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The Neuroprotective Effects of Lithium in Deafferentation-Induced Cell Death of Chick Cochlear Nucleus Neurons

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THE NEUROPROTECTIVE EFFECTS OF LITHIUM IN DEAFFERENTATION-INDUCED CELL DEATH OF CHICK COCHLEAR NUCLEUS NEURONS

By,

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<tbody>
<tr>
<td>ABC</td>
<td>avidin biotin complex</td>
</tr>
<tr>
<td>ACSF</td>
<td>artificial cerebral spinal fluid</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>AKT</td>
<td>known as protein kinase B</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>APAF-1</td>
<td>apoptosis activating factor-1</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine trisphosphate</td>
</tr>
<tr>
<td>BCL-2</td>
<td>b cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DAB</td>
<td>diaminobenzidine</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DIABLO</td>
<td>direct inhibitor of apoptosis-binding protein with low pl</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxryribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothrietol</td>
</tr>
<tr>
<td>GSK-3</td>
<td>glycogen synthase kinsase-3</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HSF</td>
<td>heat shock factor</td>
</tr>
<tr>
<td>IAPs</td>
<td>inhibitors of apoptosis proteins</td>
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</table>
ICC  immunocytochemistry

iGluR  ionotopic glutamate receptor

LiCl  lithium chloride

mGluR  metabotropic glutamate receptor

mRNA  messenger ribonucleic acid

NA  nucleus angularis

NFkB  nuclear factor kappa beta

NGS  normal goat serum

NHS  normal horse serum

NMDA  n-methyl d-aspartate

NL  nucleus laminaris

NM  nucleus magnocellularis

PBS  phosphate buffered saline

pCreb  phosphorylated calcium/cAMP response element binding protein

PEBP-2beta  polyomavirus enhancer binding protein

RNA  ribonucleic acid

Smac  second mitochondrial-derived activator of caspase

SDS  sodium dodecyl sulfate

SSC  saline-sodium citrate
ABSTRACT

The avian brainstem serves as a useful model to answer the question of how afferent activity influences viability of target neurons. Approximately 20-30% of neurons in the avian cochlear nucleus, nucleus magnocellularis (NM) die following deafferentation (i.e., deafness produced by cochlea removal). We have recently shown that chronic treatments of lithium increase neuronal survival following deafferentation. The mechanism of this neuroprotective effect is unknown, but one possibility is that lithium increases the expression of neuroprotective molecules. We have shown that the neuroprotective protein Bcl-2, is upregulated in NM neurons following lithium treatment. Interestingly, Bcl-2 mRNA (but not protein) is upregulated in a subpopulation of NM neurons following deafferentation. In both cases, it is not known what sequence of events leads to the upregulation of Bcl-2. The present experiments examine changes in molecules known to regulate Bcl-2 expression to determine whether similar factors are involved in the regulation observed following lithium administration and following deafferentation. Although Bcl-2 expression may be an important contributor to neuronal survival, it is possible that lithium is neuroprotective through its influence on other molecules. To begin to identify the mechanism of lithium’s neuroprotective effect, we also determined where in the cell death cascade lithium is having its effect.
CHAPTER 1

GENERAL INTRODUCTION

Cell death is a highly regulated and organized process during development of the nervous system. This process can be induced by many events including injury, disease states and absence or loss of normal afferent sensory inputs. This dissertation focuses on cell death in the auditory system that is observed following deafness. This deafferentation-induced cell death was first observed in chicken embryos by Levi-Montalcini (1949). Deafness-induced cell death is also observed in the post hatch chickens (Born and Rubel, 1985). Cell death following the loss of sensory input is not unique to the chicken auditory system. Similar cell death is observed in the mammalian auditory system (Hashisaki and Rubel, 1989, Tierney et al., 1997). In addition, cell death has been observed in other sensory systems following the loss of sensory input (Oppenheim, 1991). For instance, in the visual system, activity-dependent release of trophic factors from the retinotectal axon terminals, can influence cell survival of tectal neurons (Castiscas et al., 1992). In the olfactory system unilateral naris occlusion can result in death of mitral and tufted cells (Meisami and Safari, 1981). Additionally, olfactory bulb ablation transiently increases tunnel positive cells in the piriform and dentate gyrus which suggests a trans-synaptic regulation of cell survival (Pope and Wilson, 2007).

This chapter will first review some of what is known about cell death in the auditory system and our recent work on neuroprotection in this system. I will then set up the questions and hypotheses that are tested in the experimental reports contained in the chapters 2 and 3. It is hoped that in addition to understanding neuronal changes following deafness, this research will also aid in understanding the interplay of pro-life and pro-death molecules that could lead to treatments for neurodegenerative disorders such as Huntington’s and Parkinson’s diseases.
**Apoptosis**

There are numerous ways by which a neuron can die. Based on the sequence of events, cell death is commonly categorized as either programmed cell death (apoptosis) or necrosis. Necrosis is typically considered a pathological disintegration of the cell with complete emptying of its contents within the extracellular medium. It can trigger an inflammatory response that can lead to scar tissue. One of the most important early steps in necrosis is the excessive increase in cytosolic calcium (Salposky, 2001). This increase in calcium can result in an increase in mitochondrial calcium which can inhibit the respiratory energy cycle and energy production processes (Genestra, 2007). These changes lead to an increase in the reactive oxygen species (ROS) which can go on to oxidatively damage various components of the cell. Excessive calcium can also activate a number of enzymes such as calpains. Calpains and other proteases which degrade neurofilaments, seem to be involved in the late phase of the calcium activated excitotoxic cascade in ischemia-induced neuronal loss (Golstein and Kroemer, 2007). Another enzyme with potentially self-digesting properties to a cell, is the phospholipase A₂, which is activated by elevations of intracellular calcium (Salposky, 2001).

Apoptosis is commonly used to describe the type of cell death that takes place during normal development. Apoptosis, which means dropping off, is a highly regulated process believed to be critical for the elimination of supernumerary cells and cells with inappropriate synaptic connections in the developing nervous system (Oppenheim, 1991, Jacobson et al., 1997). Interestingly, some of cellular and molecular events that take place during naturally occurring developmental cell death are also observed during cell death associated with neurodegenerative disease states such as Huntington’s and Parkinson’s diseases and following injury such as stroke or even deafferentation (Norenberg and Rao, 2007; Sugwara et al., 2004).

Apoptosis is activated through two main pathways extrinsic and intrinsic (Hutchins and Barger, 1998). The extrinsic pathway begins outside the cell when pro-apoptotic ligands bind with pro-apoptotic receptors on the cell that will undergo apoptosis. The intrinsic pathway is induced by developmental cues, such as inappropriate connections (wrong signal sent to postsynaptic cell or intense cell stress such as DNA damage, defective cell cycle, hypoxia, or loss of cell survival factors).
The mitochondria are commonly thought to be involved in the regulation of cell death in other systems (Gross et al., 1999). When the mitochondrion is comprised (for example, by oxidative damage), the outer mitochondria membrane becomes more permeabilized and allows for the release of cell death signals into the cytosol. This leads to the activation of caspases, a family of cysteine proteases that are responsible for the initiation and execution of apoptosis (Festejens et al., 2006). The pathway from apoptotic trigger to the caspase activation cascade can take different routes. One of the best mapped pathways involves the release of cytochrome c into the cytoplasm. Once cytochrome c is released, it forms a complex with Apaf-1, dATP and procaspase 9, called an apoptosome (Li et al., 1997). This results in a cleavage of procaspase 9 to produce caspase 9, which, in turn, cleaves procaspase 3 to activate caspase 3. The proteolytic activity of caspase 3 culminates in an apoptotic form of cell death.

Another putative pro-apoptotic factor that is associated with the mitochondrion is the Apoptosis Inducing Factor (AIF) (Bras et al., 2005). AIF is also released following mitochondrial permeability and appears to target the nucleus, causing its condensation. A third potential apoptotic factor that is released from the permeable mitochondrion is Smac/Diablo (second mitochondria-derived activator of caspases/direct inhibitor of apoptosis binding protein with low Pi) (Kim et al., 2006). Smac/Diablo binds to inhibitors of apoptosis proteins (IAP). IAP’s bind caspases and have protective effects. By binding to the IAP’s, Smac/Diablo allows the activated caspases to have uninhibited effects.

Release of factors, such as cytochrome c, can be prevented by the anti-apoptotic protein Bcl-2. Bcl-2 is a member of a large group of proteins that regulate cell death/life. This group is divided into two types, those that rescue cells (bcl-2, bcl-xL, and bcl-w) and those that are pro-apoptotic (bax, bad, and bcl-xS) (Verma et al., 2006). Through their interactions, the relative concentrations of these two groups can tip the cell towards life or death. For example, Bcl-2 which is located in the outer mitochondrial membrane helps maintain the mitochondrial membrane integrity (Gross et al., 1999). In the presence of trophic factors, the pro-apoptotic gene Bad is phosphorylated and this event allows Bcl-2 to interact with another outer mitochondrial membrane protein, Bax. If Bax and Bcl-2 interact there will be no subsequent release of cytochrome c. However, if trophic support is taken away, as is the case in deafferentation (Yin et al., 1994), Bad remains unphosphorylated and is able to heterodimerize...
with Bcl-2 (Verma et al., 2006). This prevents Bcl-2 from binding with Bax leading to the formation of Bax homodimers. The Bax homodimers are involved in making the mitochondrial membrane permeabilized, thereby allowing the release of the caspase activating substances (e.g. cytochrome c) into the cytosol.

It is important to note that many instances of neuronal death cannot clearly be categorized as either apoptotic or necrotic types of neuronal death. Rather they have only some features of one or perhaps both types. This is the case for the type of cell death that is investigated in these studies. Deafferentation-induced cell death of auditory neurons share features of typical apoptosis such as shrinkage in neuronal size and lack of cytoplasmic membrane rupture (Born and Rubel, 1985). Yet other events consistent with characteristics of apoptosis, such as nuclear condensation and cytoplasmic blebbing, are not commonly observed. Consequently, rather than simply labeling the form of cell death as either apoptosis or necrosis, it is important to characterize the specific sequence of events that occur during the cell death process.

**Model System and Sequence of Events**

The auditory system of the chick has proven to be a useful model in examining deafferentation-induced cell death. In this system, the ipsilateral auditory or eighth nerve projects solely to two populations of neurons, nucleus angularis (NA) and nucleus magnocellularis (NM) (**Fig. 1**). The eighth (VIII) nerve provides these NM neurons with their only source of excitatory input, which comes in form of large synapses called end-bulbs of Held, or calyces. Each NM neuron then projects bilaterally to nucleus laminaris (NL). The unilateral innervation of NM by the auditory nerve is useful for studies of activity-dependent changes in the developing nervous system because unilateral manipulations of the auditory periphery only affect sensory input to ipsilateral NM, yet leave input to contralateral NM intact. This allows for within-subject comparisons of NM neurons following manipulations of auditory activity.
Figure 1.) Schematic of the chick auditory brainstem. Auditory nerve (n. VIII) which originates in the n. VIII ganglion cells projects unilaterally to the cochlear nuclei, nucleus magnocellularis (NM) and nucleus angularis (NA). These projections then cross the midline to synapse on both the ipsilateral and contralateral neurons nucleus laminaris (NL). Local GABAergic neurons (gray) and the superior olive (SO) provide inhibitory input to this system.
The most common manipulation has been to induce deafness by removing the cochlea on one side and then comparing NM neurons on the deafened side with those on the intact side. Approximately 20-30% of NM neurons die within two days following deafness (Born and Rubel, 1985; Hyde and Durham, 1994a and b; and Edmonds et al., 1999). The remaining 70% show signs of atrophy (within two days following cochlea removal (CR) but survive (Born and Rubel, 1985). The NM neurons that do survive show a decrease in cell size and maintain life albeit in a relatively lower metabolic state (Rubel et al., 1991).

There are well documented arrays of events that occur after deafferentation in the chick auditory system (Fig. 2). One of the first events observed following cochlea removal is a decrease in protein synthesis in NM neurons (Steward and Rubel, 1985). Additionally, there is a rise in intracellular calcium (Zirpel et al., 1995; 1996; 1998; 2000b). Both of these events are observed across the entire population of deafferented NM neurons within one hour following cochlea removal.

The rapid changes in calcium homeostasis and protein synthesis have allowed investigators to use an in vitro brain slice preparation to examine the possible activity-dependent signals that might be required to maintain neurons in a healthy state. Both ratiometric calcium imaging studies and studies examining ribosomal functional integrity have lead to a similar conclusion. In both cases, unstimulated NM neurons in vitro show changes that are similar to those observed following cochlea removal and these changes can be reduced by electrical stimulation of the auditory nerve (Hyson and Rubel, 1989; Hyson, 1995; Zirpel and Rubel, 1996). Pharmacological studies have shown that stimulation is only effective if it results in the activation of metabotropic glutamate receptors (mGluRs) (Zirpel et al., 2000a,b; Hyson, 1995; Nicholas and Hyson, 2004). This suggests that activity maintains NM neurons in a healthy state by some sequence of events requiring the activation of mGluRs. Without this activation, intracellular calcium levels rise to potentially toxic levels and the cell’s protein synthesis machinery is disrupted. It is noteworthy, however, that although this rise in calcium and initial change in synthesis is seen across the entire population of NM neurons, but only a fraction (about 20-30%) of NM neurons go on to die. By six hours following deafferentation, autoradiographic analysis of protein synthesis can segregate neurons in the deafferented NM into dying and surviving populations (Steward and Rubel, 1985; Born and Rubel, 1985; Born et al.,
A subpopulation of cells (20-30%) show a complete cessation of protein synthesis, and electron microscopic analyses showed a dissociation of ribosomes in this same population of neurons. These, presumably, are neurons that eventually die.

Within the first day after cochlea removal there is also a proliferation of mitochondria and an increase in several key enzymes for oxidative metabolism (Durham and Rubel, 1985; 1993; Hyde and Durham, 1994 a,b). Given the dramatic changes in mitochondria, it is possible that some of the key players in mitochondrial-related apoptosis might be involved in controlling cell death following deafferentation. Indeed, the mitochondrial protein, Bcl-2 has been implicated as having a neuroprotective effect in mammalian cochlear nucleus neurons following deafferentation (Mostapour et al., 2002). Wilkinson et al. (2002) found that mRNA for Bcl-2 is dramatically upregulated in a subpopulation of NM neurons (approximately 30%) within 6-12 hours following cochlea removal. Other key players in the typical apoptotic cell death cascade have also been shown to be upregulated in the deafferented populations of neurons. Immunostaining for both cytochrome c and active caspase 9 increase, but there does not appear to be significant release of cytochrome c into the cytosol (Wilkinson et al., 2003). Although this seems unusual, it is possible for caspase activation to be independent of cytochrome c (Marsden et al., 2004).

**Lithium**

As noted above, Bcl-2 often plays a vital part in neuronal survival. Wilkinson et al. (2002) provided us with an interesting theory based on the changes in Bcl-2 mRNA expression in NM following cochlea removal. They suggested that the subpopulation (30%) of NM neurons that show an increase of Bcl-2 mRNA is the same 30% of neurons that go on to die. This is surprising since Bcl-2 is known to have a neuroprotective influence. At this time point (6-12 hours post cochlea removal), however, protein synthesis has broken down in the dying subpopulation of NM neurons (Steward and Rubel, 1985; Born and Rubel, 1988). Consequently, these cells cannot translate the mRNA into the viable protein to sustain life.
Table 1.) Time course post cochlea removal of known cellular and molecular events in NM neurons.

<table>
<thead>
<tr>
<th>Time after Cochlea Removal</th>
<th>Observed consequences</th>
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| 1-3 hours                  | 1.) Decrease in protein and RNA synthesis  
                              2.) Increase in intracellular calcium |
| 6-12 hours                 | 1.) Increase in bcl-2 mRNA  
                              2.) Increase in cytochrome c (most likely due to the subsequent mitochondrial proliferation)  
                              3.) Increase in oxidative metabolism  
                              4.) Decrease in Y10b staining |
| 24+ hours                  | 1.) Decrease in neuronal number (approximately 20-30%)  
                              2.) Decrease in soma size  
                              3.) Decrease in oxidative metabolism |
Bush and Hyson (2006) suggested that the increase in Bcl-2 mRNA observed by Wilkinson et al. (2002) is a hint that Bcl-2 protein might be able to protect cells from deafferentation-induced cell death. They presumed that the increase in message indicated that some compensatory cellular signaling was engaged to increase the expression of neuroprotective molecules. Unfortunately for the NM neurons, those needing this protective upregulation are unable to produce the protein at the time. They hypothesize that if Bcl-2 protein was upregulated before insult (i.e. CR), then more cells would have enough of the cytoprotective protein to prevent the deafferentation-induced death. To test this hypothesis, they used chronic administration of lithium.

Lithium is the main therapeutic agent for the control of Bipolar Disorder yet its mechanism of action is not thoroughly understood. One possible mechanism of action is that lithium has been shown to increase neuronal survival (Chuang et al., 2002). Chronic lithium has been shown to reduce cell death in animal models of ischemia and the neurodegenerative disease, Huntington’s (Leegwater-Kim and Cha, 2004; Nonaka and Chuang, 1998). Bush and Hyson (2006) found that chronic lithium administration increased Bcl-2 protein in NM neurons and reduced deafferentation-induced cell death by approximately 50%.

There are various mechanisms by which lithium could have its neuroprotective effect (Fig 2). Rowe and Chuang (2004) concluded that lithium directly inhibits the kinase, Gsk-3beta. Gsk-3beta is a kinase that phosphorylates various transcription factors and tags them for degradation. In this way, Gsk-3beta affects NFkappaB, pCreb, and Beta-catenin (Grimes and Jope, 2001; Hoeflich et al., 2000; Yost et al., 1996; Ilouz et al., 2006), all of which promote cell survival. By regulating these transcription factors, Gsk-3beta can indirectly regulate the Bcl-2 family of proteins (Grimes and Jope, 2001). Inhibition of Gsk-3beta would allow for prolonged activity of these transcription factors and thus allow a greater opportunity for these factors to increase the anti-apoptotic molecule Bcl-2 (Tamutani et al., 1999; Pahl, 1999; Hammonds et al., 2007; Li et al., 2007).

Chen and colleagues (1999) reported that lithium and valproate (another bipolar disorder treatment) robustly increase the levels of Bcl-2 in frontal cortex. Lithium could increase Bcl-2 by indirectly upregulating transcription factors that promote expression of Bcl-2 such as NFkappaB (NFkB). The commonly proposed pathway suggests that lithium inhibits glycogen synthase
kinase-3beta (Gsk-3beta) which allows for a prolonged expression of NFkB which goes on to promote Bcl-2. Demarchi et al. (2003) proposed that lithium increases NFkB activity by way of its inhibition of Gsk-3beta. Gsk-3beta phosphorylates NFkB at the p105 site. When phosphorylated at this site, NFkB is targeted for degradation. So quite possibly, chronic lithium could be indirectly affecting the expression of the transcription factor NFkappaB.

Like many transcription factors, Beta-catenin function is dependent on phosphorylation. There are several studies that suggest that Gsk-3beta may be the primary kinase responsible for phosphorylation and thus degradation of Beta-catenin levels (Grimes and Jope, 2001). Gsk-3beta typically phosphorylates Beta-catenin at a specific site and tags it for degradation. Mutant mice lacking a Gsk-3beta site on Beta-catenin are more active and stable than wild-type in the presence of Gsk-3beta activity (Yost et al., 1996). By degrading Beta-catenin, Gsk-3 inactivates the transcription factor complex that Beta-catenin is associated with and thereby disrupts the expression of a variety of genes that are involved in cell cycle, cell adhesion, and cellular development. Thus inhibition of Gsk-3beta with lithium could lead to an increase in Beta-catenin. If Beta-catenin is increased then it is possible that this increase could promote neuronal survival in NM.

Interestingly, lithium also affects the transcription factor Creb (Chuang et al., 2002). Kopinsky et al. (2003) saw that Creb protein can function to promote the expression of pro-survival proteins.
Figure 2) Proposed pathway of the neuroprotective pathway of lithium pretreatment in NM neurons. Increase in lithium will give a subsequent decrease in GSK-3 (the main kinase affected by lithium pretreatment). This inhibition of GSK-3 by lithium will lead to an increase in the transcription factors NFkappaB, pCREB and Beta-catenin. All three of these transcription factors can increase the cytoprotective protein Bcl-2. Once Bcl-2 is increased there will be an increase in neuronal survival.
They showed that chronic lithium pretreatment suppresses glutamate-induced decrease in pCreb. Einat et al. (2003) showed that phosphorylated transcription factors, such as pCreb are increased and proapoptotic genes, such as BAD, are decreased when subjects are pretreated with lithium chronically. It is possible that lithium pretreatment will have the same effect on pCreb in NM neurons.

In addition to changes in gene expression that may influence how cells respond to apoptotic signals, lithium may also influence mechanisms that control the early triggers of cell death. Lithium can prevent intracellular calcium release in other systems (Ikonomov et al., 2000; Okamoto et al., 1994). Nonaka et al. (1998) reported that pretreatment with lithium markedly protected cultured rat hippocampal neurons against glutamate-induced, NMDA-receptor mediated, excitotoxicity by inhibiting calcium influx. Additionally, Shao et al. (2005) showed that lithium inhibited glutamate-induced increases in cytoplasmic free calcium concentration.

We have recently shown that chronic administration of lithium before cochlea removal upregulates the anti-apoptotic protein Bcl-2 and prevent cell loss by approximately 50% (Bush and Hyson, 2006). As reviewed above, however, lithium influences several molecular pathways that could lead to upregulation of Bcl-2 and neuroprotection and it is still unknown which of these pathways are influenced in NM. Deafferentation of NM neurons also leads to an increase in Bcl-2 mRNA labeling in a subpopulation of neurons (Wilkinson et al., 2002), but it is not known if this increase involves the same pathways as those influenced by lithium. In chapter 2, we will examine whether lithium is inhibiting the kinase Gsk-3beta and the transcription factors NFkappaB, Beta-catenin, and pCreb. Additionally, we will examine whether deafferentation influences the expression of the same molecules (proteins, kinases, and transcription factors) that are involved in the lithium neuroprotection pathway. It is hoped that these studies will provide a more precise and detailed account of which molecules are important for rescuing cells from death and increasing the cytoprotective protein Bcl-2. A second unanswered question is where in the cell death cascade is lithium having its effect? Is it preventing the initial cell death trigger, or do the changes in gene expression influence a downstream event? The experiments in Chapter 3 address this question by examining effects of chronic lithium treatment on some of the known early changes that occur following deafferentation.
CHAPTER 2
PROTEINS THAT ARE REGULATED BY CHRONIC LITHIUM AND DEAFFERENTATION IN THE CHICK COCHLEAR NUCLEUS

Introduction

Cell death in the nervous system can be induced by many events including injury, disease states and absence or loss of normal afferent sensory inputs. In the developing auditory system deafferentation produced by cochlea removal, can result in death of cochlear nucleus neurons (Born and Rubel, 1985; Hashisaki and Rubel, 1989; Tierney et al., 1997). The brainstem auditory system of the chick has proved to be a useful model for understanding this form of cell death (Fig. 1) (Rubel et al., 1990). In this system, the ipsilateral VIIIth nerve synapases on a homogenous population of auditory neurons in nucleus magnocellularis (NM) and provides NM neurons with their sole excitatory input (Lippe et al., 1980; Rubel et al., 1990; Born et al., 1991). Consequently, unilateral cochlea removal will deafferent only the ipsilateral NM, but leaves innervation to the contralateral NM unaffected. This allows for within-subject comparisons between deafferented and intact neurons. Approximately 20-30% of deafferented NM neurons die within a few days. The remaining neurons survive, albeit at a lower metabolic state and with a reduction in soma size (Parks and Rubel, 1978; Born and Rubel, 1985; Born and Rubel, 1988).

Deafferentation-induced changes in NM neurons are observed across the entire population of neurons as early as one hour after cochlea removal. At this early time there is a reduction in protein synthesis, RNA synthesis, and an increase in intracellular calcium (Steward and Rubel, 1985; Garden et al., 1995; Zirpel et al., 1995). At later time points (6-12 hours post cochlea removal) the NM neurons appear to divide into two discrete populations: those that die and those that will survive. Cells that are destined to die show a breakdown of polyribosomes (Rubel et al., 1991) and an apparent total cessation of protein synthesis (Steward and Rubel, 1985).

The deafferentation-induced cell death of NM neurons includes some features of typical apoptosis such as shrinkage in neuronal size and lack of cytoplasmic membrane rupture (Born and Rubel, 1985) (Table 1). Yet, these neurons do not typically show other characteristics of
apoptosis, such as nuclear condensation and cytoplasmic blebbing. At the molecular level of analysis, some molecules that are commonly involved in controlling apoptosis have also been shown to be altered by deafferentation. Wilkinson et al. (2003) showed that active caspase 9 was increased in deafferented NM neurons, as well as cytochrome c. One particularly intriguing set of experiments involves the cytoprotective protein Bel-2. Bcl-2 is located in the outer mitochondrial membrane and prevents the toxic release of cytochrome c into the cytosol (Festjens et al., 2006). Moustapour et al. (2002) showed that overexpression of Bcl-2 in mice protects cochlear nucleus neurons from deafferentation-induced cell death. Wilkinson et al. (2002) observed a dramatic and transient upregulation of mRNA for Bcl-2 in the deafferented NM neurons of the chick. Unexpectedly, however, the upregulation was observed in 20-30% of the neurons suggesting that this cytoprotective gene would be upregulated primarily in the dying subpopulation of NM cells. They suggested that cellular signaling mechanisms are engaged in the dying population of cells that upregulate expression of the potentially cytoprotective Bcl-2 message, but by the time the message is upregulated, the cellular protein synthesis machinery is broken down. Consequently the message is left untranslated and cannot have its protective influence.

Bush and Hyson (2006) suggested that the upregulation of Bcl-2 message was a clue that Bcl-2 protein could protect NM neurons from deafferentation-induced cell death if the protein was produced prior to cochlea removal. They used a pharmacological treatment, chronic administration of lithium, which has been shown to upregulate Bcl-2 protein in other systems (Nonaka and Chuang, 1998; Chuang and Chen, 1999; Wei et al., 2001). They found that chronic lithium administration did upregulate Bcl-2 protein in NM neurons and did protect neurons from deafferentation-induced cell death.

Lithium is a commonly used treatment for Bipolar Disorder, although its mechanism of action is unclear. It is clear, however, that lithium can alter gene expression in neurons and that it can lead to neuroprotection (Chen et al., 1999). A first step in lithium’s action, in many cases, is the inhibition of the kinase Gsk-3beta (Grimes and Jope, 2001). This kinase has been implicated in certain psychiatric disorders and neurodegenerative diseases (Chen et al., 2004). Unlike many protein kinases, Gsk-3beta is highly active in resting cells and is primarily regulated by inactivation (Grimes and Jope, 2001). Gsk-3beta in turn regulates a variety of transcription factors including, but not limited to, pCreb, NFkappaB,
and Beta-catenin (Grimes and Jope, 2001). Inhibition of Gsk-3beta by lithium can lead to increased activity of all of these transcription factors which can subsequently increase the expression of neuroprotective proteins such as Bcl-2 (Fig. 2).

The experiments by Wilkinson et al. (2002) and Bush and Hyson (2006) clearly demonstrates that Bcl-2 expression in NM neurons can be upregulated by two different conditions: 1.) deafferentation leads to a transient upregulation of mRNA in a subpopulation of cells, and 2.) chronic administration of lithium leads to an upregulation of Bcl-2 protein across the entire population of cells. There are several possible transcription factors that regulate Bcl-2 expression and it is not known which of these are involved in the regulation of expression observed in NM neurons. In addition it is possible that the transcription factors are involved in the regulation of Bcl-2 by lithium are different than those involved in the regulation observed after deafferentation. The present experiments examine both lithium and deafferentation-induced changes in concentration of the active forms of the kinase Gsk-3beta and the transcription factors pCreb, Beta-catenin, and NFkappaB in attempt to identify the possible sequence of events leading to the upregulation of Bcl-2 expression in NM neurons.

**Experimental Procedures**

*Animals and Surgical Procedures:*

Post-hatch chicks, of either sex, received daily injections of either lithium chloride (LiCl) or saline for 17 days prior to unilateral cochlea removal. Protein expression in NM neurons was examined at various time points after CR. All procedures were in accordance with Animal Care and Use Committee guidelines.

*Daily injections:*

Post-hatch chicks (P0) were started on a lithium chloride (LiCl) or saline injection regime identical to that used by Bush and Hyson (2006). Both groups of animals received a daily sub-cutaneous injection for a total of 17 days. The dose of LiCl was gradually increased across age, beginning at 1.5 mEq/kg for the first four days, followed by 2.3 mEq/kg for seven days, and finally, 3.0 mEq/kg for the last six days. This schedule was adapted from the protocols.
of both Wei et al. (2001) and Nonaka and Chuang (1998). The volume of each injection was 0.01 ml/kg. Control subjects received daily 0.01 ml/kg injections of saline. This injection schedule produced plasma lithium levels that were proportional to the injection concentration (Bush and Hyson 2006). No lithium is detectable 24 hours after the last injection suggesting that lithium was rapidly excreted from the subject (Bush and Hyson, 2006).

**Cochlea Removal Surgery:**

Subjects received unilateral cochlea removal surgery under halothane anesthesia, one hour after the last daily injection on P16. A small incision was made to widen the ear canal and the tympanic membrane was punctured. The columella was removed, followed by the extraction of the basilar papilla (cochlea) through the oval window using forceps. The extracted tissue was viewed under a dissection microscope to ensure complete cochlea removal. The middle ear cavity was packed with Gelfoam and the external incision sealed with surgical adhesive.

**Tissue preparation:**

Subjects were allowed to survive for various times (1, 3, and 6 hours or 5 days) following cochlea removal prior to tissue preparation for the determination of protein levels using immunocytochemistry. Following the survival periods, subjects were deeply anesthetized with pentobarbital and perfused with 0.9% saline followed by ice cold 4% paraformaldehyde. Braintems were blocked and post-fixed in 4% paraformaldehyde for 1–2 h followed by overnight cryoprotection at 4°C in phosphate buffered saline (PBS) containing 20% sucrose. The tissue was rapidly frozen in 2-methylbutane on dry ice and embedded in TBS tissue freezing medium for cryosectioning using a Leica CM 1850 cryostat. Sections were cut at 25 µm and were collected into ice cold PBS for later mounting. Every section containing nucleus magnocellularis was collected.

**Immunocytochemistry:**

Pairs of subjects, one treated with LiCl and one treated with saline, were processed simultaneously (n=9, three pairs per time point). Puncture marks were made in the ventral portion of the brain stem to identify treatment condition following the simultaneous processing. Cryosections containing NM were placed in a vial containing PBS. Alternate sections of the
same subject were treated against different primary antibodies. For NFκB, tissue was microwaved for 20 seconds on low power to expose the antigen. Sections were then washed 2 X ten minutes in PBS and endogenous peroxidase activity was quenched by incubating in 0.03% H2O2 in methanol for ten minutes. Following three 10 minute rinses in PBS, sections were placed in a blocking solution containing 2% Bovine Serum Albumin (BSA), 1% Normal Goat Serum (NGS) (Gsk-3 and Beta-catenin) or Normal Horse Serum (NHS) (NFκB and pCreb), 0.1% Triton-X100 (Gsk-3beta, Beta-catenin, pCreb) or 0.4% Saponin (NFκB) in PBS for an hour. Sections were transferred to various concentrations of the primary antibodies, Gsk-3beta (h-76) (1:500, Santa Cruz, sc-9166), pCreb (ser 133) (1:1000, Upstate Signaling, 06-519), NFκB (p65 subunit) (1:250, Chemicon, mab3026), and Beta-catenin (cat-5h10) (1:1000, Zymed, 18-0226) in blocking solution and incubated on a rotator overnight at 4°C. The specificity of the reaction was assured by incubating control sections in the absence of primary antibodies. The following day, the sections were removed from 4°C and allowed to incubate at room temperature for two hours. Sections were washed 3 X 10 minutes in PBS. Sections were then incubated in a 1:200 concentration of goat anti-rabbit or horse anti-mouse secondary antibodies in blocking solution for one hour. Following 3 ten-minute washes with PBS, sections were incubated in avidin-biotin- peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA) for one hour. After another round of washes, with PBS, sections were reacted with diaminobenzidine (DAB) tetrahydrochloride and 30% H2O2 for visualization. Following a final round of washes sections were mounted on slides and allowed to dry overnight. The following day slides were dehydrated in a graded series of ethanol and cleared in xylenes. Slides were coverslipped in DPX mounting medium and allowed to dry overnight.

**Objective analysis of anatomical tissue:**

Immunolabeling of NM neurons was objectively analyzed using densitometry. When the important comparison is between lithium- and saline-treated subjects, the tissue was processed simultaneously in the same reagents to prevent processing variables from affecting the results. For examining the effects of cochlea removal, the staining densities on the intact side of the brainstem were compared to those on the deafferented side of the same tissue section using NIH Image J. The light levels and contrast settings were the same for all tissue sections analyzed. On average, 50 neurons were measured in each nucleus, on each side of the section. All neurons with a clear intact visible cell
membrane were included in the analysis. Neurons were measured starting from the most medial edge of the nucleus and proceeding laterally until either all NM neurons in that section were measured or the criterion of 50 cells was reached. Approximately 3 sections were analyzed per brain. Changes were calculated on the intact sides of both the lithium and saline treated groups. Alternate sections of the same birds were labeled with antibodies at various time points. The data were analyzed using Microsoft EXCEL and SPSS.

**Results**

*Lithium control of protein expression:*

Chronic lithium altered the expression of the proteins of interest in NM. An example of the effects of lithium on Gsk-3beta expression can be seen in Figure (3). Gsk-3 labeling is observed in the cytoplasm of NM neurons and subjects treated with lithium had lighter staining than those treated with saline. Objective analysis of labeling density confirmed these visual impressions. Figure (4) displays the average gray scale density measurements of NM neurons in lithium- and saline-treated subjects on each side of the brain at the various survival times. To avoid the possible confounding effects of cochlea removal, statistical analyses of the effects of lithium were restricted to comparisons of labeling on the intact side of the brain. There was a reliable difference between lithium and saline treated subjects (t(10)=16.792, p < 0.01).

Lithium administration increased immunolabeling for NFkappaB, pCreb, and Beta-catenin. Examples of this effect can be seen in Figures (5, 7 and 9). NFkappaB (Figure 5) labeling was observed in the cytoplasm, while Beta-catenin (Figure 7) and pCreb (Figure 9) labeling was mainly in the nuclei. A comparison of labeling on the intact sides of both saline- and lithium-treated groups show greater labeling in lithium treated subjects for all three transcription factors. Objective analysis of immunolabeling confirmed the qualitative impressions. Figures (6, 8 and 10) display the mean gray scale density measurements of NM neurons for NFkappaB, Beta-catenin and pCreb respectively. The difference between lithium- and saline-treated groups was statistically reliable in all cases (t (10) = 17.211, p<0.01, t(10)=36.955, p<0.001, t(10)=9.101, p<0.05) for NFkappaB, Beta-catenin, and pCreb respectively.
Deafferentation did not result in consistent changes in immunolabeling for either Gsk-3beta or NFkappaB at any of the time points examined. This can be seen in Figures (3-6), and statistical analyses did not reveal an effect of cochlea removal (p = 0.875 and 0.507 respectively). Labeling for the transcription molecules, Beta-catenin and pCreb, however (Figures 7-10), did show differences between the intact and deafferented sides of the section. There was lower labeling for Beta-catenin on the deafferented side of the section in both lithium- and saline-treated subjects, and this difference in labeling appeared robust at 6 hours after cochlea removal (Figure 7). To compare the effects of cochlea removal between groups, scores were converted to average percentage difference between intact and deafferented sides of the same brain. A two-way (Time X Drug) ANOVA on these percent difference scores revealed a reliable effect of Time after cochlea removal (F (2, 21) = 3.1, p < 0.05) but no reliable difference of Drug (F (1, 21), p < 1) or Drug X Time interaction (F (2, 21) = 1.98, p = 0.16). Post-hoc pairwise comparisons (Newman-Kuehls) revealed that the magnitude of the effect at 6 hours following cochlea removal was greater than that at 1 or 3 hours after surgery.

Deafferentation resulted in an increase in immunoreactivity for pCreb one hour following cochlea removal in both lithium- and saline-treated subjects (Figures 9-10). A 2-way mixed ANOVA using Drug as the between-subjects variable and Side as the within-subjects variable revealed that there was a reliable effect of Side (F (1, 10) = 52.4, p < 0.001) and, as noted above, a reliable effect of Drug (F(1,10) = 9.1, p< 0.05), but no reliable Drug X Side interaction (F(1, 10) = 3.1, p= 0.11). Converting scores to percent difference between sides confirmed that both drug treatment groups had a reliable difference between sides (t (6) = 3.9 (saline) and t(4) = 5.9 (lithium), p’s <0.05). There was no statistically reliable difference in the magnitude of the effect between saline and lithium treated subjects (between-subjects t-test on the percent difference scores (t (10) = 1.4, p= 0.19).
Figure 3. Representative photomicrographs of intact and deafferented sides of saline- (top) and lithium-treated (bottom) subjects 3 hours after cochlea removal (40x). Tissue sections were immunolabeled with an antibody recognizing Gsk-3beta. A.) saline intact side. B.) saline deafferented side. C.) lithium intact side. D.) lithium deafferented side. There is decreased labeling in the lithium-treated subjects, but no difference in labeling between intact and deafferented sides of the brain.
**Figure 4.** Average gray scale density measured over individual intact and deafferented NM neurons immunolabeled for Gsk-3beta at different times following cochlea removal. Lithium-treated subjects showed reliably less labeling than saline-treated controls \( (p<0.05) \) for Gsk-3. Error bars represent standard error of the mean.
Figure 5). Representative photomicrographs of intact and deafferented sides of saline- (top) and lithium-treated (bottom) subjects 3 hours after cochlea removal (40x). Tissue sections were immunolabeled with an antibody recognizing NFκB. A.) saline intact side. B.) saline deafferented side. C.) lithium intact side. D.) lithium deafferented side. There is an increase labeling in the lithium-treated subjects, but no difference in labeling between intact and deafferented sides of the brain.
Figure 6.) Average gray scale density measured over individual intact and deafferentated NM neurons immunolabeled for NFkappaB at different time points following cochlea removal (3 and 6 hours). Lithium-treated subjects showed reliably more labeling than saline-treated controls ($p<0.01$) for NFkappaB. Error bars represent standard error of the mean.
Figure 7. Representative photomicrographs of intact and deafferented sides of saline- (top) and lithium-treated (bottom) subjects 3 hours after cochlea removal (40x). Tissue sections were immunolabeled with an antibody recognizing Beta-catenin. A.) saline intact side. B.) saline deafferented side. C.) lithium intact side. D.) lithium deafferented side. There is an increase labeling in the lithium-treated subjects than in saline-treated subjects and cochlea removal resulted in less immunolabeling in both groups.
Figure 8.) Average gray scale density measured over individual intact and deafferented NM neurons immunolabeled for Beta-catenin at different times following cochlea removal (1, 3, 6 hours). Lithium-treated subjects showed reliably more labeling than saline-treated controls \( (p < 0.001) \) for Beta-catenin. Additionally there is an intact versus deafferented side difference in both groups \( (p< 0.01) \). Error bars represent standard error of the mean.
Figure 9.) Representative photomicrographs of intact and deafferented sides of saline- (top) and lithium-treated (bottom) subjects one hour after cochlea removal (40x). Tissue sections were immunolabeled with an antibody recognizing pCreb. A.) saline intact side. B.) saline deafferented side. C.) lithium intact side. D.) lithium deafferented side. There is an increase labeling in the lithium-treated subjects in the nucleus of the NM neurons.
Figure 10.) Average gray scale density measured over individual intact and deafferented NM neurons immunolabeled for pCreb one hour post cochlea removal. Lithium-treated subjects showed reliably more labeling than saline-treated controls \((p < 0.05)\) for pCreb. Additionally there is an intact versus deafferented side difference in both groups \((p < 0.01)\). Error bars represent standard error of the mean.
Discussion

Cochlea removal results in neuronal cell death and atrophy in the avian cochlear nucleus, nucleus magnocellularis (NM) (Born and Rubel, 1985). Previous studies have shown that chronic administration of lithium increases immunolabeling for the neuroprotective molecule Bcl-2 in NM neurons and protects NM neurons from deafferentation-induced cell death (Bush and Hyson, 2006). Cochlea removal in the absence of drug treatment also leads to an upregulation of Bcl-2 mRNA (although not Bcl-2 protein) in NM neurons. The present set of experiments examined candidate molecules that might contribute to the upregulation of Bcl-2 in these two different conditions. Lithium administration decreases Gsk-3beta expression, but increases the expression of the transcription factors NFkappaB, Beta-catenin and pCreb. Cochlea removal, on the other hand, did not appear to alter the expression of either Gsk-3beta or NFkappaB and decreased the expression of Beta-catenin. As reported previously (Zirpel et al., 2000b), however, cochlea removal did upregulate expression of pCreb.

Technical Considerations

The objective densitometric analysis of immunolabeled tissue is, at best, semi-quantitative. Conclusions can be made about relative changes in expression but there is no information about absolute concentration of the proteins of interest. In addition, processing variables, such as incubation time, can dramatically influence the overall darkness of immunolabeling. To limit the influence of these processing variables, pairs of lithium- and saline-treated brains were processed simultaneously. Marks (hole punches) on the ventral surface of the brain stem were used to identify individual brains after the sections were mounted onto slides. Effects of lithium were evaluated by comparing these pairs simultaneously processed tissue sections. Although some variables, such as the quality of fixation, could not be completely controlled, the consistency of the data suggests that the matched lithium-saline comparisons used in the analyses are valid. Statistical analyses lead to identical conclusions whether the matched lithium- and saline-treatment was analyzed as a between-subject variable or if they were treated as a within-subject variable. The influence of processing variables is even less problematic for the analyses of the effect of cochlea removal. These effects are evaluated by comparing labeling on opposite sides of the same tissue section.
Lithium control of protein expression

There are several ways by which lithium could lead to upregulation of neuroprotective proteins, such as Bcl-2, and the most common pathway proposes that the kinase Gsk-3beta is involved. The present experiments confirmed that lithium reduces levels of Gsk-3beta in NM neurons. This is in accordance with others (DeSarno et al., 2002; Grimes and Jope 2001; Gould et al., 2003) who have shown that chronic lithium pretreatment down-regulates the constitutively active Gsk-3beta found in neurons. It has been relatively well established that Gsk-3beta is a primary target of lithium and is one of the mostly highly regulated molecules by lithium pretreatment. Gsk-3beta normally degrades or blocks the activation of these factors (Grimes and Jope, 2001; Hoeflich et al., 2000, Tamatani et al., 1999; Yost et al., 1996; Kopinksy et al., 2003). By inhibiting Gsk-3beta’s activity with lithium, the target transcription factors can have enhanced activity. Some studies (Hoeflich et al., 2000), however, have shown that NFkappaB could be positively or negatively correlated to Gsk-3beta inhibition. Beta-catenin is phosphorylated by Gsk-3beta, and it appears that there is an increase in Beta-catenin when lithium inhibits Gsk-3beta (Yost et al., 1996). Inhibition of Gsk-3beta also results in increased expression of pCreb in other systems (Chuang et al., 1998), and increased expression of Gsk-3beta resulted in decreased phosphorylation of the substrate Creb among others (Ilouz et al., 2006; Kopinsky et al., 2003; Einat et al., 2003). The present studies show that the transcription factors NFkappaB, Beta-catenin, and pCreb are all increased in NM neurons following chronic lithium administration. Any of these transcription factors could lead to increases in Bcl-2 (Tamatani et al., 1999; Pahl, 1999; Hammonds et al., 2007; Li et al., 2007) or other neuroprotective molecules, and ultimately account for the neuroprotection observed following chronic lithium administration.

Deafferentation control of expression

Only two of the candidate molecules, Beta-catenin and pCreb, show a deafferentation-induced difference in immunolableing. Beta-catenin was reduced by 6 hours following cochlea removal. This is in line with the overall reduction in protein synthesis that is observed in NM neurons following deafferentation.(Steward and Rubel, 1985). The transcription factor pCreb, however, is transiently incresed one hour after cochlea removal, and there were statistically reliable differences between the intact and deafferented sides in both lithium- and saline-treated
groups. This result suggests that although lithium increases the overall levels of pCreb immunoreactivity in NM neurons, it cannot prevent the further increase in pCreb observed following deafferentation. pCreb labeling was only evaluated one hour following cochlea removal since previous studies (Zirpel et al., 2000b) showed that no pCreb was observed at later time points. The present studies confirmed this transient expression (data not presented) and further show that this transient increase in pCreb is also observed in lithium-treated subjects.

Synthesis

Current knowledge supports that lithium is neuroprotective in different animal models of disease states and hypoxia/stroke paradigms. This protection requires long term pretreatment which suggests that changes in gene expression are necessary (Chaung et al., 2000; Silverstone and Romans, 1996). Bcl-2 mRNA is also robustly upregulated in a subpopulation (approximately 20-30%) of NM neurons following cochlea removal (Wilkinson et al., 2002). This is an intriguing finding because 20-30% of NM neurons go on to die after deafferentation (Born and Rubel, 1985). It was hypothesized that the upregulation of Bcl-2mRNA in a dying subpopulation of neurons was an indicator of cell that were destined to die, and that the potentially neuroprotective Bcl-2 message was unable to be translated into protein in these neurons because protein synthesis machinery was compromised by the time the message had increased. Bush and Hyson (2006) showed that chronic lithium pretreatment increased immunolabeling for Bcl-2 protein in NM neurons and reduced deafferentation-induced cell death by approximately 50%.

The present studies suggest that the upregulation of Bcl-2 expression by lithium may involve a different sequence of events than the upregulation of Bcl-2 mRNA in NM neurons following cochlea removal. Lithium, but not cochlea removal, reduces immunolabeling for Gsk-3beta. Consequently, there was an increase in expression of all three of the transcription factors examined (NFkappaB, Beta-catenin, and pCreb). Cochlea removal did not alter NFkappaB and reduced levels of Beta-catenin. Only pCreb showed an increase in expression following cochlea removal (see also, Zirpel et al., 2000b). This suggests that, of the possible promoters examined to date, pCreb is the most likely candidate for upregulating Bcl-2 mRNA in NM neurons following deafferentation. One difficulty with this conclusion, however, is that it appears that pCreb is upregulated in the majority of deafferented neurons, but Bcl-2 mRNA s upregulated in
only 20-30% of the neurons. Additionally, pCREB upregulation is observed only very soon after cochlea removal (one hour) whereas Bcl-2 mRNA upregulation is observed 6-12 hours post cochlea removal. It is possible that pCREB is responsible for the upregulation of Bcl-2 mRNA following deafferentation, but there is a delay between the time at which pCREB promotes gene transcription and the time at which the fully transcribed and edited mRNA is present and able to be detected by the oligonucleotide probe. If this were the case, then it would still be mysterious as to why the mRNA is only observed in a subpopulation of neurons. One could speculate that there is some threshold of pCREB activation that must be achieved or there is a competing (repressive) influence that has not yet been identified in those cells that do not increase Bcl-2 expression. Alternatively, it is possible that the apparent upregulation of Bcl-2 mRNA in NM neurons is attributable to reduced degradation of the mRNA rather than increased production.
CHAPTER 3

THE INFLUENCE OF CHRONIC LITHIUM ADMINISTRATION ON DEAFFERENTATION-INDUCED CELLULAR CHANGES IN THE CHICK COCHLEAR NUCLEUS

Introduction

It is generally accepted that sensory experience plays an important role in the development of the brain. This idea is supported by studies showing changes in innervation patterns or even cell survival, following the loss of sensory innervation in young animals (e.g. Catsicas et al., 1992; Pope and Wilson, 2007; Mesaimi and Safari, 1981). One sensory system, in which the effects of sensory deprivation have been extensively studied, is the brainstem auditory system of the chick (Rubel et al., 1990). Loss of sensory input, produced by cochlea removal, results in the death of approximately 20-30% of the neurons in the cochlear nucleus, nucleus magnocellularis (NM) (Born and Rubel, 1985).

The brainstem auditory system of the chick has proven be a fruitful model system for examining the effects of sensory deprivation, in part, because of its relatively simple organization. In this system, the ipsilateral auditory or eighth nerve (n. VIII) provides the sole excitatory input to NM neurons (Figure 1) (Parks and Rubel, 1978). Consequently, a unilateral manipulation of the auditory periphery affects sensory input to ipsilateral NM, yet leaves input to contralateral NM intact. This allows for within-subject comparisons of deprived and intact NM neurons following unilateral manipulations of auditory activity. The most common manipulation has been to induce deafness by removing the cochlea on one side and then compare NM neurons on the deafened and intact sides of the brain.

A second advantage of studying the chick brainstem is that the effects of sensory input are relatively rapid. This allows one to track the sequence of events that occur between the onset of deafness and the ultimate death of the neurons. As noted, approximately 20-30% of NM neurons die within two days following deafness (Born and Rubel, 1985). The remaining neurons show decreased soma size and reduced metabolism, but survive. Changes in NM are observed,
however, at much earlier times following deafferentation. As early as one hour following cochlea removal, all deafferented NM neurons show a rise in intracellular calcium (Zirpel et al., 1995, 1996) and a reduction in overall protein synthesis (Steward and Rubel, 1985). At later time points (6-12 hours post deafferentation) deafferented NM neurons divide into two populations based on changes in ribosomal structure and function. The majority of neurons show some recovery of protein synthesis, but 20-30% of the neurons appear to show a complete disruption of protein synthesis (Steward and Rubel, 1985) that appears to be attributable to a disassociation of their polyribosomes (Rubel et al., 1991; Garden et al., 1994). Presumably, those cells that show a complete disruption of their ribosomes are those that will eventually die within the next 48 hours. The rapid changes in ribosomal function that are observed after cochlea removal are mirrored by changes in antigenicity for a ribosomal epitope that is recognized by the antibody Y10b (Garden et al., 1994; Hyson and Rubel, 1995; Hyson, 1997, 1998) (Table 1).

Analyses of molecular changes that may regulate the death or survival of NM neurons following deafferentation revealed that some molecules known to be involved in the control of traditional apoptotic cell death are also regulated by deafferentation (Wilkinson et al., 2003). Unexpectedly, however, Wilkinson et al. (2002) found an increase in mRNA expression for the neuroprotective protein, Bcl-2 in the, presumably, dying population of deafferented NM neurons. They suggested that although mRNA increased for this neuroprotective molecule, the protein synthesis machinery had broken down by the time the mRNA was expressed. Consequently, it was too late for Bcl-2 to have its known neuroprotective influence.

Bush and Hyson (2006) hypothesized that if Bcl-2 protein was upregulated prior to cochlea removal, then NM neurons might be protected from deafferentation-induced cell death. To test this hypothesis, they chronically administered lithium to posthatch chicks prior to cochlea removal. Lithium has been shown to increase expression of Bcl-2 (Chen et al., 1999) and to increase neuronal survival in a variety of animal models of disease and stroke/ischemia (Chuang et al., 2002; Leegwater-Kim and Cha, 2004; Manji et al., 2002; Martin et al., 1998). Additionally, lithium has been shown to be effective in preventing toxic increases in intracellular calcium (Okamoto et al., 1994). Bush and Hyson (2006) found that chronic lithium
administration was effective at increasing Bcl-2 protein expression in NM neurons and this treatment protected approximately 50% of the neurons from deafferentation-induced cell death.

Although upregulation of Bcl-2 protein following lithium administration is correlated with cell survival in NM neurons, it is possible that lithium could be influencing other aspects of the cell death cascade. Lithium’s neuroprotective effect could be through preventing the triggers for cell death, or by preventing cell death cascades that are downstream of the initial trigger. The present series of experiments examine different events in the cell death cascade in NM neurons. Early occurring (calcium levels), mid-occurring (Y10b immunolabeling), and late-occurring (Bcl-2 mRNA expression) events are investigated in an attempt to determine which aspects of the cell death cascade are being influenced by lithium treatment.

**Experimental Procedures**

*Calcium Imaging:*

The subjects were administered an *in ovo* injection of 0.15M LiCl or saline 60 hours after the beginning of incubation through a hole in shell onto the chorioallantoic membrane. The lithium dose was based on egg weight, assuming 80% water content, to bring the final concentration to 0.3M LiCl (see Ikonomov et al., 2000). We assumed that the single lithium application resulted in a stable concentration throughout the experiment, since the lithium ion is not metabolized or excreted and egg weight changes less than 1%. Incubation was continued until E18. Tissue from eight subjects (5 lithium and 3 saline-treated) prepared for Fura-2 ratiometric imaging as described by Zirpel et al. (1995, 1996). The embryo was anesthetized by cooling and decapitated. A 300 µm thick coronal section that was midway between the rostral and caudal extent of NM was obtained as previously described (Hyson and Rubel, 1995; Hyson, 1997, 1998). NM neurons were loaded with Fura-2 by incubating slices in oxygenated artificial cerebral spinal fluid (ACSF) containing 6 µm of Fura-2 (Molecular Probes, Eugene, OR), 1.7% anhydrous demethylsulfoxide (DMSO), and 0.03% Pluronic (Molecular Probes, Eugene, OR) for 20-30 minutes at room temperature. ACSF contained (in mM) sodium chloride, 130; sodium bicarbonate, 26; potassium chloride, 3; calcium chloride, 2; magnesium chloride, 2; sodium phosphate, 1.25; and D-glucose, 10 and oxygenated using a 95% oxygen/5% carbon dioxide gas mixture.
mixture. Slices were then placed in an imaging chamber where they were superfused with oxygenated ACSF for 5 minutes before data acquisition. Fura-2 loaded neurons were alternately excited with 340 nm and 380 nm wavelengths of light from a xenon source using a computer controlled shutter and filter wheel. Paired 340/380 excitation images were acquired every 5 minutes for 1 hour and analyzed with Metaflour software. At the end of this test period ACSF containing 75 mM KCl was applied to the slice. Only cells showing a reliable response to KCl were included in the final analyses (see Zirpel, 1995). Data was analyzed as the change in ratio over time by ANOVA using SPSS software.

**Daily injections:**

All procedures were in accordance with Animal Care and Use Committee guidelines. Post-hatch chicks (P0), of either sex, received daily sub-cutaneous injections of either lithium chloride (LiCl) or saline for 17 days, prior to unilateral cochlea removal surgery. Both groups of animals received a daily sub-cutaneous injection for a total of 17 days. The dose of LiCl was gradually increased across age, beginning at 1.5 mEq/kg for the first four days, followed by 2.3 mEq/kg for seven days, and finally, 3.0 mEq/kg for the last six days. This schedule was identical to that used by Bush and Hyson (2006) and adapted from the protocols of both Wei et al. (2001) and Nonaka and Chuang (1998). The volume of each injection was 0.01 ml/kg. Control subjects received daily 0.01 ml/kg injections of saline.

**Cochlea Removal Surgery:**

One hour after the final injection subjects were anesthetized with Halothane. A small incision was made to widen the ear canal and the tympanic membrane was punctured. The columella was removed, followed by the extraction of the basilar papilla (cochlea) through the oval window using forceps. The extracted tissue was viewed under a dissection microscope to ensure complete cochlea removal. The middle ear cavity was packed with Gelfoam and the external incision sealed with surgical adhesive. Subjects were then allowed to survive for 3 hours, 6 hours, or 5 days.
Tissue preparation:

At the predetermined survival period following cochlea removal, subjects were deeply anesthetized with pentobarbital and perfused with 0.9% saline followed by ice cold 4% paraformaldehyde. Brainstems were blocked and post-fixed in 4% paraformaldehyde for 1–2 h followed by overnight cryoprotection at 4°C in phosphate buffered saline (PBS) containing 20% sucrose. The tissue was rapidly frozen in 2-methylbutane on dry ice and embedded in TBS tissue freezing medium for cryosectioning using a Leica CM 1850 cryostat.

Immunocytochemistry:

Sections for Y10b immunoreactivity were cut at 25 µm and were collected into ice cold PBS for later mounting. Every section containing NM was collected. Pairs of subjects, one treated with LiCl and one treated with saline, were processed simultaneously. Puncture marks were made in the ventral portion of the brain stem to identify treatment condition following the simultaneous processing. Sections containing NM, were placed in a vial containing PBS. Sections were then washed 2 X ten minutes in PBS and endogenous peroxidase activity was quenched by incubating in 0.03% H₂O₂ in methanol for ten minutes. Following three 10 minute rinses in PBS, sections were placed in a blocking solution containing 1% Normal Horse Serum (NHS) in PBS for an hour. Sections were then transferred to a 1:500 concentration of the primary antibody, Y10b (Garden et al., 1994; Hyson 1998), which recognizes ribosomal epitope, in blocking solution. Control sections receiving the same treatment but with no primary antibody were processed to ensure specificity of the reaction. Following overnight incubation on a rotator, sections were washed 3 X 10 minutes in PBS. Sections were then incubated in a 1:200 concentration of horse anti-mouse antibody in blocking solution (NHS) for one hour. Following 3 ten-minute washes with PBS, sections were incubated in avidin-biotin- peroxidase complex (ABC; Vector Laboratories, Burlingame, CA, USA) for one hour. After another round of washes, with PBS, sections were reacted with diaminobenzidine (DAB) tetrahydrochloride and H₂O₂ for visualization. Following a final round of washes sections were mounted on slides and allowed to dry overnight. The following day slides were dehydrated in a graded series of alcohols and cleared in xylenes for 10 minutes. Slides were coverslipped in DPX mounting medium and allowed to dry overnight.
In Situ Hybridization:

The in situ hybridization methods were identical to those used by Wilkinson et al. (2002). Survival time for this experiment was 6 hours post cochlea removal. Briefly, cryosections (30 µm) are collected in ice-cold diethyl-polycarbonate (DEPC)-treated, 2x sodium chloride. Sodium citrate buffer (SSC) in autoclaved scintillation vials. After the buffer is removed, the tissue was covered with pre-hybridization buffer containing 3.25 ml formamide, 2x SSC, 10% dextran, 1x Denhardt’s, 50ml DTT and 250µl of stock salmon sperm DNA (about 1ml/ 40 sections). We used a Bcl-2 oligonucleotide probe complementary to nucleotides 1147-1191 of the chicken Bcl-2 sequence (Invitrogen). The oligonucleotide was end-labeled with $^{35}$S, and is added to prehybridization after 1 hour and incubated at 39°C overnight. The following day, sections were washed with diluted serial concentrations of SSC (2x, 1x, and 0.5x for 15mins at 39°C). Sections were then be mounted and dried with gelatin onto slides. Sections were exposed to x-ray film (Amersham) for 2 days and then dipped in photographic emulsion and stored in light-tight boxes for a period ranging from 2-4 weeks. The slides were then developed with Kodak D-19 developer. Anatomical analysis of the sections was made possible by counterstaining of tissue using standard Thionin.

Objective analysis of anatomical tissue:

Immunolabeling of NM neurons will be objectively analyzed using densitometry. For some of the experiments, the staining densities on the intact side of the brainstem were compared to those on the deafferented side of the same tissue section. When the important comparison is between lithium- and saline-treated subjects, the tissue was processed simultaneously in the same reagents to prevent processing variables from affecting the results. The light levels and contrast settings remained the same for all tissue sections analyzed on NIH Image J. On average, 50 neurons in each nucleus (intact and deafferented; saline and lithium) were measured. All neurons with a clear intact visible cell membrane were included in the analysis. Neurons were measured starting from the most medial edge of the nucleus and proceeding laterally until either no other neurons were present in NM or the criterion of 50 cells was reached. Approximately 4 sections were analyzed per brain. To prevent bias, the identities of whether the subject was treated with lithium or saline was not revealed until all measurements were completed.
For analysis of *in situ* hybridization emulsion dipped slides were analyzed using NIH Image J. A gray scale threshold was visually set to identify developed silver grains. This threshold remained constant for all measurements. The grain density, defined as the percentage of soma area that was covered by silver grains, was measured for approximately 40 cells within each NM in a given tissue section. The data were analyzed using Microsoft EXCEL and SPSS

**Results**

*Calcium Ratiometric imaging:*

Intracellular calcium levels increased over time in NM neurons maintained *in vitro* (see also Zirpel, 1995). This was true both for subjects treated with lithium and saline-treated control subjects. For statistical analysis, ratios during a baseline 10 minute period were compared to those after 60 minutes in vitro (*Figure 11*). A mixed ANOVA, using time as the within-subjects variable and drug treatment as the between-subjects variable, revealed a reliable effect of Time (*F*(1,7) = 14.6, *p* < 0.01), but no reliable effect of Treatment, nor a Treatment X Time interaction (*F*(1,7) = 2.5 and 0.7, respectively, *p* > 0.1). Although there was a trend for lithium-treated subjects to have a higher baseline ratio, this effect was not statically reliable. Regardless of starting ratio, however, subjects in both groups showed increased ratios over time (paired *t*(4) = 3.0 and 2.96, for saline and lithium groups, respectively, *p* < 0.05).
Figure 11.) Average 340/380 nm excitations of NM neurons from saline- and lithium-treated subjects during Fura-2 loading. Both groups show an increase of intracellular calcium over time (p < 0.01). There is a pre-post difference that was revealed using ANOVA. The difference between lithium- and saline-treated groups was not statistically reliable. Error bars represent standard error in the mean.
Immunocytochemistry:

Deafferentation leads to a reduction in immunolabeling for the ribosomal epitope recognized by the antibody Y10b. Chronic lithium appeared to increase the overall antigenicity for Y10b, but it did not prevent this deafferentation-induced reduction in immunolabeling (Figure 12). Objective analyses of the gray scale values confirmed these visual impressions (Figure 13). A 3-way mixed ANOVA, using drug, treatment, and survival time as between-subjects variables and side of the brain as a within-subjects variable, showed that there was a reliable difference between sides (F(1, 18) = 149, p < 0.001 and a reliable effect of drug treatment (F (1,18) = 14.1, p < 0.001), but there was no reliable drug treatment X side interaction. Individual paired t-tests between the intact and deafferented sides of the brain showed reliable differences for each of the 6 drug treatment and survival time groups (p < 0.05). The overall analysis also appeared to show that tissue from different survival times had different baseline levels of labeling (F (2, 18) = 23.9, p < 0.001). This difference, however, might be attributable to processing variables since subjects at different survival times were processed separately. To remove this source of variance, scores were converted to percentage difference scores (100* (density of deafferented cells- density of intact cells)/density of intact cells) for each brain. A two-way (Drug X Survival time) between-subjects ANOVA revealed a reliable effect of survival time (F (2, 18) = 21.1, p < 0.001), but no reliable effect of drug treatment nor a drug treatment X survival time interaction (Fs < 1.0). Post-hoc pairwise comparisons (Newman-Keuls) revealed that the percentage difference in labeling between the two sides was most robust 6 hours following cochlea removal, regardless of drug treatment.
Figure 12.) Representative photomicrographs of intact and deafferented sides of saline- (top) and lithium-treated (bottom) subjects 3 hours after cochlea removal (40x). Tissue sections were immunolabeled with an antibody recognizing Y10b. A.) saline intact side. B.) saline deafferented side. C.) lithium intact side. D.) lithium deafferented side. There is an increase labeling in the lithium-treated subjects than did the controls (saline) but cochlea removal decreased labeling in both groups.
Figure 13.) Average gray scale density measured over individual intact and deafferented NM neurons immunolabeled for Y10b (3 and 6 hours and 5 days) post cochlea removal. Lithium-treated subjects showed reliably more labeling than saline-treated controls ($p<0.001$) for Y10b immunolabeling. Additionally there is an intact versus deafferented side difference in both groups ($p<0.001$). Error bars represent standard error of the mean.
In situ Hybridization:

As reported previously (Wilkinson et al., 2002), there was an increase in labeling for Bel-2 mRNA in deafferented neurons in the saline-treated subjects. This deafferentation-induced difference appeared to be blocked by chronic lithium treatment. Statistical analyses of the grain density measurements confirmed the visual impressions. A two-way mixed ANOVA on the grain density measurements, using side of the brain as a within-subjects variable and drug treatment as a between-subjects variable, revealed a reliable effect of drug treatment ($F(1, 6) = 15.5$, $p < 0.01$), a marginal effect of drug treatment ($F(1, 6) = 5.26$, $p < 0.062$) and importantly, a side X drug treatment interaction ($F(1, 6) = 9.9$, $p < 0.05$). Post-hoc pairwise comparisons (Newman-Keuls) revealed that the grain densities on the deafferented side of the saline-treated group were reliably higher than the grain densities of the intact side on the saline-treated group and higher than either side of the lithium-treated group. No other comparisons showed reliable differences.

Since processing variables dramatically influence overall levels of labeling, the raw grain density scores for each brain were converted to z-scores based on the mean and standard deviation of the distribution of grain densities measured on the intact side of the tissue section. The pooled distribution of these z-scores for the deafferented sides of the saline- and lithium-treated groups are shown in Figure (14). As can be seen, the distribution for the saline-treated group was much broader and suggests a possible bimodal distribution. Using a previously-used cutoff of 4 standard deviations above the mean (see Wilkinson et al., 2002), 23.9% of NM neurons would be considered “heavily-labeled” in the saline-treated subjects. This same criterion would account for only 0.8% of deafferented neurons in the lithium-treated subjects.
Figure 14.) Grain density distribution from subjects treated with lithium or saline. Grain density measurements were converted to z scores based on the mean and standard deviation of grain densities measured over NM neurons on the intact sides of the tissue. There were higher silver grain densities on the deafferented side of the saline-treated subjects (p < 0.01).
Discussion

Previous work (Bush and Hyson, 2006) showed that chronic lithium administration protected NM neurons from deafferentation-induced cell death, but did not prevent the deafferentation-induced reduction in soma size. The present sets of experiments examined different events that occur in the cell death cascade in deafferented NM neurons to determine which of these events are influenced by chronic lithium administration. This work focused on a subset of the known array of effects that occurs following cochlea removal (Rubel et al., 1990). Chronic lithium did not appear to block the rise in intracellular calcium levels that occurs within one hour following cochlea removal, nor did it appear to block the changes in ribosomes (decreased Y10b immunolabeling) that are observed across the entire population of NM neurons in the first few hours following cochlea removal. It did, however, appear to prevent changes in gene expression (increased Bcl-2 mRNA) that are observed predominately in a subpopulation of cells 6-12 hours following cochlea removal.

Intracellular calcium

Zirpel and colleagues (Zirpel et al., 1995) have shown that intracellular calcium levels in NM neurons rise within an hour following cochlea removal. This rise can be observed over time in vitro and is not observed if the auditory nerve is electrically stimulated (Zirpel et al., 1996). The rapid change in intracellular calcium levels following cochlea removal suggests that calcium could be an early trigger in the cell death cascade, as observed in other systems (Brauchi et al., 2006; Doonan and Cotter, 2004). One difficulty with this simple hypothesis, however, is that calcium levels rise in all deafferented NM neurons, but only a subset (20-30%) of the neurons die. Thus, although it is possible that increases in calcium play a role in cell death following deafferentation, it does not appear that an increase in calcium commits the cell to die. The present studies replicated the work of Zirpel et al. (1995) in showing a rise in intracellular calcium in NM neurons in vitro. Consequently, if calcium is the important trigger for cell death, then lithium is not preventing the cell death-“trigger”, but must be affecting a later downstream consequence of this trigger.

One interesting finding was that there was a trend for lithium-treated subjects to have a higher baseline ratio values than did saline-treated subjects. Increased baseline calcium levels
following chronic lithium have been observed previously (Wasserman et al., 2004). It is possible that such an increase in baseline calcium levels could play a role in some of the changes in gene expression that are observed following chronic lithium administration (Jope and Song, 1997). NM neurons also show increased immunoreactivity for the transcription factors pCreb, NFkappaB, and Beta-catenin following lithium administration. This increase in expression might be the result of lithium’s inhibition of the kinase Gsk-3beta (Grimes and Jope, 2001, Chapter 2), but it is possible that an increase in basal intracellular calcium levels could also modulate the activity of the transcription factors. At present, however, it is unknown how chronic lithium leads to the increases in baseline calcium levels, so it’s relationship to the changes in gene expression is simply correlational.

Conclusions based on the present results must also be qualified to some extent by one technical limitation. It appears that only embryonic NM neurons reliably load with the Fura-2 calcium indicator dye. This required that lithium be administered in ovo for the calcium imaging studies, while all other experiments examining the effects of lithium in this system have been following chronic injections in post-hatch birds. Although the serum lithium levels were similar after both procedures (Ikonomov et al., 2000 and Bush and Hyson, 2006), one cannot be certain that embryonic and post-hatch NM neurons respond identically to lithium treatment. Nevertheless, the lack of effect of lithium on the activity-dependent changes in intracellular calcium levels are not likely to be due to insufficient levels of lithium during the in ovo treatment.

*Y10b immunolabeling*

One of the first changes in the cellular functions of NM neurons that is observed following cochlea removal is a change in protein synthesis (Steward and Rubel, 1985). This change in ribosomal activity appears to correspond with changes in antigenicity to the antibody Y10b, which recognizes a ribosomal epitope (Garden et al., 1994, 1995). The present studies replicated previous reports showing a decrease in Y10b labeling within the first few hours following cochlea removal. Chronic administration of lithium did not affect this difference between deafferented and intact NM neurons. Once again, it appears that deafferentation-induced changes that occur across the entire population of NM neurons are not affected by lithium pretreatment.
Similar to the trend noted in basal calcium levels, it appears that chronic lithium increased baseline levels of Y10b antigenicity. When tissues from lithium- and saline-treated brains were processed simultaneously, NM neurons in the lithium-treated brains were more darkly labeled with Y10b. If this antigenicity is somehow related to ribosomal function, then this result suggests that NM neurons in lithium-treated subjects may have a greater protein synthesis capacity than those in saline-treated birds. This hypothesis is supported by lithium-induced increases in immunolabeling for proteins such as Bcl-2 (Bush and Hyson, 2006), pCreb, NFkappaB, Beta-catenin (Chapter 2). Perhaps chronic lithium is maintaining the integrity of the ribosomes in all NM neurons allowing for prolonged protein synthesis, or it somehow inhibits the total breakdown of protein synthesis that is observed 6-12 hours following cochlea removal. If this is the case, then lithium could be allowing more NM neurons to make whatever cytoprotective proteins they need to remain viable (e.g. Bcl-2, (Bush and Hyson, 2006)).

*Bcl-2 mRNA*

Approximately 20-30% of the deafferented NM neurons die within 2 days following cochlea removal. Indicators of which cells will die and which will survive, however, can be observed as early as 6-12 hours following deafferentation. At this time, a subpopulation of neurons appears to have a complete cessation protein synthesis (Steward and Rubel, 1985). In addition, changes in gene expression also appear to dissociate NM neurons into two populations 6-12 hours after cochlea removal. Wilkinson et al. (2002) reported that approximately 20-30% of the NM neurons show a dramatic upregulation Bcl-2 mRNA expression. They suggested that signaling mechanisms in the dying subpopulation of neurons leads to an upregulation of this potentially neuroprotective molecule, but that all these cells go on to die anyway, partly because they are unable to translate the message into protein by this time. The present experiments replicated the upregulation of Bcl-2 mRNA in deafferented neurons in saline-treated subjects. Wilkinson et al. (2002) used the criterion that a “labeled” cell was one with a grain density that was greater than 4 standard deviations above the mean grain density measured over neurons on the intact side of the brain section. They reported that, on average, 26.4% of the deafferented NM neurons would be considered “labeled” by this criterion in tissue processed 6 hours following cochlea removal. The present data set did not visually appear as distinctly bimodal as that of Wilkinson et al. (2006), but using the same criterion, 23.9% of the deafferented cells
would be considered “labeled” in the saline-treated subjects. Importantly, chronic administration of lithium prevented this upregulation in Bcl2 mRNA expression. Only 0.8% of the deafferented NM neurons would be considered labeled between deafferented and intact side of the brain. Since this same lithium treatment protects cells from deafferentation-induced cell death (Bush and Hyson, 2006), these results support the hypothesis that upregulation of Bcl-2 mRNA is an indicator of cells that are likely to die.

Synthesis

The present results blend well with previous work showing that chronic lithium administration protects NM neurons from deafferentation-induced cell death, but does not prevent the deafferentation-induced reduction in soma size. The rapid deafferentation-induced changes that occur in all NM neurons, increased calcium levels and changes in Y10b antigenicity are still observed in lithium-treated subjects. On other the other hand, the upregulation of Bcl-2 mRNA, a change that is observed when cells begin to divide into surviving and dying populations, is prevented by chronic lithium administration. Lithium administration tended to increase basal intracellular calcium levels and basal antigenicity of Y10b. These data are consistent with the increased expression of Bcl-2 protein in lithium-treated subjects (Bush and Hyson, 2006). Chronic lithium did not prevent the presumed initial trigger (influx of calcium) that occurs following cochlea removal. Perhaps the changes in gene expression produced by chronic lithium administration (see Chapter 2), results in higher levels of neuroprotective molecules (e.g., Bcl-2) in the NM neurons at the time of cochlea removal. This might allow more of the neurons to survive challenges, such as a rise in intracellular calcium levels. Such changes in gene expression would take time to be effective and this may explain why lithium is therapeutic only following chronic administration.
CHAPTER 4

SUMMARY AND GENERAL DISCUSSION

The chick auditory brainstem model has proven to be an exquisite model to begin to elucidate the mechanism by which chronic lithium pretreatment is neuroprotective in a non-invasive naturally-occurring cell death model. One purpose of this research was to further enhance the understanding of the molecular and cellular signaling mechanisms that lithium regulates in this model system. This involved detailing which genes are involved in both lithium neuroprotection and deafferentation-induced cell death. One molecule that may be involved in both situations is Bcl-2. At 6-12 hours post cochlea removal, NM neurons appear to subdivide into two distinct populations: those that die and those that will survive. One indicator of this division is an upregulation of Bcl-2 mRNA. Lithium also upregulates Bcl-2, and it is possible that this upregulation plays a role in its neuroprotective action.

The first study examined key genes that are thought to be involved in lithium neuroprotection, such as the kinase Gsk-3-beta, and some of the transcription factors that it regulates (NFkappaB, Beta-catenin, and pCreb) (Grimes and Jope, 2001; Chuang et al., 2000). Lithium decreases immunoreactivity for Gsk-3beta, yet produced no difference between the intact and deafferented sides for either lithium or saline-treated subjects following cochlea removal. Lithium increases expression of the transcription factors NFkappaB, Beta-catenin, and pCreb as judged by immunocytochemistry. Cochlea removal did not influence immunoreactivity for NFkappaB, but there were differences between deafferented and intact sides for both Beta-catenin and pCreb. Chronic lithium did not prevent the deafferentation-induced difference between sides for these transcription factors.

Lithium increased basal levels of Beta-catenin in NM neurons, but there was decreased immunolabeling for Beta-catenin by 6 hours following cochlea removal. Thus, if Beta-catenin is involved in regulating Bcl-2 expression following lithium, it appears this transcription factor is not involved in the cascade that increases Bcl-2 mRNA in deafferented NM neurons. Phospho-Creb, on the other hand, is upregulated by both lithium and deafferentation. Increased levels of pCreb were observed on the deafferented sides of brain one hour post cochlea removal.
in both lithium- and saline-treated groups. Thus, lithium increased the baseline levels of pCreb in NM neurons and an even greater increase was observed in deafferented NM neurons in the presence of lithium. Since pCreb is increased by both lithium and deafferentation, it may seem plausible that pCreb is responsible for the upregulation of Bcl-2 in both cases. This simple interpretation seems unlikely, however since deafferentation only results in transient pCreb upregulation at approximately one hour after cochlea removal. Bcl-2 mRNA, in contrast is observed 6-12 hours following cochlea removal. Secondly, pCreb upregulation appears in the majority of NM neurons following cochlea removal, whereas Bcl-2 upregulation is predominantly observed in a minority subpopulation.

There are a variety of events that happen in NM neurons following deafferentation. These events are broken into early (1 hour post cochlea removal), mid (3-6 hours post cochlea removal), and late (6+ hours cochlea removal) time points. The second set of experiments attempted to take advantage of the key times in an effort to understand where lithium is being advantageous. The presumed trigger for cell death, influx of calcium, is seen in all NM neurons within one hour after the loss of auditory nerve activity (Zirpel et al., 1995). It was hypothesized that lithium might influence calcium homeostatic mechanisms and protect cells by preventing the rise in intracellular calcium after deafferentation. Lithium did not attenuate the influx of calcium, however, suggesting that the initial cue for cell death is still present. Lithium must be acting downstream of this event, possibly by increasing cytoprotective proteins that increase neuronal viability in the absence of trophic support.

A second indicator that lithium may be having downstream effects is that it increases total ribosomal “integrity” as judged by Y10b labeling. Y10b was darker in the subjects that were treated with lithium than those that were given the control saline treatment. It is believed that a breakdown in ribosomal function is a key in determining which NM cells will die and which will survive following cochlea removal. A decrease in Y10b labeling was observed 6 hours after cochlea removal in both lithium- and saline-treated subjects, but here was an overall higher level of labeling in the lithium-treated group. It is possible that lithium is preserving the health of the ribosomes following cochlea removal and ultimately, giving NM neurons the extra “push” that they need to synthesize the necessary cytoprotective proteins, such as Bcl-2.
A third indicator that lithium is having a downstream effect in protecting neurons from cell death is that despite the increased calcium levels and deafferentation-induced decrease in Y10B labeling, lithium prevented the upregulation of Bcl-2 message. Upregulation of Bcl-2 mRNA is believed to be an indicator of NM cells dividing into surviving and dying populations of neurons. This suggests that chronic lithium has resulted in changes in gene expression such that all cells can withstand the same trigger (presumably increased calcium) and effects on some of the ribosomes. These changes in gene expression may be required for that lithium to be neuroprotective, and this may explain why lithium needs to be administered chronically in order to be therapeutic (Manji et al., 1999).

Unanswered Questions

This dissertation was aimed at understanding the interplay of the molecular cascade that takes place in NM neurons following deafferentation and molecular that follows chronic lithium administration. It is hoped that information gained from this dissertation can be used in aiding the understanding of other forms of cell death such as those observed in stroke/ischemia and neurodegenerative diseases.

Additive support was found for the therapeutic effects of lithium. This dissertation accomplished the goal of identifying some of the molecules that are regulated both by lithium and by deafferentation in our model system. It was hoped that simple conclusions could be made to rationally explain how lithium promotes cell survival. However, this research still leaves some important questions unanswered.

Since there was not one transcription factor in our study that showed an increase in a subpopulation of deafferented NM neurons, the logical question is what are the transcription factors that are causing a subpopulation of NM neurons to have robust Bcl-2 mRNA labeling? This study only looked at a chosen few factors that were extensively studied in other cell death cascades (Grimes and Jope, 2001; Nonaka et al., 1998). There are other transcription factors, which have yet to be studied in our animal model system, such as those involved in the ERK pathway, Twist, JNK, and Reticulon 3 that could increase Bcl-2 protein (Pardo et al., 2003; Einat
et al., 2003; Zhande and Karson, 2007; Zhang et al., 2007; Wan et al., 2007; Wanpen et al., 2007).

Another question that needs to be answered is whether all of the examined molecules (i.e. Gsk-3beta, NFkappaB, Beta-catenin, and pCreb) are linked together in the lithium neuroprotective cascade. This report makes some correlative assumptions and states that in order for neuronal protection to occur through chronic lithium, there has to be a decrease in Gsk-3beta and, thus, an increase in these other transcription factors. This link has not been experimentally tested. A possible way to approach this question is to look at the effects of lithium in knockout mice. By knocking out certain genes one can gain further insight into what is necessary for lithium to be effective. Unfortunately, mice do not show deafness-induced cell death in the cochlear nucleus unless they are younger than approximately 2 weeks of age. This makes it difficult to provide chronic lithium administration prior to cochlea removal. Nevertheless, knockouts could be used to examine how neurons respond to lithium and possibly determine which factors are directly affecting the cytoprotective gene Bcl-2.

The most difficult question that has been left unanswered is, what is making the 30% of NM neurons die after cochlea removal? This research looked at global changes in NM and what was effective in protecting some cells from apoptosis. However, there is still no clear understanding as to why, or what causes only a subpopulation to die. This dissertation examined molecules that could logically be linked to lithium’s neuroprotective influence, yet no one key gene or group of molecules have emerged to be the key to unlocking what is causing only a portion of cells to die following deafferentation. Our lab is continuing its effort to aid in the understanding of what is necessary for cell survival and what is the key factor involved in the separation of deafferented NM neurons into surviving and dying populations.
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