to spinal cord injury. Recent studies suggest that reactive astrocytes exert beneficial effects on spinal cord injury (Lee et al., 1998; Madiai et al., 2003; Faulkner et al., 2004). Thus if EPO promotes growth of reactive astrocytes, this could be beneficial to spinal cord injury.

My results suggest that EPO promotes neural precursor cell growth in vitro (Figure 6). EPO stimulation of EPO receptor and nestin RNA expression in spinal cords after injury brings up the possibility that EPO may also stimulate neural precursor growth in spinal cord after injury, although as I explained before, various other possibilities exist. In the case that EPO promotes neural precursor cell growth in the spinal cord after injury, next we may try to explore whether EPO combined with neural precursor transplantation can benefit spinal cord injury and whether EPO may promote survival or proliferation of transplanted neural precursor cells. To be able to track the transplanted cells, we can
transplant GFP-expressing neural precursor clone RG3.6 or primary neural precursor cells isolated from GFP transgenic rat to wild type rats.

4. Characteristics of Blood-Derived N01.1 Cells

Our laboratory generated a very interesting cell clone, N01.1, from neonatal rat blood. As I described before, these cells express nestin and EPO receptor, and upon serum withdrawal, form spherical aggregates similar to neurospheres formed by neural precursor cells. We were very excited about the possibility that they might be neural precursors. However, treatment with retinoic acid failed to differentiate them into GFAP-expressing astrocytes, βIII-tubulin-expressing neurons, or GALC-expressing oligodendrocytes, suggesting that they are not really neural precursor cells. Nestin expression is present in about 60% of CD133-positive blood stem cells in human umbilical cord blood, but not in human adult blood (Tokumine et al., 2003). Thus it is possible that N01.1 is a blood stem cell clone with some neural precursor cell potential.

My experimental results suggest that EPO activates ERK pathway in N01.1 cells through a c-Raf-independent mechanism, which is a mechanism different from that in erythroid precursor cells (Carroll et al., 1991). In addition, EPO tended to increase RNA levels of Bcl-w but not Bcl-xL or Bcl2 in N01.1 cells, whereas in erythroid precursor cells, EPO up-regulates Bcl2 and Bcl-xL to promote cell survival (Silva et al., 1996; Mori et al., 2003). Thus N01.1 cells do not seem to be erythroid precursor cells. Immunocytochemistry with various blood cell lineage markers such as CD133, CD34, Sca-1 (stem cell antigen 1), etc, will help define the N01.1 cells in more detail.
GFP-expressing N01.1 cells did not form tumor after they were transplanted into normal rat adult spinal cords, suggesting that they are not tumor-prone cells, and are safe for transplantation. We can try to explore their differentiation potential in the neural lineage by transplanting the GFP-expressing N01.1 cells into rat developing CNS since developing CNS is more permissive for transplanted cells to survive and differentiate.

5. Lithium Treatment Combined with Blood-Derived Cell Transplantation May Stimulate Neurotrophic Factor Expression in the Spinal Cord to Benefit Spinal Cord Injury

Neurotrophic factors play various important roles in the CNS, such as promoting neural cell survival and growth, neurite outgrowth, and myelination, synapse modulation, etc (Ip and Yancopoulos, 1996). Delivery of exogenous neurotrophic factors such as BDNF, GDNF, NT-3, NGF and LIF has been shown to improve neurological functions in various animal models of CNS injury and disease, including spinal cord injury (Kim et al., 1996; Namiki et al., 2000; Zang and Cheema, 2003; Tang et al., 2004). However, continuous delivery of neurotrophic factors is expensive and represents a technical challenge in human patients. Interestingly, results of our laboratory showed that transplantation of blood-derived N01.1 cells tended to increase RNA levels of neurotrophic factors BDNF, GDNF, LIF, NT-3, and NGFγ, in rat spinal cords two weeks after spinal cord injury, although the increase was not statistically significant (Figure 14). More interestingly, lithium treatment combined with N01.1 cell transplantation further significantly increased RNA levels of GDNF, LIF, NT-3, NGFβ and NGFγ, in rat spinal cords two weeks after spinal cord injury (Figure 14). These results suggest that blood-
derived cell transplantation combined with lithium treatment may stimulate neurotrophic factor expression in the spinal cords to benefit spinal cord injury.

In rats without lithium treatment, most of the transplanted N01.1 cells died two weeks after spinal cord injury (Figures 10-12), yet the spinal cords with N01.1 cell transplantation tended to contain higher levels of several neurotrophic factor RNA, compared to the spinal cords without N01.1 cell transplantation, although the difference was not statistically significant (Figure 14). These suggest that the transplanted N01.1 cells may improve the microenvironment of the spinal cord and the improved microenvironment may stimulate endogenous spinal cord cells to produce more neurotrophic factors. As I discussed in section 1 of chapter II, we next should check whether N01.1 cell transplantation promotes neurotrophic factor expression more profoundly and more significantly at earlier times when there are more surviving N01.1 cells, and we should check neurotrophic factor expression at the protein level.

We next could examine the potential beneficial effects of N01.1 cell transplantation and combined treatment with lithium and N01.1 cell transplantation in more detail. For example, we could compare lesion volume, cavity formation, glial scar formation, neuronal and glial cell death, neural precursor and reactive astrocyte proliferation, axonal degeneration and regeneration, demyelination and remyelination, inflammatory infiltrates, blood-brain barrier breakdown, locomotor function, etc, in rats treated with lithium, N01.1 cell transplantation, lithium plus N01.1 cell transplantation, and control saline, at various times after spinal cord injury. After we get more detailed characterization of the N01.1 cells, we may try to obtain cells with similar characteristics from neonatal rat blood or human umbilical cord blood, and transplant these cells into
injured rat spinal cords to check whether transplantation of these cells alone or combined with lithium treatment could benefit spinal cord injury similarly.

6. Lithium Treatment May Provide a Powerful Method of Promoting Transplanted Cell Survival or Proliferation

Transplantation of cells such as neural precursors, bone marrow stromal cells, umbilical cord blood cells, etc, has become an intriguing approach to CNS tissue repair. However, due to the unfavorable environment of the adult CNS, especially after CNS injury, the survival rate of the transplanted cells is often low. As an example, when N01.1 cells were transplanted into injured rat spinal cords, most of the N01.1 cells die two weeks after spinal cord injury (Figures 10-12). Amazingly, when the rats received lithium treatment (100 mg/kg/day), the number of N01.1 cells survived two weeks after spinal cord injury dramatically increased (Figures 10-12). This suggests that lithium promotes survival, proliferation, or both of N01.1 cells transplanted into the injured rat spinal cords.

We next should determine whether the increase in the number of N01.1 cells transplanted into injured rat spinal cords could partially arise from lithium’s promotion of N01.1 cell proliferation. We will check proliferation of the transplanted N01.1 cells at various time points. If we find that lithium promotes proliferation of N01.1 cells transplanted into injured rat spinal cords, we will need to monitor this lithium effect at longer time period to eliminate the possibility of tumor formation, although from the experience we had in our laboratory, tumor formation is unlikely. Dr. Tsutomu Iseda in our laboratory also did the experiments in a chronic spinal cord injury setting. He
transplanted N01.1 cells into injured rat spinal cords four weeks after spinal cord injury and started daily lithium treatment at the same time. When the transplanted cells were checked eight weeks after cell transplantation, the cells were not detectable even in the lithium-treated rat spinal cords (data not shown). This suggests that even though lithium may promote N01.1 cell proliferation at some time point, without immunosuppression, the transplanted cells still die eventually at longer period of time, most likely due to the host immune response.

Lithium robustly increased numbers of N01.1 cells transplanted into injured rat spinal cords (Figures 10-12), and remarkably increased RNA levels of several neurotrophic factors in the spinal cords with N01.1 cell transplantation (Figures 14). We originally suspected that neurotrophic factor stimulation in the spinal cord might account for one of the mechanism by which lithium promotes transplanted N01.1 cell survival or proliferation. After close inspection of the results, I feel that this may not be the case. Neurotrophic factor RNA levels tended to be higher in the injured rat spinal cords transplanted with N01.1 cells than in the injured spinal cords without N01.1 cell transplantation (Figure 14), yet most of the transplanted cells died. Further increase in neurotrophic factors is unlikely to make such a big difference in transplanted N01.1 cell survival.

The results of our laboratory suggest a powerful method of promoting transplanted cell survival or proliferation in the CNS. A recent study showed that lithium treatment significantly increased numbers of neural progenitors transplanted into normal rat spinal cords (Su et al., 2007). This suggests that lithium’s promotion of transplanted cell survival or proliferation in the CNS is not restricted to N01.1 cells, but also applies to
neural progenitors. We next could explore whether lithium can promote survival or proliferation of other types of cells transplanted into the diseased or injured CNS, for example, neural precursor cells, bone marrow stromal cells, umbilical cord blood cells, olfactory ensheathing glial cells, etc.

7. Lithium Promotes Proliferation of Blood-Derived Cells and Neural Precursor Cells in Vitro

Preliminary results of our laboratory showed that lithium remarkably stimulated cell number increase in cultures of blood-derived N01.1 cells (Figure 9). Since the survival rate of the N01.1 cells in the culture condition is very high, lithium may not have much influence on N01.1 cell survival. Lithium-induced cell number increase in these N01.1 cell cultures therefore is mostly likely due to lithium’s promotion of cell proliferation. While lithium may promote N01.1 cell proliferation before they reach 100% confluency in culture, from my observation, lithium may also render the N01.1 cells to overcome contact inhibition and continuously grow after reaching 100% confluency. While lithium promotes N01.1 cell proliferation in vitro, lithium robustly promoted survival or proliferation of N01.1 cells acutely transplanted into injured rat spinal cords (Figure 10-12). Thus lithium may promote survival or proliferation of cells, depending on the situation. Acutely after spinal cord injury, the spinal cord is under a very stressed condition, which also put the transplanted cells into a very stressed condition. Lithium may act on various kinds of cells in the body, attenuating the stress of the transplanted cells, promoting the transplanted cell survival.
My studies also showed that lithium stimulated cell number increase in cultures of neural precursor cells, including mouse primary neural precursor cells, rat primary neural precursor cells, and radial glial (neural precursor) cell clone RG3.6 cells (Figures 15-18). Lithium selectively induces expression of a plethora of cell proliferation-related genes but not cell survival-related genes in cultured neural precursor clone RG3.6 cells (Table 4). In addition, lithium promotes proliferation but not survival of RG3.6 cells in vitro (Figures 19-21). Consistent with my studies, a recent study showed that lithium promoted proliferation of primary neural precursor cells both in vitro and after transplanted into normal rat spinal cords (Su et al., 2007). Altogether, these studies provide important insight into the therapeutic effects of lithium in the CNS.

8. Lithium Promotes Neural Precursor Proliferation through GSK-3β Inhibition and Subsequent NFAT Activation

The mechanism by which lithium stimulates neural precursor cell proliferation is currently poorly understood. My experimental results suggest that lithium’s promotion of neural precursor proliferation does not necessarily involve neurotrophic factors (Figures 22-23), or inositol depletion (Figure 24), but may involve GSK-3β inhibition (Figures 25-26) and subsequent NFAT activation (Figures 29-30). I also found that lithium-induced down-regulation of Dscr111, a potential calcineurin/NFAT suppressor, may provide a positive feedback for NFAT activation (Figures 27-28, 31 and 33). These findings not only provide mechanistic insights into the clinical effects of lithium, but also suggest an alternative therapeutic strategy for manic depressive illness and other neural diseases by targeting GSK-3β-NFAT signaling.
To substantiate these findings, we will first ascertain the role of GSK-3β inhibition in lithium’s promotion of neural precursor clone RG3.6 cell proliferation by checking whether more specifically knocking down GSK-3β by specific GSK-3β SiRNA, or overexpressing constitutively active GSK-3β, could mimic or counteract lithium’s promotion of RG3.6 cell proliferation, respectively. Next, we could examine which NFAT member(s) is activated by lithium in RG3.6 cells. We could determine this by checking the nuclear translocation of NFAT(s), using immunocytochemistry and western blot analysis with separated cytosol and nuclear fractions. After we find out which NFAT member is activated by lithium, we could then specifically knock down expression of that NFAT member using SiRNA technology to check whether this could block lithium’s promotion of neural precursor clone RG3.6 cell proliferation and lithium-induced Dscr1ll1 and Sez6 gene expression down-regulation. We may also check whether overexpressing constitutively active form of that NFAT can mimic lithium’s effects in RG3.6 cells. Both Dscr1ll1 and Sez6 gene contain a conserved NFAT binding motif in their promoter regions (Figures 31 and 32). We could use electrophoretic mobility shift assay, chromatin immunoprecipitation assay, and reporter assay to check whether NFAT can directly bind to the promoter regions of these two genes. To ascertain that GSK-3 inhibition leads to NFAT activation in RG3.6 cells, we could check whether the specific GSK-3 inhibitors, SB415286 or SB216763, could also activate that NFAT member(s) in RG3.6 cells. We may also check whether specifically knocking down GSK-3β using GSK-3β specific SiRNA could activate the NFAT member(s) in RG3.6 cells.
Lithium down-regulates Dscrl11 RNA expression in RG3.6 cells. We next would like to ascertain whether Dscrl11 down-regulation plays a role in NFAT activation and RG3.6 cell proliferation. We first should examine Dscrl11 expression at the protein level to check whether lithium down-regulates DSCR1L1 protein expression in RG3.6 cells. Next we will need to determine whether DSCR1L1 protein interacts with calcineurin. We could use immunoprecipitation assay to check whether calcineurin antibody can pull down DSCR1L1 or whether DSCR1L1 antibody can pull down calcineurin. We could then use an NFAT activity reporter or examine NFAT nuclear translocation to check whether overexpressing Dscrl11 or suppressing Dscrl11 could inhibit or activate NFAT, respectively. Finally, we could check whether overexpressing Dscrl11 or specifically suppressing Dscrl11 expression could counteract or mimic lithium’s promotion of RG3.6 cell proliferation.

After we define the mechanism by which lithium promotes RG3.6 cell proliferation more thoroughly, we will try to confirm the results in purified primary neural precursor cells. We could also check whether this mechanism applies to blood-derived N01.1 cells. In addition, we could investigate the involvement of GSK-3 inhibition in lithium’s promotion of transplanted cell survival or proliferation in the CNS by checking whether specific GSK-3 inhibitors such as SB415286 or SB216763 could similarly promote survival or proliferation of N01.1 cells transplanted into injured rat spinal cords.

My experiments suggest that lithium promotes neural precursor cell proliferation through GSK-3β inhibition but not inositol depletion. But it seems highly plausible that inositol depletion and inhibition of GSK-3 both contribute to the therapeutic actions of
lithium, because either inositol depletion or GSK-3β deficient animals exhibit phenotypes similar to those induced by lithium treatment (O'Brien et al., 2004; Bersudsky et al., 2008). Given an important role of inositol in various signaling pathways including the GSK-3 pathway and an essential role of GSK-3 in the optimal de novo synthesis of inositol (Azab et al., 2007), the two different mechanisms possibly also together with other mechanisms collaboratively fine-tune animal’s responses to lithium.

It is likely that the relative importance of each mechanism is also context dependent. For example, inositol depletion and GSK-3β inhibition are responsible for lithium-induced growth cone spread in dorsal root ganglion sensory neurons and neuronal polarity of hippocampal neurons, respectively (Williams et al., 2002; Jiang et al., 2005; Yoshimura et al., 2005). In the hippocampal neuron polarity, collapsin response mediator protein-2 (CRMP-2), a factor critical for specifying axon/dendrite fate by promoting neurite elongation via microtubule assembly, is the major target of GSK-3β (Yoshimura et al., 2005). Interestingly, my experimental results suggest that inhibition of GSK-3β, but not inositol depletion, is the mechanism accounting for lithium-induced cell proliferation. In addition, NFAT activation is involved in lithium-induced neural precursor cell proliferation. My studies together with those from others suggest that lithium utilizes different mechanisms to achieve the same or different effect on different cells. These effects are further integrated, which will decide the final outcome of lithium administration. Thus, understanding when and where each mechanism is most effective will help to fine-tune therapeutic strategies to promote or constrain specific arms of lithium.
9. Cyclosporin A May Suppress Neural Precursor Cells and Antagonize the Effects of Lithium

Cyclosporin A (CsA) is used to suppress host immune response to allogeneic transplants in human patients and animals. The use of CsA frequently induces neurological side effects in up to 40% of human patients (Gijtenbeek et al., 1999). The symptoms may include tremor, headache, seizures, altered mental functioning, impaired consciousness, visual disturbances (including cortical blindness), loss of motor function, and movement disorders, etc. The causes of the CsA-induced neurological side effects are not clear. My experiments showed that CsA at doses of 10 μM and above was detrimental to RG3.6 cells. This suggests that EPO may suppress neural precursors and possibly other neural cells in the CNS, which may contribute to some of the adverse effects of CsA in the CNS.

CsA prolongs survival of allogeneic transplants mainly by suppressing cell-mediated immune reactions, and to a less extent, some humoral immunity. The experimental results of our laboratory suggest that lithium can also promote survival or proliferation of cells transplanted into the CNS after injury (Figures 10-12), possibly through a different mechanism. On the other hand, my experiments suggest that CsA can antagonize lithium’s effects on neural precursor cells (Figure 30). This brings up the possibility that CsA may also antagonize lithium’s effects on cells transplanted into the CNS. Since there is no clinical need for using CsA together with lithium, the drug interaction between CsA and lithium has not been studied yet. It would be interesting to check whether administering lithium together with CsA at the same time will attenuate or boost lithium’s effects on transplanted cells. If CsA attenuate lithium’s effects on
transplanted cells, it suggests that NFAT activation may also contribute to lithium’s promotion of transplanted cell survival or proliferation in the CNS, and that we should avoid using lithium and CsA together at the same time.
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PUBLICATIONS (Publications in Chinese journals are not included)

8. Zhaoxia Qu, Dongming Sun, and Wise Young. Erythropoietin stimulates expression of erythropoietin receptor and neural stem cell marker nestin in injured rat spinal cords. (In preparation)
9. Tsutomu Iseda, Masayuki Hashimoto, Zhaoxia Qu, Dongming Sun, and Wise Young. Lithium robustly increases numbers of blood-derived cells transplanted into injured rat spinal cord. (In preparation)
10. Tsutomu Iseda, Masayuki Hashimoto, Zhaoxia Qu, Dongming Sun, and Wise Young. Lithium treatment combined with transplantation of blood-derived cells stimulates neurotrophic factor expression in injured rat spinal cords. (In preparation)
11. Zhaoxia Qu, Dongming Sun, Wise Young. Lithium promotes neural precursor cell proliferation: evidence for the involvement of the non-canonical GSK-3β-NF-AT signaling. (In preparation)
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