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Billy You Bun Lau

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The Dissertation Committee for Billy You Bun Lau Certifies that this is the approved version of the following dissertation:

Molecular Mechanisms of Neural Plasticity after Spinal Cord Injury in the Lamprey Central Nervous System

Committee:

Jennifer R Morgan, Supervisor

Theresa J O'Halloran

Nicholas J Priebe

Kimberly Raab-Graham

Wesley J Thompson

Harold H Zakon

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by

Billy You Bun Lau, B.S.

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Dedication

To my family.

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Molecular Mechanisms of Neural Plasticity after Spinal Cord Injury in the Lamprey Central Nervous System

Billy You Bun Lau, Ph.D.

The University of Texas at Austin, 2012

Supervisor: Jennifer R Morgan

Spinal cord injury induces anatomical plasticity throughout the nervous system, including distant locations in the brain. Several types of injury-induced plasticity have been identified, such as neurite sprouting, axon regeneration and synaptic remodeling. However, the molecular mechanisms involved in anatomical plasticity after injury are unclear, as is the extent to which injury-induced plasticity in the brain is conserved across vertebrate lineages. Here, I used lampreys to identify the molecular mechanisms in mediating anatomical plasticity, because lampreys undergo anatomical plasticity and functional recovery after a complete spinal cord transection. Due to their robust roles in neurite outgrowth during neuronal development, I examined synapsin and synaptotagmin for their potential involvement in anatomical plasticity after injury. I found increased synapsin I mRNA throughout the lamprey brain as well as increased protein levels of synapsin I, phospho-synapsin (Ser 9) and synaptotagmin in the lamprey hindbrain after injury, suggestive of anatomical plasticity. Anatomical plasticity was confirmed at the ultrastructural level, where I found increased neurite density in the lamprey hindbrain after injury. Other molecular mechanisms that promote anatomical plasticity have been

previously identified, such as cyclic AMP (cAMP). However, the cellular mechanisms and the molecular targets of cAMP in mediating anatomical plasticity are unclear. My investigation of cAMP revealed that cAMP enhanced the number of regenerated axons beyond the lesion site in lampreys after injury. For the first time in a spinal cord injury model, I found cAMP prevented the death of axotomized neurons that normally have a high tendency to die after injury. In addition, cAMP promoted more regenerating axons to re-grow in straighter paths rather than turning rostrally towards the brain stem. At the molecular level, I found cAMP increased synaptotagmin protein level at the regenerating axon tips, suggestive of enhanced axon elongation. Taken together, my results show that neurite sprouting in the brain and the cAMP-enhanced axon regeneration are conserved responses in vertebrates after spinal cord injury. In addition, my results suggest that at least some developmental pathways are activated during injury-induced and cAMP-enhanced anatomical plasticity. Further understanding of these pathways will provide insights for improving recovery after spinal cord injury.

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Chapter 1: General Introduction

1.1 SPINAL CORD INJURY

1.1.1 Spinal cord injury is detrimental to human health

The spinal cord, along with the brain, makes the central nervous system in all vertebrate species. It contains both ascending and descending nerve fibers that are essential for relaying sensory and motor information between the brain and the body. People suffering from spinal cord injury may become permanently paralyzed, because severed axons exhibit a poor capacity to regenerate across the injury site and form the synapses necessary for recovery.

According to the report from National Spinal Cord Injury Statistical Center (NSCISC, 2012), there is approximately 265,000 Americans living with spinal cord injury, and at least 12,000 new cases arise annually. In addition, the average age of people with spinal cord injury is 40.7 years old, and 80.7% of the injured population is male. Moreover, the number one cause for spinal cord injury is motor vehicle accidents (40.4%) followed by accidental falls (27.9%) and violence (15.0%). Interestingly, some people with spinal cord injury do spontaneously recover some of their deficits, though the recovery is incomplete (Fawcett et al., 2007). This observation raises the questions of how people with spinal cord injury are able to spontaneously recover without medical interventions. Specifically, what are the injury-induced anatomical and molecular changes associated with the recovery? Answers to these questions will provide new

insight for improving recovery and developing new therapies for people suffering from spinal cord injury.

1.1.2 Spinal cord injury induces anatomical plasticity

Evidence from mammalian studies of spinal cord injury suggests that spontaneous recovery may be supported by compensatory anatomical plasticity throughout the nervous system. Injury-induced anatomical plasticity includes spontaneous axon sprouting from injured and uninjured neurons located within the brain and spinal cord (Blesch and Tuszynski, 2009; Bradbury and McMahon, 2006; Maier and Schwab, 2006) (Fig. 1.1). In the spinal cord, injury to the mouse dorsal corticospinal tract (CST) at the mid-thoracic level induces axon sprouting of both injured dorsal CST as well as uninjured dorsal-lateral and ventral CST. Subsequently, sprouted axons form new connections with motorneurons (Bareyre et al., 2005). In monkeys, transection of the right CST at the cervical level induces spontaneous sprouting of the left uninjured corticospinal axons in the spinal cord, resulting in improved coordinated muscle movement, hand function and locomotion (Rosenzweig et al., 2010). Within the brain, transection of the right CST of the rat at the level of the hindbrain induces sprouting of axotomized brain neurons, which then form new connections with the uninjured brain neurons, resulting in partial recovery of descending activity of the forelimb (Z'Graggen et al., 2000). In monkeys, at the mid-cervical level, transection of the right CST that are responsible for hand function induces sprouting of uninjured brain neurons that are responsible for innervating the face. Subsequently, these new "face" axons make

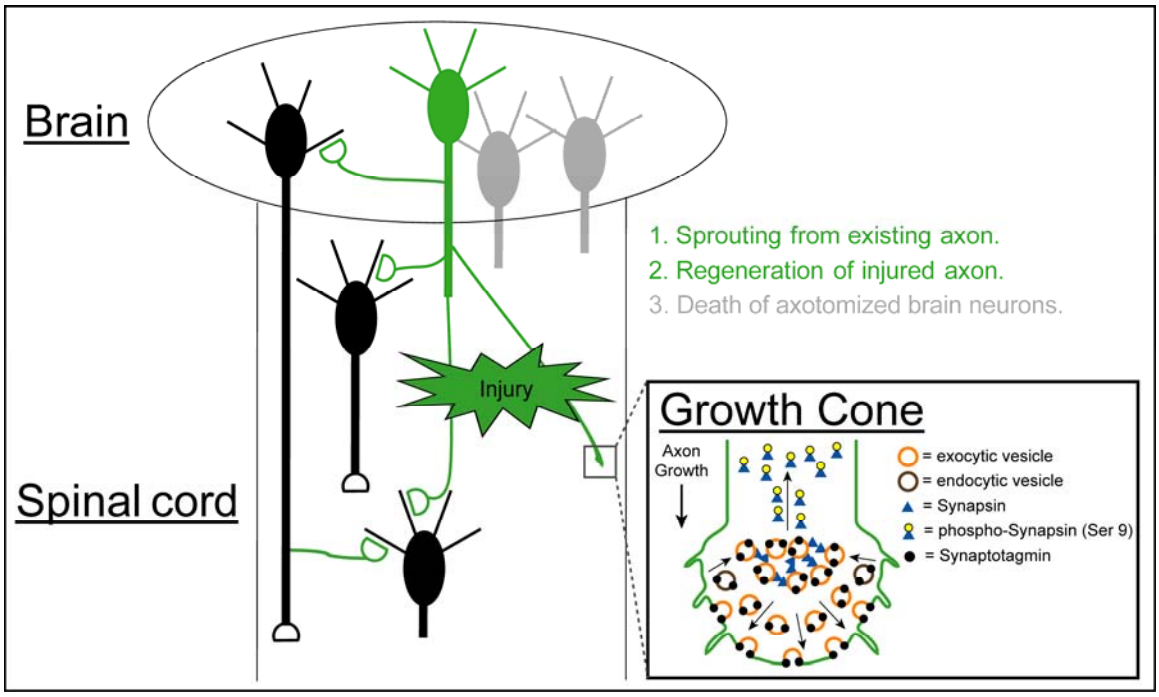

Figure 1.1. Spinal cord injury induces anatomical and cellular plasticity.
(1) Spinal cord injury induces sprouting of axotomized brain neurons (green) that subsequently form new synapses (green semi-circles) with other uninjured brain neurons as well as spinal neurons (black). In addition, uninjured brain neurons can sprout within the spinal cord and make new connections with spinal neurons. Both of these sprouting events may provide some functional recovery. (2) When provided with a permissible environment at the lesion site (Injury), axotomized brain neurons can regenerate their axons across the lesion site and form new connections with spinal neurons. Axon regeneration of axotomized neurons may also provide some functional recovery [adapted from Blesch and Tuszynski, 2009]. (3) In addition to injury-induced anatomical plasticity, spinal cord injury also induces death of axotomized brain neurons (grey) (Busch and Morgan, 2012; Hains et al., 2003; Shifman et al., 2008; Zottoli et al., 1984), which may impede recovery. (Inset) Diagram showing the growth cone of a regenerating axon. During axon regeneration, vesicles mainly undergo exocytosis for membrane addition, but endocytosis also occurs. Exocytic events during axon regeneration may utilize the same mechanism as developmental neurite growth. In this case, synapsin sequesters a pool of vesicles at the growth cone. When synapsin becomes phosphorylated at serine 9 by PKA, it releases the exocytic vesicles and disperses into the axon. The "free" exocytic vesicles move towards the growing end of the growth cone and undergo exocytosis with the assistance of synaptotagmin, thereby, providing the membrane material needed for axon elongation [adapted from Bloom and Morgan, 2011; Bonanomi et al., 2005].

connections with the axotomized "hand" neurons in the brain (Jain et al., 2000). Spinal cord injury also induces sprouting and remodeling within the propriospinal circuit of the peripheral nervous system (Courtine et al., 2008). Thus, spontaneous axon sprouting is a form of compensatory anatomical plasticity that might support some recovery after spinal cord injury.

Another form of injury-induced compensatory anatomical plasticity is the regeneration of injured axons across the lesion site (Fig. 1.1). Most vertebrate axons have poor capacity to self-regenerate after injury, because the injury site develops a glial scar that contains inhibitory molecules such as Nogo, myelin-associated glycoproteins (MAG), oligodendrocyte myelin glycoproteins and chondroitin sulfate proteoglycans (CSPGs), which prevent axon regeneration (Hannila and Filbin, 2008). However, injured axons retain the intrinsic capability to grow and regenerate across the injury site if a permissible environment is provided in the inhibitory scar. For example, in rats and rabbits, when a peripheral tissue graft is placed at the injury site to bridge the damaged spinal cord, injured axons can regenerate into and out of the graft, resulting in partial recovery (David and Aguayo, 1981; Richardson et al., 1980; Yoshii et al., 2009). In rats, a combined treatment of peripheral tissue graft with chondroitinase ABC (ChABC), an enzyme that degrades CSPGs within the scar, promotes regeneration of injured axons and the formation of new synapses (Tom et al., 2009). Thus, providing a permissive environment at the lesion site to permit axon regeneration may support some recovery.

In addition to the injury-induced axon sprouting and regeneration, spinal cord injury also modifies the dendritic processes of the injured brain neurons. This has been

demonstrated in the rat motor cortex where the axotomized motor neurons have increased density, length and size of dendritic spines (Kim et al., 2006). These results suggest a highly dynamic structural remodeling of the brain motor circuits after injury, which may provide the capacity to support some recovery.

Taken together, spinal cord injury induces compensatory anatomical plasticity within the brain and spinal cord that may support some recovery. These findings raise the questions of whether enhancing axon sprouting and regeneration as well as synaptic remodeling will improve recovery. While some of the neuronal pathways undergoing anatomical plasticity have been identified, the molecular mechanisms mediating such plasticity remain poorly understood. These questions will be the main focus of my dissertation.

1.2 SYNAPSIN POSITIVELY REGULATES NEURITE GROWTH, SYNAPSE FORMATION AND SYNAPTIC TRANSMISSION

1.2.1 Synapsin is evolutionarily conserved

Synapsin is a protein that has the potential to play an important role in the injury-induced anatomical plasticity because of its multifaceted function in neurons. Synapsin is an abundant neuronal phosphoprotein (De Camilli et al., 1983a; Greengard, 1987; Johnson et al., 1972). It is localized at the presynaptic termini where it interacts with filamentous actin (F-actin) and the cytoplasmic portion of synaptic vesicles (Bahler and Greengard, 1987; De Camilli et al., 1983a; De Camilli et al., 1983b; Fletcher et al., 1991). Synapsin is evolutionarily conserved across vertebrate and invertebrate species (Cesca et al., 2010; Kao et al., 1999). In mammals, the synapsin family has three genes

(I, II and III) (Kao et al., 1998) (Fig. 1.2). Other organisms have synapsin I and II (e.g. *Drosophila melanogaster*) (Diegelmann et al., 2006; Klagges et al., 1996) while some have only synapsin I (e.g. *Caenorhabditis elegans*) (Kao et al., 1999) or an ancestral version of the genes (e.g. *Loligo pealei*) (Hilfiker et al., 1998) (Fig. 1.2). Synapsin I and II genes also give rise to different isoforms through alternative splicing. For example, mammals have synapsin Ia and Ib, as well as synapsin IIa and IIb (Kao et al., 1998) (Fig. 1.2). All synapsins have several highly conserved structural domains and amino acid residues (Kao et al., 1999) (Fig. 1.2). In particular, the highly conserved domain A contains a serine residue at either position 9, 10 or 11 that is a target for phosphorylation by protein kinase A (PKA) (Czernik et al., 1987) and CamKI/IV (Nairn and Greengard, 1987), as well as de-phosphorylation by protein phosphatase 2A (PP2A) (Jovanovic et al., 2001). Phosphorylation of synapsin at serine 9 by PKA dissociates synapsins from synaptic vesicles (Hosaka et al., 1999), and it is essential for proper development of neurons and synaptic function at mature synapses (discussed in section 1.2.2).

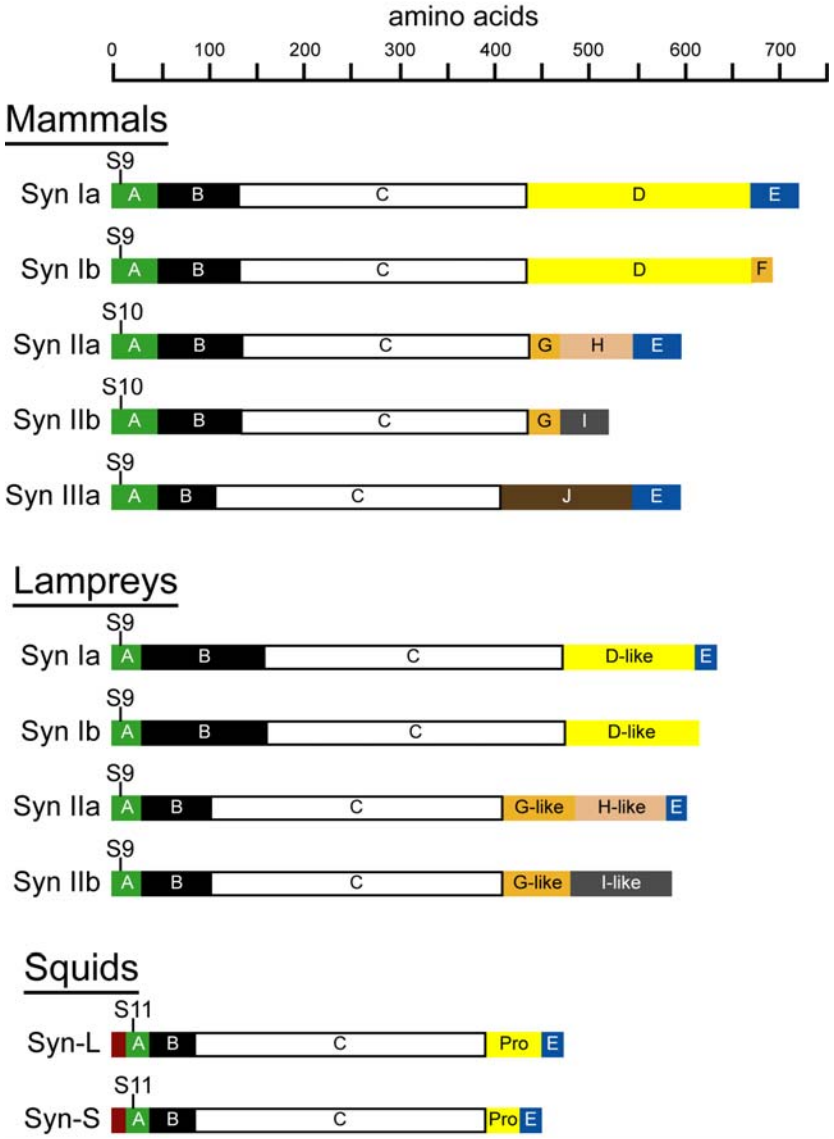

Figure 1.2. Domain model of vertebrate and invertebrate synapsins.
Synapsins are evolutionarily conserved. Examples of the mammalian, lamprey and squid synapsins are shown. Different structural domains are color-coded and indicated by the letters A-J. Conserved domains have the same color codes and letters. For squids: Syn-L = synapsin-long, Syn-S = synapsin-short, Pro = proline-rich domain, which has the characteristic of the D domain. The evolutionarily conserved serine residue in domain A (S9, S10 or S11) is phosphorylated by PKA and CaMKI/IV, as well as de-phosphorylated by PP2A (see text for full detail). Amino acid scale is indicated at the top [modified from Cesca et al., 2010; Kao et al., 1999].

1.2.2 Synapsin regulates axon growth, synapse formation and synaptic transmission

Synapsin positively regulates developmental axon growth and synapse formation. For example, when synapsin I is knocked out from hippocampal neurons, these neurons develop shorter axons and show a delay in synapse formation (Chin et al., 1995; Ferreira et al., 1998). Neurons lacking synapsin II exhibit delays in both axon and synapse formation, and their axons are shorter and fail to elongate during early development (Ferreira et al., 1998). Neurons lacking synapsin III have defects in axon formation and growth (Ferreira et al., 2000). However, synapse formation is not affected if synapsin III is suppressed after axon differentiation. In overexpression studies, cultured fibroblast cells transfected with either synapsin I or II develop long, thin and branched processes that resemble neurites (axons and dendrites) (Han and Greengard, 1994). Overexpression of synapsin I or II in cultured NG108-15 neuronal cells enhances synapse formation (Zhong et al., 1999). Thus, all synapsins positively regulate several aspects of axon and/or synapse growth and development.

Synapsin-mediated developmental axon growth and synapse formation is dependent on the phosphorylation of serine 9 by PKA. For example, in *Xenopus* studies, injecting embryos with synapsin IIa RNA with mutated serine 9 induces abnormal axon growth (Kao et al., 2002). Specifically, when serine 9 is mutated to alanine to prevent phosphorylation, nerve fibers throughout the nervous system of the developing embryos are much shorter than uninjected embryos. When serine 9 is mutated to glutamate to mimic constitutive phosphorylation, nerve fibers are much longer. The synapsin-

mediated axon growth is also dependent on PKA activity (Kao et al., 2002). *Xenopus* cultured neurons develop with fewer and shorter axons when PKA activity is pharmacologically suppressed. Conversely, pharmacological activation of PKA induces neurons to develop with many longer axons. Thus, PKA-dependent phosphorylation of synapsin at serine 9 is essential for proper neuronal development.

One potential mechanism of how synapsin regulates axon growth is by regulating a pool of synaptic vesicles that are destined to fuse with the plasma membrane for axon extension (Bonanomi et al., 2005) (Fig. 1.1). At the growing end of the axon (growth cone), the non-phosphorylated synapsin clusters a pool of synaptic vesicles by binding to F-actin and the membrane of synaptic vesicles. Upon stimulation by either pharmacological activation of PKA or with high potassium chloride, synapsin becomes phosphorylated at serine 9. Then, it dissociates from synaptic vesicles and disperses throughout the growth cone. The "free" synaptic vesicles move towards the plasma membrane, where they undergo exocytosis for membrane addition and axon extension, as well as release of secreted molecules that promote growth (Bonanomi et al., 2005; Cesca et al., 2010).

In addition to its positive regulation of axon growth, synapsin also positively mediates synaptic transmission by its similar mechanisms for axon growth (Gitler et al., 2008; Pieribone et al., 1995). The phosphorylation of synapsin at serine 9 by PKA is also essential for proper synaptic transmission (Chi et al., 2001; Menegon et al., 2006). Additional roles for synapsin at mature synapses are the regulation of vesicle recycling

during endocytosis (Bloom et al., 2003; Ryan et al., 1996), kinetics of exocytosis (Hilfiker et al., 1998) and short-term plasticity (Rosahl et al., 1993).

Taken together, synapsin is a great candidate for having an essential role in injury-induced anatomical plasticity because of its multifaceted functions in axon and synapse growth during development and at mature synapses. However, before my studies, the role of synapsin after spinal cord injury was not clear. As a first step towards understanding the role of synapsin in the injury-induced anatomical plasticity, Chapter 2 will examine the expression levels of synapsin, as well as its phosphorylation status before and after spinal cord injury.

1.3 cAMP PROMOTES DEVELOPMENTAL NEURITE GROWTH AND CELL SURVIVAL, AND ENHANCES AXON REGENERATION AFTER SPINAL CORD INJURY

1.3.1 Introduction of cAMP: its targets and role in developmental neurite growth

As synapsin has the potential to mediate injury-induced anatomical plasticity, other molecular pathways have recently been identified to enhance such plasticity, including cyclic adenosine monophosphate (cAMP) (Bhatt et al., 2004; Ghosh-Roy et al., 2010; Pearse et al., 2004). However, the cellular mechanisms and the molecular targets of cAMP in mediating anatomical plasticity are unclear. cAMP is a second messenger converted from ATP by adenylate cyclase (Fig. 1.3). cAMP regulates many molecular pathways that are essential for proper biological functions, including pathways that regulate developmental neurite growth (Aglah et al., 2008; Cai et al., 2001), neuronal survival (Hanson et al., 1998; Li et al., 2000; Meyer-Franke et al., 1995; Rydel and Greene, 1988) by potentially activating mitogen-activated protein kinase (MAPK;

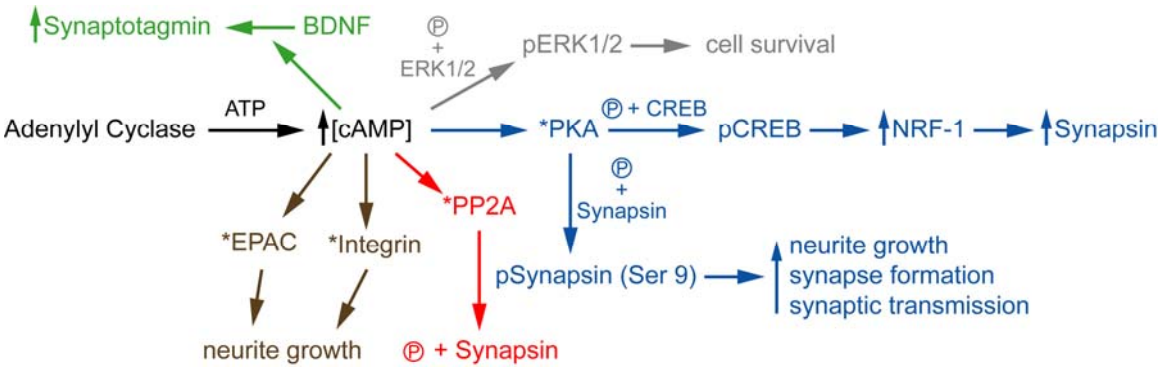

Figure 1.3. cAMP regulates many biological pathways for proper cell function. Examples of the cAMP-regulated biological pathways discussed in this chapter are illustrated. cAMP is converted from ATP by adenylyl cyclase. cAMP regulates synapsin protein level as well as the phosphorylation of synapsin at serine 9 [pSynapsin (Ser 9)] via PKA activation (*PKA) (blue pathway). These events lead to increased neurite growth and synapse formation during neuronal development and increased synaptic transmission at mature synapses. cAMP activates protein phosphatase 2A (*PP2A), which then de-phosphorylates pSynapsin (Ser 9) (red pathway). cAMP also regulates neurite growth by activating exchange proteins activated by cAMP (*EPAC) and integrin (*integrin) (brown pathway). In addition, cAMP regulates cell survival by phosphorylating mitogen-activated protein kinase (pERK1/2) (grey pathway). Moreover, cAMP modulates synaptotagmin protein level by interacting with the brain-derived neurotrophic factor (BDNF) pathway (green pathway). pCREB = phosphorylated cAMP response element-binding protein, NRF-1 = nuclear respiratory factor-1. These pathways are drawn based on published findings [see text for references].

ERK1/2) (Troadec et al., 2002) and synaptic activity (Menegon et al., 2006) (Fig. 1.3).

One of the main targets of cAMP is PKA (Taylor et al., 1990) (Fig. 1.3). Cyclic AMP activates PKA by binding to the regulatory subunits of PKA, thereby, releasing the catalytic domains responsible for phosphorylating downstream targets. One of the targets subjected to PKA regulation is synapsin (Fig. 1.3). It has been suggested that PKA regulates synapsin protein level by phosphorylating and activating the transcription factor, cAMP response element-binding protein (CREB) (Delghandi et al., 2005). The activated CREB positively regulates the protein level of the transcription factor, nuclear respiratory factor-1 (NRF-1) (Suliman et al., 2010), and NRF-1 has been demonstrated to positively regulate synapsin protein level (Wang et al., 2009). PKA also regulates the activity of synapsin by phosphorylating it at serine 9, as described in section 1.2 (Bonanomi et al., 2005; Chi et al., 2001; Meneagon et al., 2006). In addition to the regulation of synapsin, cAMP also positively modulates the protein level of synaptotagmin by interacting with the brain-derived neurotrophic factor (BDNF) pathway (Tartaglia et al., 2001) (Fig. 1.3). Synaptotagmin is a transmembrane synaptic vesicle protein that regulates both exo- and endocytosis at mature synapses (Sudhof and Rizo, 1996; Yao et al., 2012). During neuronal development, synaptotagmin is enriched on growth cone vesicles that may be responsible for membrane addition as axons elongate (Ide, 1996; Igarashi et al., 1997; Mikoshiba et al., 1999) (Fig. 1.1). Overexpression of synaptotagmin in PC12 cells lines enhances the length of growing neurites (Fukuda and Mikoshiba, 2000). Other targets of cAMP are exchange proteins activated by cAMP (EPAC) (de Rooij et al., 1998), integrins (Ivins et al., 2004) and protein phosphatase 2A

(Feschenko et al., 2002) (Fig. 1.3), but their functional roles in axon growth are not well understood.

During neuronal development, endogenous cAMP level in rat dorsal root ganglion cells (RGCs) is initially high, which coincide with the ability of the young RGCs to grow in cultures containing myelin-associated glycoprotein (MAG) (Cai et al., 2001), which is a known inhibitor of axon growth in mature neurons (Mukhopadhyay et al., 1994). As RGCs mature, endogenous cAMP level drops and parallels with the inability of RGCs to grow neurites on cultures containing MAG. Interestingly, increasing cAMP level in the mature neurons, using a membrane-permeable cAMP analogue, dibutyryl-cAMP (db-cAMP), promotes neurite growth in the same environment (Cai et al., 2001). The cAMP-enhanced neurite growth is mediated through the activation of PKA, as inactivation of PKA with the membrane-permeable inhibitor H89 prevents neurite growth (Aglah et al., 2008; Cai et al., 2001). cAMP has also been shown to promote axon growth in cell lines of the rat serotoninergic raphe cells RN46A (Rumajogee et al., 2006), mouse NG108-15 (Weeks et al., 1991) and mouse neuroblastoma cells Neuro-2A (Wong et al., 2002). Thus, cAMP positively mediates neurite growth during neuronal development.

1.3.2 cAMP promotes axon regeneration after spinal cord injury and cell survival in non-injury context

Because cAMP enhances developmental neurite growth, recent studies of spinal cord injury have focused on cAMP as a potential candidate for enhancing anatomical plasticity after spinal cord injury. After spinal cord injury, the mammalian lesion site develops a glial scar that contains molecules known to prevent axon growth (refer to

section 1.1.2). As cAMP promotes normal developing neurons to grow neurites in an inhibitory environment (Cai et al., 2001), recent studies have explored for the potential of cAMP to promote axon regeneration after spinal cord injury. For example, it has been shown in rats that spinal cord injury decreases cAMP level in regions of the nervous system that respond to the injury, such as the spinal cord, brainstem and sensorimotor cortex (Pearse et al., 2004). This decrease in cAMP level also coincides with fewer regenerated axons and the lack of behavioral recovery. However, enhancing cAMP with db-cAMP and rolipram, a phosphodiesterase inhibitor, along with Schwann cell grafts, increases the number of regenerated axons distal to the lesion site, which coincides with improved functional recovery, though the recovery remains incomplete (Pearse et al., 2004). The effect of cAMP-promoting axon regeneration after injury has been demonstrated in other species as well. For example, injection of db-cAMP into axotomized zebrafish Mauthner neurons induces the ability of these neurons to extend their axons across the lesion site (Bhatt et al., 2004). In lampreys, bath application of db-cAMP onto the transected spinal cords prevents axon retraction and increases axon regeneration towards the lesion site (Jin et al., 2009). Finally, axotomized posterior lateral microtubule (PLM) sensory neurons of the worms re-grow their axons after forskolin treatment (Ghosh-Roy et al., 2010), a compound that activates adenylyl cyclase, which converts ATP to cAMP. Taken together, elevating cAMP is an evolutionarily conserved mechanism for enhancing axon growth and regeneration after spinal cord injury.

Despite the knowledge that cAMP enhances axon regeneration in a variety of vertebrate models, the other cellular changes associated with the cAMP-enhanced growth and regeneration are not clear. In addition to its positive role in promoting axon growth of the developing neurons, cAMP has also been demonstrated to promote survival of developing neurons. For example, elevation of cAMP in cultures containing immature rat superior cervical and dorsal root ganglion cells prolongs the survival of these cultured developing neurons (Rydel and Greene, 1988). The survival-promoting effect by cAMP has also been demonstrated in cultures containing developing rat spinal motorneurons (Hanson et al., 1998), cerebellum granule cells (Li et al., 2000) and retinal ganglion cells (Meyer-Franke et al., 1995). Thus, cAMP promotes cell survival in a non-injury environment.

After spinal cord injury, it has been documented that axotomized neurons located in the brain undergo cell death after the injury (Fig. 1.1). For example, the injury-induced cell death has been observed from the axotomized pyramidal neurons in the rat primary motor cortex (Hains et al., 2003) and the Mauthner neurons of the goldfish (Zottoli et al., 1984). In lampreys, a distinct subset of descending giant reticulospinal (RS) neurons within the brain undergoes intrinsic delayed cell death after a complete spinal cord transection (Busch and Morgan, 2012; Shifman et al., 2008). Thus, spinal cord injury causes death of axotomized neurons. Since cAMP has a positive role in neuronal survival during development, it is conceivable that cAMP would also have a positive role in promoting neuronal survival after injury. However, whether cAMP promotes cell survival of axotomized neurons after spinal cord injury is not clear. In

addition, whether the cAMP-regulated axon-growth molecules are increased at the regenerating axon tips is also not clear. Chapter 3 will determine if cAMP enhances the survival of axotomized neurons after spinal cord injury. In addition, Chapter 3 will examine if cAMP enhances the expression level of the axon-growth gene, synaptotagmin, at the regenerating axon tips after spinal cord injury.

1.4 LAMPREY AS A MODEL ORGANISM FOR SPINAL CORD INJURY

To study the molecular mechanisms mediating injury-induced and cAMP-enhanced anatomical plasticity, it is difficult to use the current mammalian models of spinal cord injury, because neurite sprouting and regeneration are rare events, and it is difficult to distinguish injured from uninjured neurons because of their small sizes. The basal extant vertebrate, sea lamprey (*Petromyzon marinus*), provides a great opportunity to study the molecular mechanisms mediating injury-induced anatomical plasticity for the reasons discussed in the next three sections.

1.4.1 Lampreys recover normal swimming behavior after spinal cord injury

The late larval sea lamprey, *Petromyzon marinus*, between 5-7 years old and 11-14 cm long, provides a robust vertebrate model to study the molecular mechanisms mediating injury-induced anatomical plasticity. After a complete spinal cord transection at the level of the 5th gill, injured lampreys are completely paralyzed below the transection site within the first week of injury (Cohen et al., 1986; Oliphint et al., 2010) (Fig. 1.4). For the next few weeks, movement of the distal body gradually returns

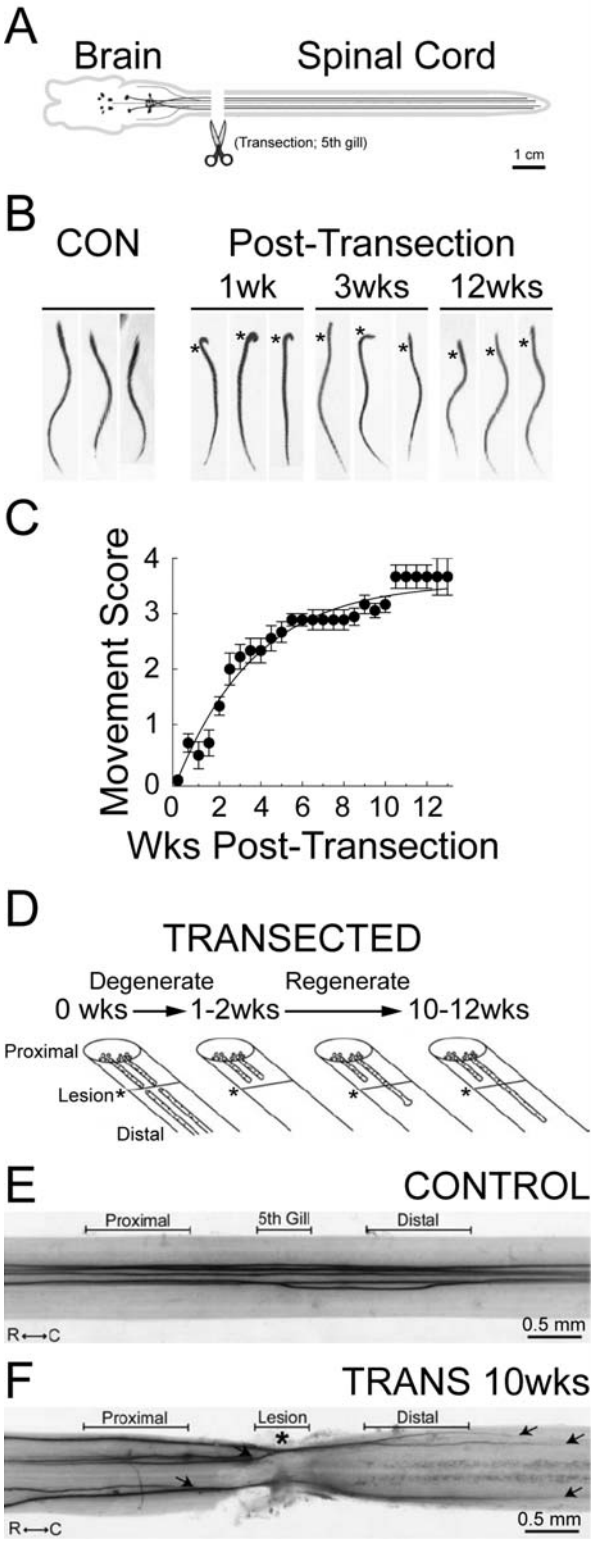

Figure 1.4. Lampreys recover normal swimming behavior that coincides with axon regeneration after spinal cord transection.

(A) Drawing of lamprey brain and spinal cord as well as site of transection at the level of 5th gill. (B) Images of individual control lampreys (CON) and spinal-transected lampreys at 1 week (1wk), 3 weeks (3wks) and 12 weeks (12wks) post-transection. Undulatory movements are impaired at 1 and 3 weeks post-transection, but return to normal at 12 weeks post-transection. Asterisks indicate site of transection. (C) Time course of recovery of swimming behavior. Recovery is nearly normal by 10 weeks post-transection. Each point represents mean movement score ± S.E.M. for 9 spinal-transected lampreys. (D) Time course of axon retraction and regeneration after spinal-transection. After spinal-transection (Lesion; *) and for the first 2 weeks (1-2wks), proximal portion of axons retracts and distal portion degenerates. During the next few months, some injured axons begin to grow towards the lesion site and regenerate across it by 10-12 weeks post-transection (10-12wks). (E-F) Bright field images of uninjured control (E) and 10 weeks post-transected (F; TRANS 10wks) spinal cords in which reticulospinal (RS) axons were injected with horseradish peroxidase. Each dark line represents one RS axon. About 50% of the RS axons regenerated across the lesion site by 10 weeks post-transection. Arrows mark the tips of RS axons that terminated proximal and distal to the lesion site [modified from Oliphint et al., 2010].

(Davis and McClellan, 1993; Oliphint et al., 2010; Selzer, 1978) (Fig. 1.4). By 10-12 weeks after injury, injured lampreys recover a normal swimming behavior that is comparable to uninjured lampreys (Ayers, 1989; Cohen et al., 1986; Davis and McClellan, 1993; Oliphint et al., 2010; Rovainen, 1976; Selzer, 1978) (Fig. 1.4).

1.4.2 Spinal cord injury induces anatomical plasticity in the lamprey central nervous system

1.4.2.1 Spinal cord injury induces axon regeneration in lamprey spinal cord

Spinal cord transection severs both descending and ascending axons, including the reticulospinal (RS) axons that are known to provide the major descending motor commands for regulating lamprey swimming behaviors (Deliagina et al., 2000; Dubuc et al., 2008; Fagerstedt et al., 2001; Orlovsky et al., 1992; Zelenin, 2011). These axons include a subset of the giant RS neurons, the Müller and Mauthner cells, whose cell bodies are situated within the midbrain and hindbrain. In uninjured lampreys, the giant Müller axons project ipsimedially and the giant Mauthner axons project contralaterally along the length of the lamprey spinal cord (Rovainen, 1979), where they make *en passant* ("in passing") synapses (Oliphint et al., 2010; Pieribone et al., 1995). During the first two weeks after a complete spinal cord transection, the proximal portion of the injured giant RS axons predominantly retracts and some axotomized RS neurons will begin to regenerate thereafter (Roederer et al., 1983; Yin and Selzer, 1983; Zhang et al., 2005) (Fig. 1.4). During this period, the regenerating RS axon tips are readily identified proximal to the lesion site (Jin et al., 2009). The distal portion of the injured giant RS axons undergoes degeneration. By 10-12 weeks after the injury, about 50% of the giant

RS axons have regenerated across the lesion site (Armstrong et al., 2003; Davis and McClellan, 1994; Oliphint et al., 2010; Rovainen, 1976; Selzer, 1978; Yin and Selzer, 1983) (Fig. 1.4). Approximately 40% of the regenerating giant RS axons also branch new axons both proximal and distal to the lesion site (Oliphint et al., 2010). The projection paths of regenerated RS axons are also different than their uninjured counterparts: 1) some giant Müller axons have lateral projections rather than their normal ipsimedial projections, 2) some RS axons will make rostral turns, and 3) some will cross the midline. Members of our lab, including myself, have also shown that regenerated RS axons form presynaptic structures distal to the lesion site, albeit with fewer numbers (Oliphint et al., 2010). Surprisingly, other labs have shown synaptic strength is enhanced at synaptic connections between regenerated RS axons and target spinal neurons (Cooke and Parker, 2009; Mackler and Selzer, 1987). Regeneration of ascending axons of spinal neurons has also been observed in lampreys after injury (Armstrong et al., 2003). Thus, a great deal of anatomical plasticity occurs within the lamprey spinal cord that correlates with the robust recovery of swimming behavior after spinal cord injury, providing a model for identifying the molecular mechanisms mediating injury-induced anatomical plasticity.

1.4.2.2 Spinal cord injury induces cellular changes of lamprey giant RS neurons

Within the lamprey midbrain and hindbrain, there are 17 pairs of giant RS cell bodies that reside in stereotypical locations. These cells include the Müller cells of the mesencephalic (M), isthmic (I) and bulbar (B) brain regions, as well as the Mauthner

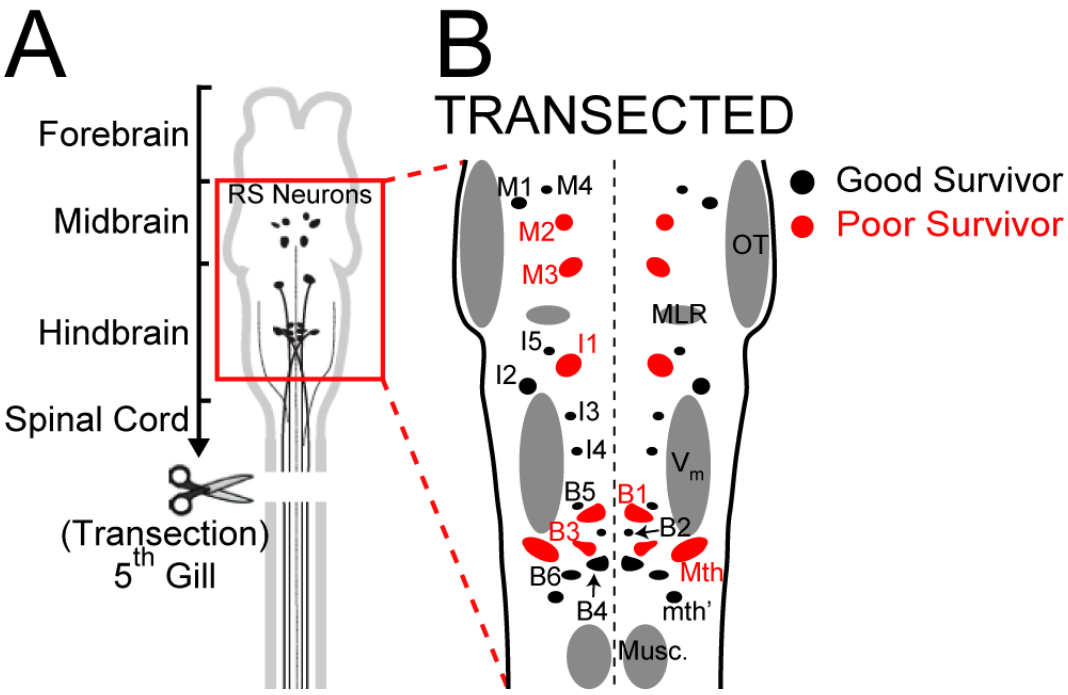

Figure 1.5. A distinct subset of giant RS neurons undergoes delayed cell death after spinal cord injury.

(A) Schematic drawing of the lamprey central nervous system showing the brain, the locations of the giant RS neurons with their axon projection paths in the spinal cord, and the site of spinal cord transection. (B) Schematic drawing of the midbrain and hindbrain of a transected lamprey showing the giants RS neurons that are likely to survive (black circles) and those that undergo delayed cell death (red circles) after spinal cord transection. The afferent neurons known to make direct synaptic connections with the giant RS neurons are illustrated in grey. OT = optic tectum, MLR = mesencephalic locomotor region, V_m = trigeminal motor nucleus, Musc. = muscarinoceptive neurons, M = mesecenphalic, I = isthmic, B = bulbar, Mth = Mauthner and mth' = auxillary Mauthner [adapted from Busch and Morgan, 2012; Jacobs et al., 1997; Shifman et al., 2008; Smetana et al., 2010; Viana Di Prisco et al., 2005; Zompa and Dubuc, 1996].

(Mth) and auxillary Mauthner (mth') cells in the hindbrain (Fig. 1.5). The large size of the RS cell bodies provides a great tool to understand their normal cell morphology, as well as the morphological changes that occur after spinal cord transection (Busch and Morgan, 2012; Hall and Cohen, 1988; Jacobs et al., 1997; Shifman et al., 2008). Within the brain, the giant RS neurons receive direct synaptic inputs from the optic tectum (OT) (Zompa and Dubuc, 1996) and trigeminal motor nucleus (V_m) (Viana Di Prisco et al., 2005) as well as mesencephalic locomotor region (MLR) and muscarinoceptive neurons (Musc.) (Smetana et al., 2010) (Fig. 1.5).

After a complete spinal cord transection, many giant RS neurons can survive and regenerate their axons (Busch and Morgan, 2012; Jacobs et al., 1997; Shifman et al., 2008) (Fig. 1.5). These "good survivors" are the M1 and M4 cells of the midbrain, the I2-I5, B2, B4-B6 and mth' cells of the hindbrain. They have characteristics of a healthy cell: 1) they maintain their normal body size and shape (Busch and Morgan, 2012; Jacobs et al., 1997; Shifman et al., 2008), 2) their cell bodies maintain a dark uniform Nissl staining (Busch and Morgan, 2012; Shifman et al., 2008), which marks ribosomal RNA in the endoplasmic reticulum, 3) their cell bodies are negative for Fluoro-Jade C staining (FJC) (Busch and Morgan, 2012), an anionic fluorescent dye that labels degenerating neurons with an unidentified mechanism (Ehara and Ueda, 2009; Schmued et al., 2005), and 4) their cell bodies are also negative for terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-fluorescein nick and labeling (TUNEL) staining (Shifman et al., 2008). In contrast to these surviving neurons, starting at 3 weeks post-injury, a distinct subset of the giant RS neurons undergoes delayed cell death and has a

poor capacity to regenerate (Busch and Morgan, 2012; Jacobs et al., 1997; Shifman et al., 2008) (Fig. 1.5). These "poor survivors" are the M2 and M3 cells of the midbrain, and I1, B1, B3 and Mth cells of the hindbrain. They have characteristics of dying cells: 1) they have a swollen appearance (Busch and Morgan, 2012; Jacobs et al., 1997; Shifman et al., 2008), 2) they have high tendency to lose Nissl staining (Busch and Morgan, 2012; Shifman et al., 2008), and 3) they have staining for both FJC (Busch and Morgan, 2012) and TUNEL (Shifman et al., 2008). These effects persist for at least 12 weeks after injury (Busch and Morgan, 2012; Jacobs et al., 1997; Shifman et al., 2008). Thus, spinal cord injury induces cell death of a subset of the lamprey giant RS neurons, an event that is consistently found in other models of spinal cord injury (Hains et al., 2003; Zottoli et al., 1984). The intrinsic cell death property of the identifiable giant RS neurons provides an opportunity to ask whether cAMP can promote neuronal survival after spinal cord injury. This question will be explored in Chapter 3.

In addition to the injury-induced cell death, injury also alters the dendritic trees of the giant RS neurons. After a complete spinal cord transection at the level of the 5th gill, some axotomized giant RS neurons will retract their dendrites (Hall and Cohen, 1988). Dendritic retraction is characterized by the narrowing of the major dendritic branches. Eventually, some of these dendrites will disappear. Using this method of injury, dendritic sprouting and branching are rarely seen. Assuredly, the loss of dendrites would also mean the loss of synaptic inputs coming into the giant RS neurons. Therefore, the loss of presynaptic connections will also most likely trigger anatomical and molecular changes

within those target neurons, such as neurons in the OT, MLR, V_m and Musc. nuclei. This idea has not been tested to date and will be addressed in Chapter 2.

1.4.3 Lampreys have conserved molecules that regulate axon growth, synapse formation and synaptic transmission

While lampreys undergo anatomical plasticity after spinal cord injury, a mechanism that is conserved across vertebrates (Blesch and Tuszynski, 2009; Bradbury and McMahon, 2006; Maier and Schwab, 2006), lampreys also have conserved molecules that are involved in mediating axon growth and synapse formation. The lamprey synapsin I and II genes have been cloned previously (Kao et al., 1999) (Fig. 1.2). Both synapsin sequences are highly identical to other species. In particular, the serine 9 residue is well conserved. Synapsin I protein is expressed at the presynaptic termini of the lamprey synapses, where it is associated with synaptic vesicles that are positive for synaptic vesicle glycoprotein 2 (SV2) (Bloom et al., 2003; Oliphint et al., 2010; Pieribone et al., 1995). In addition, the lamprey synapsin regulates the size of the reserve vesicle pool (Pieribone et al., 1995). However, the expression exchanges of synapsin after spinal cord injury in lampreys have not been explored. Chapter 2 will address the expression changes of synapsin before and after spinal cord injury.

Axon growth and regeneration after spinal cord transection in lampreys are likely to utilize the common cAMP/PKA pathway as seen in other models of spinal cord injury. For example, axotomized lamprey RS axons treated with db-cAMP show less retraction and a greater rate of regeneration towards the lesion site at early time points, such as 2 weeks post-injury (Jin et al., 2009). However, it remains unknown whether db-cAMP

increases the number of regenerated RS axons distal to the lesion site at the time of behavioral recovery, which occurs by 10-12 weeks post-injury. In addition, whether db-cAMP increases the expression of axon growth-associated genes, such as synaptotagmin, in growing axons is also unknown. Chapter 3 will attempt to address these questions.

Chapter 2: Increased synapsin expression and neurite sprouting in lamprey brain after spinal cord injury[1]

2.1 INTRODUCTION

Spinal cord injury induces compensatory anatomical plasticity in both injured and spared neurons throughout the vertebrate nervous system (Blesch and Tuszynski, 2009; Bradbury and McMahon, 2006; Maier and Schwab, 2006). For example, spontaneous sprouting of both lesioned and unlesioned corticospinal axons has been observed after spinal cord injury in mammals, supporting recovery of movements via reconnections with propriospinal neurons (Bareyre et al., 2005; Courtine et al., 2008; Rosenzweig et al., 2010; Weidner et al., 2001). In addition, regeneration of injured spinal axons through the lesion site has been demonstrated for several ascending and descending tracts in vertebrates ranging from lampreys to mammals (Armstrong et al., 2003; Bradbury and McMahon, 2006; Mladinic et al., 2009; Oliphint et al., 2010; Rovainen, 1976; Yin and Selzer, 1983). Spinal cord injury also induces remodeling of injured neuronal connections at distant locations in the brain. For example, dorsal spinal cord lesions induce axon sprouting in the mammalian brainstem (Jain et al., 2000), which likely plays a role in functional reorganization of the somatotopic map in cortex (Jain et al., 1997; McKinley et al., 1987). Spinal cord injury also induces changes in the density, size, and length of dendritic spines in the motor cortex, indicating a remodeling of synaptic

[1] A great portion of the work is published as Lau, B. Y. B., Foldes, A. E., Alieva, N. O., Oliphint, P. A., Busch, D. J., and Morgan, J. R., 2011. Increased synapsin expression and neurite sprouting in lamprey brain after spinal cord injury. Experimental Neurology 228**,** 283-293. Lau and Morgan designed experiment and wrote the manuscript. Lau performed most of the experiments. Foldes and Oliphint contributed to EM studies. Alieva contributed to RT-PCR studies. Busch contributed to Nissl staining studies.

connections in the brain (Kim et al., 2006). Taken together, these findings indicate that spinal cord injury induces compensatory anatomical plasticity of neurites (i.e. axons and dendrites) throughout the vertebrate nervous system at locations both near to and far from the lesion site. Despite our growing understanding of the pathways that undergo anatomical plasticity after spinal cord injury, the molecular mechanisms underlying such plasticity remain poorly understood.

Synapsin has the potential to play an important role in injury-induced plasticity, due to its robust developmental roles in neurite outgrowth and synapse formation. Synapsin is an abundant neuronal phosphoprotein that is highly conserved across vertebrate and invertebrate species (De Camilli et al., 1983; Kao et al., 1999). The mammalian synapsin family consists of three genes (synapsin I, II, and III), while other organisms typically express synapsin I (and sometimes synapsin II) or an ancestral version of the gene (Cesca et al., 2010; Kao et al., 1999). During development of the mammalian nervous system, synapsins I and II regulate initial neurite formation, axon specification and growth, and synapse formation (Chin et al., 1995; Ferreira et al., 1998; Ferreira et al., 1995; Han et al., 1991; Lu et al., 1992; Schaeffer et al., 1994). Synapsin III plays a role in axon growth, but has little effect on synapse formation (Ferreira et al., 2000). Overexpression of synapsin I or II induces non-neuronal cells to sprout thin, elongated, branched processes resembling neurites, suggesting that synapsins are sufficient for neurite sprouting (Han and Greengard, 1994). The extent of synapsin-mediated neurite outgrowth is positively regulated by cAMP-dependent protein kinase A (PKA)-mediated phosphorylation at serine 9, as shown in developing *Xenopus* neurons

(Kao et al., 2002). In mature neurons, synapsin is highly expressed in synaptic boutons where it regulates the size of the vesicle pool (Gitler et al., 2008; Pieribone et al., 1995), exocytosis kinetics (Hilfiker et al., 1998), vesicle trafficking (Bloom et al., 2003; Ryan et al., 1996), and synaptic plasticity (Rosahl et al., 1993). Thus, the multifaceted roles for synapsins in neurite outgrowth, as well as synapse structure and function, in both developing and mature neurons make it an excellent candidate for mediating some aspects of injury-induced structural plasticity. However, this has not been tested in any model to date.

Here, we examined synapsin expression before and after spinal cord injury as a first step toward gaining a better understanding of its potential role in injury-induced plasticity. We utilized the nervous system of lampreys for several reasons. First, lamprey nervous system undergoes an exceptional degree of anatomical plasticity after spinal cord injury, including spontaneous regeneration of descending and ascending axons (Armstrong et al., 2003; Davis and McClellan, 1994; Oliphint et al., 2010; Rovainen, 1976; Selzer, 1978; Yin and Selzer, 1983), synapse regeneration (Oliphint et al., 2010; Wood and Cohen, 1979), and synapse remodeling (Cooke and Parker, 2009). The injury-induced anatomical plasticity temporally coincides with robust behavioral recovery (Cohen et al., 1986; Davis et al., 1993; Oliphint et al., 2010). Second, the lamprey synapsins have already been sequenced and are well characterized in the uninjured nervous system (Bloom et al., 2003; Kao et al., 1999; Pieribone et al., 1995). We report that spinal cord injury induced a robust upregulation of synapsin I mRNA and protein, as well as increased levels of phospho-synapsin (serine 9), in the lamprey brain.

Synapsin upregulation in response to spinal cord injury coincided with a significant increase in neurite density in the lamprey hindbrain. Taken together, these data indicate the existence of spinal cord injury-induced neurite remodeling in lamprey brain, as occurs in other vertebrates. The data are also consistent with a role for synapsin and phospho-synapsin (serine 9) in the injury-induced neurite sprouting mechanism, suggesting that at least some developmental processes are recapitulated after injury.

2.2 METHODS

2.2.1 Spinal cord transections and brain dissections

Late larval stage sea lampreys (*Petromyzon marinus*; 11-14 cm) housed at room temperature (RT; 25ºC) were used for all experiments. For spinal transections and sham operations, lampreys were anesthetized using MS-222 (0.1 g/L tank water; Argent Labs; Redmond, WA). Complete spinal cord transections were made at the level of the 5th gill, as previously described (Fig. 2.1A) (Jacobs et al., 1997; Oliphint et al., 2010). Alternatively, sham operated lampreys underwent the same anesthesia, skin and muscle incision, and suturing, but the spinal cord was not severed. Both spinal transected and sham operated lampreys were allowed to recover at RT for 1, 3, or 10-12 weeks (collectively called "11 wks"). Then, the lampreys were re-anesthetized, and the brains were removed by dissection for further experimentation. For this study, we used a total of 23 uninjured control lampreys, 42 spinal transected lampreys, and 21 sham operated lampreys. All procedures were approved by the Institutional Animal Care and Use

Committee at The University of Texas at Austin in accordance with standards set by the NIH.

2.2.2 Nissl stain

Nissl staining was performed as previously described with a few minor modifications (Selzer, 1979; Shifman et al., 2008). Briefly, lamprey brains were fixed in 4% paraformaldehyde (PFA) in 0.1M PBS (pH 7.4). Then, the brains were stained with toluidine blue O (EM Sciences; Hatfield, PA) containing 1% Borax and no boric acid, followed by differentiation in Bodian's fixative for 20 min., dehydration in 95% and 100% ethanol for 5 min. each, clearing in cedarwood oil, and mounting with Permount (EM Sciences; Hatfield, PA). Imaging was performed using a Leica DFC420C camera attached to a Leica MZ10F stereoscope (Leica Microsystems Inc.; Bannockburn, IL).

2.2.3 Semi-quantitative reverse transcriptase PCR (RT-PCR)

Using an RNAqueous Micro Kit (Applied Biosystems, Ambion Inc.; Austin, TX), total RNA was isolated from lamprey midbrain and hindbrain regions containing descending giant reticulospinal (RS) neurons and several brain nuclei that project to the RS neurons (Fig. 2.1A-B). Then, mRNA was reverse transcribed to generate first strand cDNA libraries, as previously described (Matz, 2002). The reverse transcription reaction utilized 500 ng of total RNA and 1 μM of the poly dT-containing TRsa primer [5'-AAGCAGTGGTATCAACGCAGAGTCGCAGTCGGTAC$(T)_{13}$-3']. The reaction was inactivated at 70ºC for 15 min., followed by digestion of all RNA with 1 U RNase H

(Invitrogen Corp.; Carlsbad, CA) at 37ºC for 20 min. These methods ensured production of first strand cDNA that was representative of mRNA levels in the original sample.

For semi-quantitative RT-PCR experiments, we used as templates first strand cDNA generated from 3-8 control, spinal transected or sham operated lampreys at each time point of interest. Gene-specific primers (Table 2.1) were designed using available sequence information obtained from the lamprey EST database (NCBI) or from the 5.9X assembly of the lamprey genome (Pre Ensembl). Each primer set amplified a fragment of the synaptic vesicle- associated gene of interest (Fig. 2.1C, indicated in red). All primer sets produced a single product of the predicted size, which was subsequently cloned and sequenced to confirm the gene identity. Prior to running the semi-quantitative RT-PCR reactions, all cDNA libraries were normalized against a housekeeping gene, GAPDH, such that when 30 ng of cDNA template were used in the PCR reactions, the GAPDH band intensities were similar among all libraries and within the linear range of amplification. Each RT-PCR reaction contained: 30 ng of first strand cDNA, 0.2 μM each of forward and reverse primers, 0.2 mM dNTPs, 2.5 units of DNA Taq Polymerase (New England Biolabs; Ipswich, MA), and 1X Standard Taq Buffer. For each gene of interest, RT-PCR reactions were performed simultaneously using cDNA templates generated from control and either spinal transected or sham operated lampreys. Equal volumes of PCR products were run on an agarose gel and imaged using an AlphaEaseFC AlphaImager System (Cell Biosciences; San Leandro, CA). The mean intensity of each gel band from individual lampreys was measured and background subtracted using ImageJ (NIH). The gene:GAPDH ratio was obtained for each animal and normalized to

the average intensity obtained from all uninjured controls. This allowed us to directly compare the relative fold changes in mRNA levels between control and either spinal transected or sham operated lampreys at 1, 3, and 11 weeks post-injury. All statistical analyses and graphs were generated using Origin Pro 7.0 (OriginLab; Northampton, MA).

2.2.4 *In situ* hybridization

Synapsin I mRNA probes were generated against the same fragment of synapsin I used in the RT-PCR experiments (Fig. 2.1C; Table 2.1). This synapsin I fragment was cloned into pGEM T-Easy vector (Promega Corp.; Madison, WI). Sense and anti-sense mRNA probes were transcribed from linearized synapsin I-containing vector using T7 and Sp6 RNA polymerases, respectively (Roche Diagnostics Corp.; Indianapolis, IN). Whole mount *in situ* hybridizations were performed on brains of control and spinal transected lampreys using the methods previously described with several modifications (Swain et al., 1994). Proteinase K digestion was for 10 min. at RT (20 µg/mL; Roche), and probe hybridization was for 48 hrs at 50ºC. Post-hybridization washes were for 60 min. at 55°C in 2X Standard Sodium Citrate buffer (2X SSC: 0.3 M NaCl, 0.03M sodium citrate), followed by RNase A (20 µg/ml) for 30 min. at 37°C and 0.2X SSC for 60 min. at 55°C. Remaining washes, antibody incubations, and chromogenic reactions were performed using the procedures published in Shifman and Selzer (2000). Within each experiment, brains of control and transected lampreys were hybridized, developed,

and imaged under identical conditions. Brains were imaged at 40x or 80x magnification using a 1.0 PlanApo lens on a Leica MZ10F stereoscope.

2.2.5 Immunofluorescence and image analysis

Brains were dissected, fixed with 4% PFA in 0.1M PBS (pH 7.4), and cryoprotected with 12-18% sucrose. Then, the brains were embedded in Tissue-Tek® O.C.T. Compound (EM Sciences; Hatfield, PA) and sectioned horizontally (14 μm; -20°C). Immunofluorescence was performed as described previously (Oliphint et al., 2010). Briefly, brain sections were incubated for 1 hour at RT in Blocking Buffer (2% filtered goat serum, 0.3% Triton X-100, 450 mM NaCl, and 20 mM sodium phosphate in 0.1M PBS). Sections were then incubated for 2 hours at RT with primary antibodies diluted in Blocking Buffer. The primary antibodies and their working concentrations were: synapsin I (1:300), SV2 (1:100), or phospho-synapsin Ia/b (1:100). The synapsin I rabbit polyclonal antibody was kindly provided by Drs. Ona Bloom and Paul Greengard, and it was raised against a GST-fusion of lamprey synapsin I domain D (a.a. 470-635) (Bloom et al., 2003). The SV2 mouse monoclonal antibody was made by Dr. Kathleen Buckley and obtained from the Developmental Studies Hybridoma Bank, and it was raised against purified synaptic vesicles from the electric organ of *Discopyge ommata* (NICHD/University of Iowa) (Buckley and Kelly, 1985). The synapsin and SV2 antibodies recognize the appropriate target proteins in lamprey nervous system, as shown by Western blotting and immunofluorescence (Bloom et al., 2003; Oliphint et al., 2010; Pieribone et al., 1995). The phospho-synapsin rabbit polyclonal antibody was obtained

from Santa Cruz Biotechnology Inc., and it was raised against a peptide containing phosphorylated serine 9 (Ser 9) of rat synapsin Ia/b (sc-135710; Santa Cruz, CA). The phospho-synapsin antibody recognizes a band of the appropriate molecular weight in lamprey brain extracts, as shown in this study by Western blotting (Fig. 2.5A). After primary antibody incubations, the brain sections were washed in Wash Buffer (20 mM Sodium Phosphate, pH 7.4, 0.3% Triton-X100, 450 mM NaCl) for 3 x 15 min. at RT and then incubated for 1 hour at RT with either AlexaFluor® 488- or AlexaFluor® 594-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Invitrogen; Carlsbad, CA) at a dilution of 1:300.

After immunostaining, the hindbrain sections containing the bulbar giant reticulospinal neurons were identified, and images were acquired from the regions of interest (ROIs) located between bulbar neurons 3 and 4 and the Mauthner (Mth) neuron (Fig. 2.4A). Imaging was performed using a Zeiss Pascal Exciter attached to an Axioskop 2FS (40x, 1.3 NA EC Plan-NEOFLUAR, oil immersion lens). Image acquisition settings were identical for brains of control and spinal transected lampreys (n=4 for each condition/antibody). Within each ROI, two smaller (25 μm x 25 μm) areas were randomly selected from which the mean intensity and density for all fluorescently-labeled synapsin I-, SV2- and phospho-synapsin-positive puncta were measured using ImageJ. All measurements were made independently, and only those puncta that were >1.7x brighter than background were used in the analyses. We note that the intensity and density measurements represent semi-quantitative analyses of the amount of each protein per punctum and the number of puncta, respectively, since the signals produced by

fluorescent secondary antibodies are non-linear. All statistical analyses and graphing were performed with Origin Pro 7.0.

2.2.6 Western blotting

For Western blotting experiments, 2-3 µg of lamprey brain extracts and 15 µg of rat brain extracts were separated on a 10% SDS-polyacrylamide gel and transferred for 1 hour at 4°C (100 V) to nitrocellulose membranes. After blocking the membranes overnight at 4ºC with Blocking Buffer (5% dry milk in TBST: 20 mM Tris-base, 140 mM NaCl, 0.1% Tween-20, pH 7.6), blots were incubated for 1 hour at RT with 1:1000 rabbit phospho-synapsin (S9) antibody or rabbit anti-synapsin I domain D antibody. After primary antibody incubations, blots were washed 3 x 10 min. with TBST, incubated for 1 hour with 1:4000 peroxidase-conjugated goat anti-rabbit IgG (H+L) (ImmunoPure; Thermo Scientific; Rockford, IL), and developed using Pierce Thermo Scientific enhanced chemiluminescence substrates.

2.2.7 Electron Microscopy

Brains from control lampreys (n=3) and spinal transected lampreys (11 weeks; n=2) were fixed in 3% glutaraldehyde, 2% PFA, and 2% sucrose in 0.1 M sodium cacodylate buffer (pH 7.4). Then, the fixed brains were processed for electron microscopy, embedded in Embed-812, counterstained and sectioned, as previously described (Oliphint et al., 2010). First, thick (1 µm) brain sections were stained with toluidine blue O to identify the region containing the B3 bulbar and Mauthner (Mth)

neurons, as was done for the immunofluorescence analysis. Then, ultrathin (70 nm) sections were obtained, and small ROIs (5 x 5 μm) were imaged in the region between Mth and B3 neurons (Fig. 2.6, boxes) at 16,500x for analysis of neurite and synaptic bouton density (#/μm^2), as well as neurite size (circumference). Individual synaptic boutons were imaged at 26,500x for analysis of synaptic vesicles. All micrographs were coded and then analyzed by a researcher blinded to the experimental conditions using ImageJ. Only those neurites having a complete border within the image frame were analyzed. Statistical analyses and graphing were performed with Origin Pro 7.0.

2.3 RESULTS

2.3.1 Synapsin I mRNA is selectively upregulated in lamprey brain after spinal cord injury

To begin assessing the molecular basis for injury-induced anatomical plasticity, we transected lamprey spinal cords and then measured gene expression changes in the midbrain and hindbrain, because these are the brain regions most likely to respond to a distant spinal cord transection. Midbrain and hindbrain contain the cell bodies of descending reticulospinal (RS) neurons, whose axons are severed upon spinal cord transection (Fig. 2.1A). Amongst these are ~30 giant RS neurons, including the Müller neurons of the mesencephalic (M), isthmic (I) and bulbar (B) brain regions, as well as the Mauthner (Mth) neuron (Fig. 2.1B, left). Lamprey midbrain and hindbrain also contain several brain regions that project to the injured giant RS neurons and that are likely to respond to the observed injury-induced changes in RS neuron activity (McClellan et al., 2008). These brain regions include the optic tectum (OT), mesencephalic locomotor

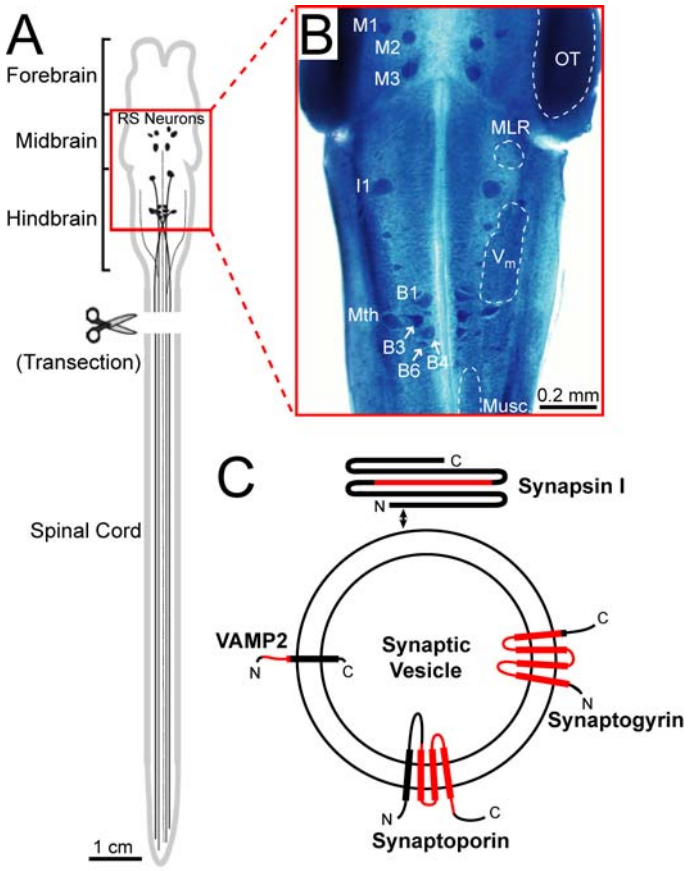

Figure 2.1. Lamprey nervous system and genes examined in this study.
(A) Diagram of the lamprey nervous system showing the general architecture of the brain, the locations of giant reticulospinal (RS) neurons, and the site of spinal cord transection. Red box highlights the brain regions from which cDNA libraries were generated. (B) Nissl stained brain from an uninjured control lamprey. The neurons and brain regions shown were predicted to respond to spinal cord injury with changes in gene expression. (Left) Several of the identified giant RS neurons are labeled, including mesencephalic neurons (M1-3), isthmic neuron (I1), bulbar neurons (B1, B3, B4, B6), and Mauthner (Mth) neuron, all of which are bilaterally localized in the same position across individual lamprey brains. These neurons are severed upon spinal cord transection. (Right) Also shown are the optic tectum (OT), mesencephalic locomotor region (MLR), trigeminal motor nucleus (Vm), and muscarinoceptive neurons (Musc.), which project to RS neurons. (C) Diagram of a synaptic vesicle and the associated proteins whose gene expression levels were examined in this study. Red region on the protein corresponds to the gene fragment that was amplified by semi-quantitative RT-PCR.

region (MLR), trigeminal motor nucleus (Vm), and muscarinoceptive neurons (Musc.) (Fig. 2.1B, right) (Smetana et al., 2010; Viana Di Prisco et al., 2005; Zompa and Dubuc, 1996). Therefore, we predicted that mRNA expression profiles would be altered within the axotomized RS neurons and/or their afferent neurons in response to spinal cord injury. Indeed, several other studies have already reported changes in gene expression within RS neurons in response to spinal cord transection (Jacobs et al., 1997; McClellan et al., 2008).

We started by examining the effect of spinal cord injury on synapsin I mRNA levels in lamprey brain using semi-quantitative reverse transcriptase PCR (RT-PCR). To do so, we generated cDNA libraries from the combined midbrain and hindbrain regions of uninjured control and spinal transected lampreys (Fig. 2.1A-B). For the spinal transected lampreys, the brain cDNA libraries were generated at 1, 3, and 11 weeks post-injury, because these time points reflect paralysis, mid-recovery, and late recovery of locomotor function, respectively (Oliphint et al., 2010). In addition, synapsin I-specific primers were generated to amplify a 402 bp internal region of the gene (Fig. 2.1C; Table 2.1) (Kao et al., 1999). Semi-quantitative RT-PCR revealed that synapsin I mRNA levels increased more than 2-fold after spinal cord transection, relative to expression levels observed in uninjured control lampreys (Fig. 2.2A, left). After an initial delay, the upregulation of synapsin I was present by 3 weeks post-transection and was sustained until at least 11 weeks post-transection, which was the latest time point examined (Fig. 2.2A-B; Table 2.2). At 3 and 11 weeks after spinal cord transection, synapsin I mRNA levels were significantly higher in the brains of spinal transected lampreys than in either

GENE	PRIMER SEQUENCE		SIZE
	FW, 5'→3'	REV, 5'→3'	(bp)
GAPDH	GGAGTAAACCACGAGAAGTA	AAGATGGACGAGTGGGTGTC	485
Synapsin I	AAGACTACCGCAGCATTGTCA	AGGTTCTCATGTAGGCCTTGT	402
Synaptogyrin	TCATGAAGCAACCTCAGACCAT	CGAGCGCTGTCGTGAGAGTA	382
Synaptoporin	TTCTCCTCGTCGGTCGAGTT	CCCGTCTCCTTGAACACGAA	389
VAMP2	CCCAACCTCACGAGCAACAA	TGCAGTTCTTCCACCAGTACTT	211

Table 2.1. Gene-specific primers used for semi-quantitative RT-PCR experiments. For each gene of interest, the forward and reverse primer sequences are listed, as is the expected size of the PCR product in base pairs (bp).

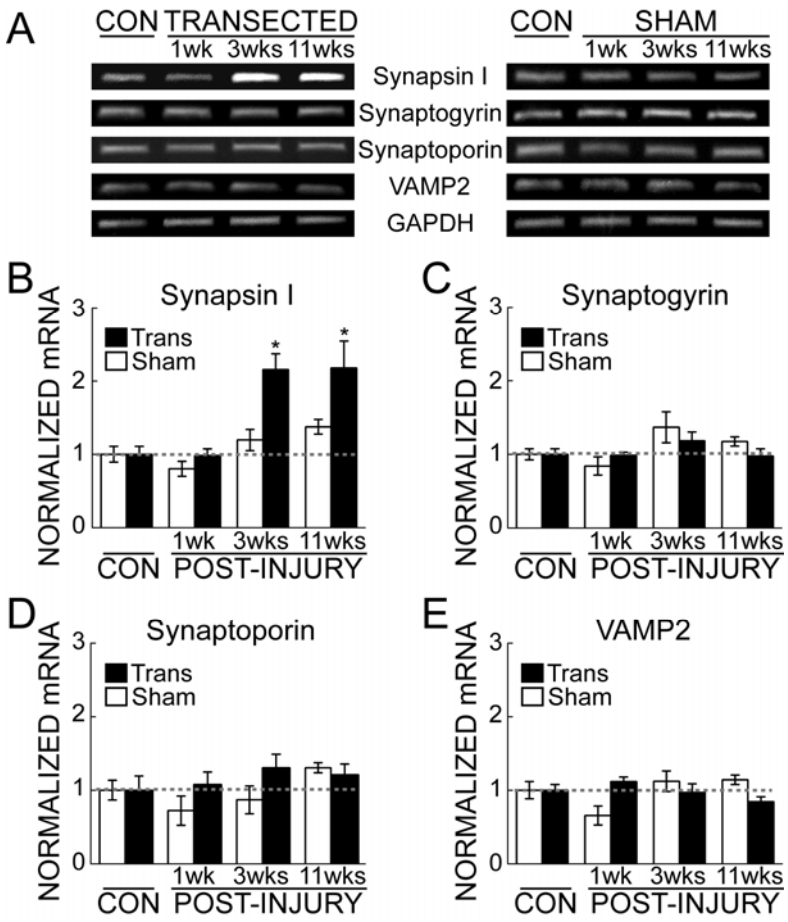

Figure 2.2. Synapsin I is upregulated in lamprey brain after spinal cord injury.
(A) Semi-quantitative RT-PCR on several synaptic vesicle-associated genes was performed using lamprey brain cDNA libraries generated from uninjured control (CON), spinal transected and sham operated lampreys at several time points post-injury in weeks (wks). GAPDH was used as a control against which other gene expression levels were normalized. Only synapsin I is upregulated after spinal cord transection. (B-E) Synapsin I was upregulated >2-fold after spinal transection, but not after sham operations (B). In contrast, synaptogyrin (C), synaptoporin (D), and VAMP2 (E) expression levels did not change significantly after spinal transection or sham operations. Bars indicate mean ± S.E.M. Asterisks indicate statistical significance between transection conditions and uninjured controls or shams (*ANOVA; $p<0.05$*).

GENE	CON	POST-TRANSECTION		
		1 wk	3 wks	11 wks
Synapsin I	1.0 ± 0.1 (7)	0.98 ± 0.09 (6)	2.2 ± 0.2 (7)*	2.17 ± 0.4 (6)*
Synaptogyrin	1.0 ± 0.1 (7)	0.98 ± 0.04 (6)	1.2 ± 0.1 (7)	0.97 ± 0.1 (7)
Synaptoporin	1.0 ± 0.2 (6)	1.1 ± 0.2 (6)	1.3 ± 0.2 (7)	1.2 ± 0.1 (6)
VAMP2	1.0 ± 0.1 (7)	1.1 ± 0.1 (6)	1.0 ± 0.1 (7)	0.84 ± 0.1 (7)

GENE	CON	SHAM-OPERATED		
		1 wk	3 wks	11 wks
Synapsin I	1.0 ± 0.1 (6)	0.80 ± 0.1 (6)	1.2 ± 0.1 (7)	1.4 ± 0.1 (8)
Synaptogyrin	1.0 ± 0.1 (4)	0.84 ± 0.1 (5)	1.4 ± 0.2 (6)	1.2 ± 0.1 (8)
Synaptoporin	1.0 ± 0.1 (7)	0.72 ± 0.2 (6)	0.87 ± 0.2 (7)	1.3 ± 0.1 (8)
VAMP2	1.0 ± 0.1 (3)	0.65 ± 0.1 (4)	1.1 ± 0.1 (6)	1.1 ± 0.1 (8)

Table 2.2. Changes in gene expression after spinal cord injury.
Summary of the data obtained from semi-quantitative RT-PCR experiments. All gene expression data were normalized to the levels observed in uninjured control (CON) lampreys. Synapsin I expression was significantly increased at 3 and 11 weeks post-transection. Asterisks denote statistical significance (*ANOVA; Tukey's post hoc, $p<0.05$*). In contrast, synapsin I did not significantly change after sham operations. Synaptogyrin, synaptoporin, and VAMP2 expression levels remained fairly stable after both spinal transection and sham operations, and none of the differences observed reached statistical significance. The number of individual lamprey cDNA libraries used for each condition or time point is shown in parentheses.

uninjured control lampreys or in sham operated lampreys (Fig. 2.2A-B, Table 2.2). Synapsin I mRNA levels were not significantly changed in sham operated lampreys at any of the time points examined (Fig. 2.2A, right; Fig. 2.2B; Table 2.2). To determine the specificity with which synapsin I is upregulated after spinal cord transection, semi-quantitative RT-PCR was repeated for three additional synaptic vesicle-associated genes: synaptogyrin, synaptoporin, and VAMP2 (Fig. 2.1C). In summary, synaptogyrin, synaptoporin, and VAMP2 mRNA levels were not significantly altered after spinal cord transection or sham operations (Fig. 2.2A, C-E; Table 2.2). Thus, spinal cord transection induced a selective, robust, and long-lasting upregulation of synapsin I mRNA in the lamprey brain.

2.3.2 Synapsin I mRNA is increased globally throughout lamprey brain after spinal cord injury

To visualize the brain regions where synapsin I is upregulated, whole mount *in situ* hybridizations were performed on brains of control and spinal transected lampreys using probes specific for synapsin I mRNA. In uninjured control lampreys, synapsin I mRNA was expressed in relatively low levels and was diffuse throughout the midbrain and hindbrain (Fig. 2.3A). In comparison, at 3 and 11 weeks post-transection, synapsin I mRNA increased globally throughout most regions of the midbrain and hindbrain, as determined by darker signal intensity (Fig. 2.3B-C). The regions where the *in situ* signal intensity appeared darker included the optic tectum (OT), mesencephalic locomotor region (MLR), trigeminal motor nucleus (Vm) and the muscarinoceptive neurons (Musc.) (Fig. 2.3B-C). In addition, smaller neurons in the neuropil surrounding the transected

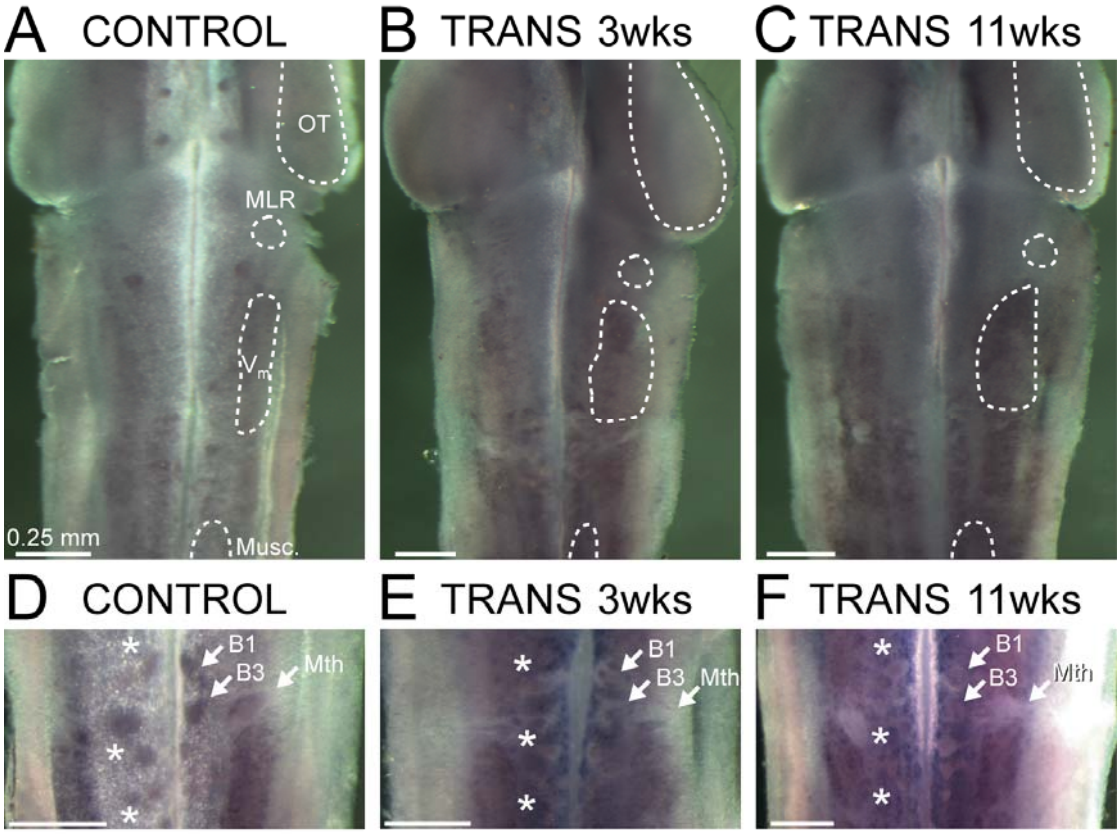

Figure 2.3. Synapsin is upregulated globally throughout lamprey midbrain and hindbrain after spinal cord injury.

(A-C) Whole mount in situ hybridizations for synapsin I mRNA in lamprey brain. In the brains of uninjured control lampreys (A), synapsin I mRNA is low and diffuse throughout midbrain and hindbrain. In contrast, at 3 and 11 weeks post-transection, the signal intensity increased throughout the midbrain and hindbrain (B, C), including optic tectum (OT), mesencephalic locomotor region (MLR), trigeminal motor nucleus (Vm), and muscarinoceptive neurons (Musc.) (dashed lines). (D-F) High magnification images showing synapsin I mRNA expression in a different set of lamprey brains. Region shown contains the bulbar (B) and Mauthner (Mth) neurons for reference. Note the presence of increased signal intensity in the neuropil surrounding the giant RS neurons at 3 and 11 weeks post-transection (asterisks). The giant RS neurons themselves appear to lose synapsin expression. Scale bar = 0.25 mm in all panels.

giant RS neurons exhibited increased synapsin I mRNA expression after spinal cord transection (Fig. 2.3D-F, asterisks). At this level of resolution, it appeared that synapsin I mRNA expression actually decreased in some of the axotomized giant RS neurons themselves, such as Mauthner, B1, and B3, but this appearance may be due to the fact that these particular neurons undergo general chromatolysis and delayed cell death after injury (Shifman et al., 2008). Taken together, the *in situ* hybridizations revealed a general global and widespread, rather than site-specific, upregulation of synapsin I mRNA throughout the lamprey midbrain and hindbrain.

2.3.3 Synapsin I protein levels are increased in lamprey brain after spinal cord injury

Next, we examined whether spinal cord injury similarly affects synapsin I protein levels in the brain. In theory, upregulation of synapsin I mRNA might reflect an increased need for recruiting more synapsin I protein to individual synapses, an increase in the size of synaptic vesicle clusters, increased neurite or synapse sprouting, or a combination of these possibilities. To begin addressing these scenarios, we performed immunofluorescence for synapsin I protein on hindbrain sections from control and spinal transected lampreys. We compared synapsin I immunofluorescence to that of SV2, which is an integral membrane protein on synaptic vesicles that serves as a reliable marker of synapses in all vertebrates, including lampreys (Bloom et al., 2003; Buckley and Kelly, 1985; Oliphint et al., 2010). We focused our analyses on the neuropil in the hindbrain surrounding the bulbar (B3) and Mauthner (Mth) neurons, because synapsin I mRNA was upregulated in this region after spinal cord transection (Fig. 2.3D-F) and

because these giant RS neurons are always located in a stereotypical position, providing landmarks for consistent analysis (Fig. 2.4A).

In the brains of uninjured, control lampreys, there were many synapsin I-positive structures, which were largely synaptic in nature as determined by colocalization with SV2 (Fig. 2.4B). In comparison, at 11 weeks post-transection, the synapsin I-positive puncta that colocalized with SV2 at synapses appeared brighter (Fig. 2.4C, arrows). In addition, there were more synapsin I-positive puncta after transection, some of which lacked detectable SV2 (Fig. 2.4C, arrowheads). Semi-quantitative analysis on these immunostained brain sections revealed a significant increase in the fluorescence intensity of synapsin I-positive puncta at 11 weeks after spinal cord transection (Fig. 2.4D) (*Control*: 58.0 ± 7.7 AU, n = 1586 puncta, 4 animals; *Transected*: 89.6 ± 8.5 AU, n = 2294 puncta, 4 animals; $p < 0.05$; *T-test*). In addition, the density of synapsin I-positive puncta significantly increased after transection (Fig. 2.4E) (*Control*: 0.16 ± 0.02/μm^2, n = 16 ROIs, 4 animals; *Transected*: 0.23 ± 0.01/μm^2, n = 16 ROIs, 4 animals; $p < 0.05$; *T-Test*). In contrast, no significant differences were detected for either the intensity or the density of SV2-positive puncta (Fig. 2.4B-C, F-G) (Intensity - *Control*: 70.2 ± 6.6 AU, n = 2033 puncta, 4 brains; *Transected*: 89.5 ± 23.0 AU, n = 2735 puncta, 4 brains; $p > 0.05$; *T-Test*; Density - *Control*: 0.20 ± 0.02/μm^2, n = 16 ROIs, 4 animals; *Transected*: 0.27 ± 0.03/μm^2, n = 16 ROIs, 4 animals; $p > 0.05$; *T-test*). These observations revealed two separate features of injury-induced changes in synapsin protein expression. First, the increased intensity of synapsin I immunofluorescence at SV2-positive synapses suggests

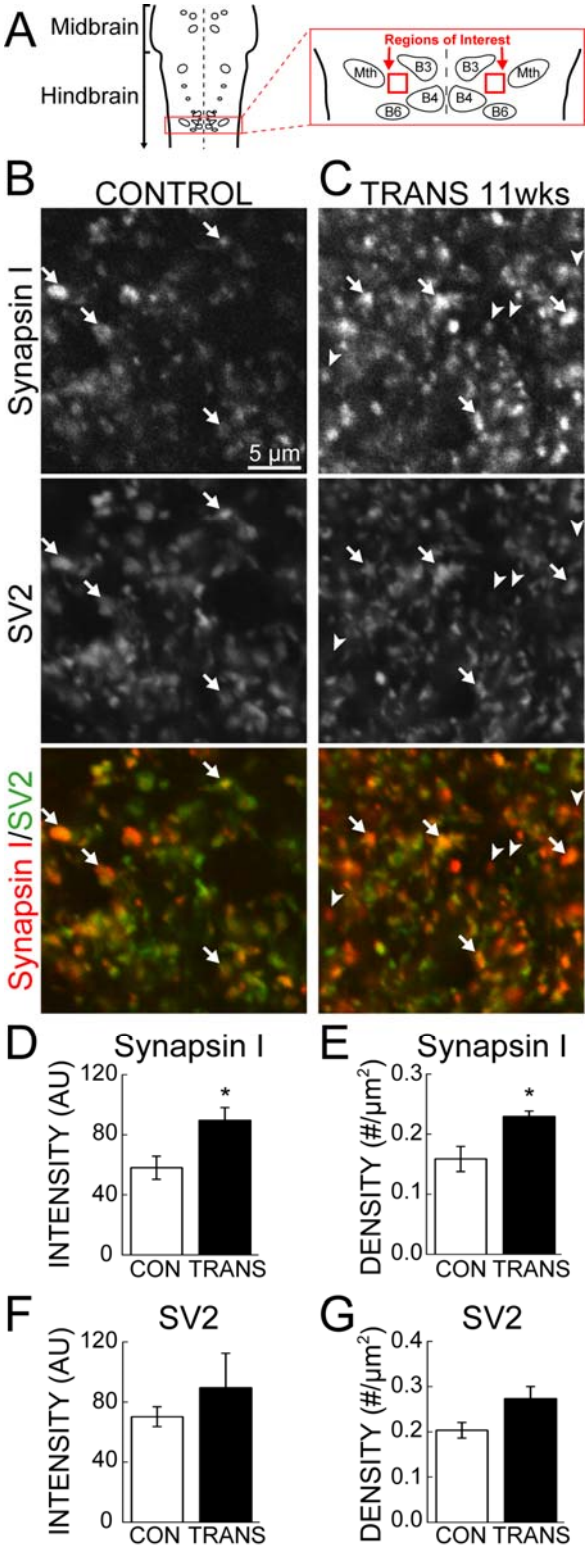

Figure 2.4. Synapsin protein levels increase in the hindbrain after spinal cord injury.
(A) Drawing of lamprey brain showing regions of interest used for synapsin and SV2 immunofluorescence analyses. (B-C) Confocal images showing synapsin- and SV2-positive structures in lamprey hindbrain. In control brains (B), synapsin I is found in modest levels at synapses, as determined by colocalization with SV2 (arrows). At 11 weeks post-transection (C), synapsin I intensity is greater at individual synapses (arrows), and there are also more synapsin-positive puncta, some of which do not contain SV2 (arrowheads). Scale bar applies to all images. (D-G) Both the intensity and density of synapsin I-positive structures significantly increased after spinal cord transection (D, E) In contrast, SV2 was not significantly altered (F, G). Bars indicate mean ± S.E.M for 1586-2735 puncta (intensity) or 16 regions of interest (density) measured from 4 animals. Asterisks indicate statistical significance (*T-test; $p<0.05$*).

a greater abundance of synapsin I at individual synapses following spinal cord injury. Second, the increased density of synapsin I-positive structures, some of which lack SV2, suggests an enhancement of neurite sprouting.

2.3.4 Phosphorylation of synapsin at serine 9 increases in lamprey brain after spinal cord injury

Phosphorylation of synapsin at serine 9 by PKA increases neurite outgrowth during development (Kao et al., 2002) and enhances transmitter release at mature synapses (Chi et al., 2001; Menegon et al., 2006), indicating that phospho-synapsin (Ser 9) is a good marker for synapsin-mediated neuronal plasticity. We therefore examined the phosphorylation state of synapsin using an antibody raised against a peptide containing phosphorylated serine 9 from rat synapsin Ia/b. Serine 9 is conserved in lamprey synapsins (Kao et al., 1999). *In vitro* studies indicated that PKA phosphorylates lamprey synapsin: pharmacological inactivation and activation of PKA decreased and increased, respectively, the level of phospho-synapsin (Ser 9) in lampreys (Appendix Figure A1.1).[2] Western blot analysis revealed that the phospho-synapsin Ia/b antibody detected a single protein band in lamprey brain lysate that was the same size as both the major phospho-synapsin (Ser 9) band in rat brain lysate and total synapsin I in lamprey brain lysate (Fig. 2.5A). Immunofluorescence on hindbrain sections from uninjured control lampreys revealed only a few structures that were positive for phospho-synapsin (Ser 9) (Fig. 2.5B). In contrast, at 11 weeks post-transection, semi-quantitative analysis revealed a significant increase in the density of phospho-synapsin (Ser 9)-positive puncta,

[2] This was not part of the original Lau et al., 2011 publication.

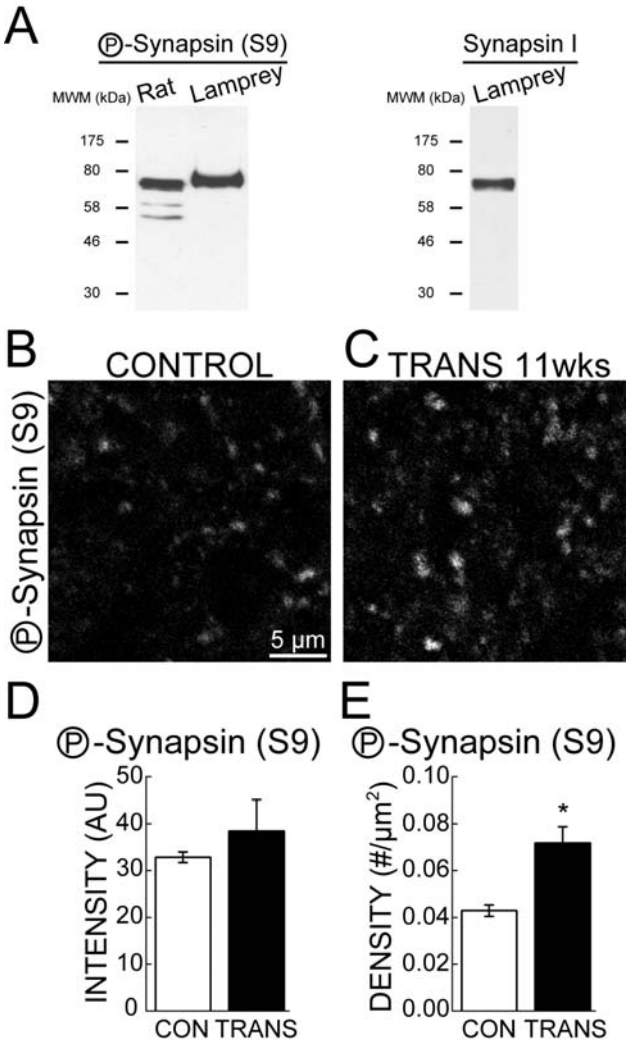

Figure 2.5. Phospho-synapsin (Ser9) levels increase in lamprey hindbrain after spinal cord injury.

(A, left) Western blot for phospho-synapsin (Serine 9; S9) in rat and lamprey brain lysates. (Right) Western blot for total synapsin I in lamprey brain lysates. Note that the phospho-synapsin (S9) antibody recognized a single protein of the correct molecular weight for synapsin I in lamprey brain lysates. (B, C) Confocal images showing phospho-synapsin (S9) in lamprey hindbrain. At 11 weeks post-transection, more structures containing phospho-synapsin (S9) were observed than in the control brains. Scale bar applies to both panels. (D, E) While the average intensity of phospho-synapsin (S9)-positive structures remained unchanged after spinal cord transection (D), the density of phospho-synapsin (S9)-positive structures statistically increased (E). Bars indicate mean ± S.E.M. for 429-635 puncta (intensity) or 14-16 regions of interest (density) measured from 4 animals. Asterisks indicate statistical significance (*T-test; $p<0.05$*).

relative to controls (Fig. 2.5C, E) (*Control*: 0.043 ± 0.002/μm^2, n = 16 ROIs, 4 animals; *Transected*: 0.072 ± 0.007/μm^2; n = 14 ROIs, 4 animals; $p < 0.05$; *T-test*). The increased density of phospho-synapsin (Ser 9) in the brain began as early as 6 weeks post-transection (Appendix Figure A1.2), and phospho-synapsin (Ser 9) expression was also significantly increased at regenerated RS synapses at 11 weeks post-transection (Appendix Figure A1.3.1) but it was not significantly altered by PKA activation (Appendix Figure A1.3.2).[3] No significant difference in the intensity of phospho-synapsin (Ser 9) positive puncta was detected at 11 weeks post-transection (Fig. 2.5C-D) (*Control*: 32.9 ± 1.1 AU, n = 429 puncta, 4 animals; *Transected*: 38.5 ± 6.6 AU, n = 635 puncta, 4 animals; $p > 0.05$; *T-test*). Thus, spinal cord transection increased the number of structures positive for phospho-synapsin (Ser 9), which is suggestive of enhanced neurite sprouting (Kao et al., 2002).

2.3.5 Spinal cord injury has little effect on the ultrastructural features of synaptic boutons in lamprey brain

Next, we examined the ultrastructural changes in lamprey brain that might be correlated with increased synapsin I and phospho-synapsin (Ser 9) levels. We focused on a brain region where injury-induced increases in synapsin I and phospho-synapsin (Ser 9) were observed, specifically within the neuropil located between the bulbar neuron B3 and the Mauthner neuron (Fig. 2.6, boxes). Within these sites, a synaptic bouton was defined as any structure containing a synaptic vesicle cluster. A neurite was defined as any structure that lacked a synaptic vesicle cluster and predominantly contained microtubules

[3] This was not part of the original Lau et al., 2011 publication.

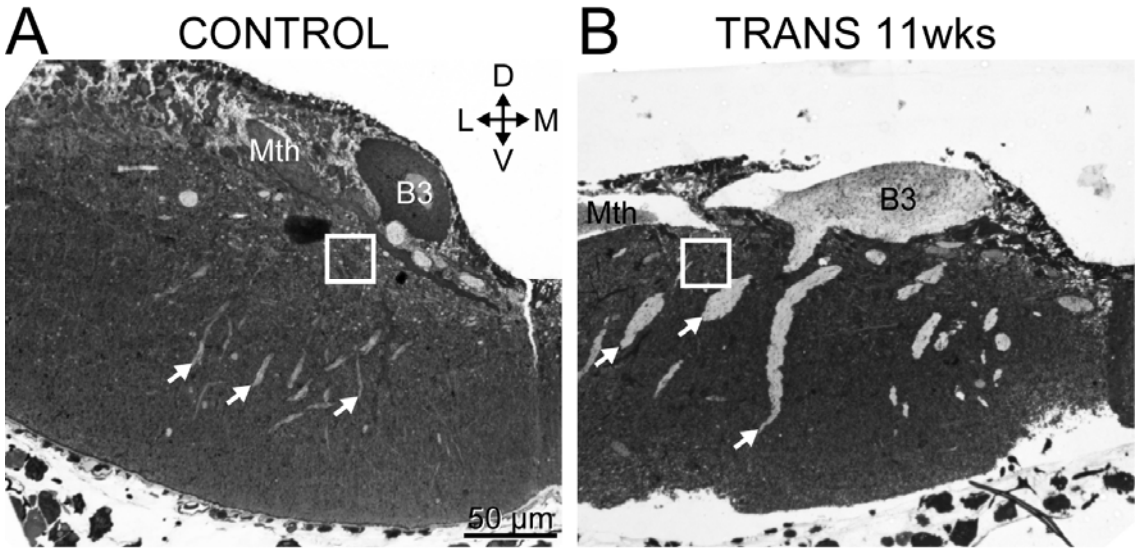

Figure 2.6. Identification of hindbrain regions used for ultrastructural analysis.
(A, B) Nissl stained transverse sections of the left hemisphere of the hindbrain from a control lamprey (A) and a spinal transected lamprey at 11 weeks (B). For the remaining ultrastructural analyses, the neuropil between B3 bulbar cell and Mauthner (Mth) was examined (box), as was done for the immunofluorescence analyses. Several large dendrites of the giant RS neurons are visible (arrows). Dorsal (D), ventral (V), lateral (L), medial (M) orientations are indicated. Midline of brain is to right of each image. Scale bar applies to both panels.

and/or intermediate filaments, which are characteristic of axons and dendrites. The presumed neurites also lacked the tightly-packed (~17 nm apart) intermediate filaments that are characteristic of keratin-rich glial processes, which have been well described in lamprey spinal cord (Lurie et al., 1994; Merrick et al., 1995; Pijak et al., 1996). Any alterations in the ultrastructural features in this location could theoretically represent changes in the dendrites of axotomized giant RS neurons and/or sprouting of small neurites belonging to unaxotomized neurons.

At 11 weeks post-transection, the average size (circumference) of synaptic boutons in the hindbrain remained similar to that observed in control brains (Fig. 2.7A-C) (*Control*: 2.8 ± 0.1 μm, n = 54 boutons, 2 lampreys; *Transected*: 3.0 ± 0.1 μm, n = 50 boutons, 2 lampreys; $p > 0.05$; *T-test*). Likewise, the average number of synaptic vesicles per bouton remained unchanged after spinal cord transection in the hindbrain region examined (Fig. 2.7A-B, D) (*Control*: 69.1 ± 6.3, n = 54 boutons, 2 lampreys; *Transected*: 66.1 ± 10.0, n = 50 boutons, 2 lampreys; $p > 0.05$; *T-test*). The density of synaptic boutons also remained altered (Fig. 2.7E) (*Control*: 0.22 ± 0.02/μm^2, n = 21 ROIs, 2 lampreys; *Transected*: 0.25 ± 0.02/μm^2, n = 20 ROIs, 2 lampreys; $p > 0.05$; *T-test*). Thus, the number, size, and ultrastructural features of synaptic boutons themselves were not drastically changed at 11 weeks after spinal cord transection, suggesting that increased synapsin expression was not drastically affecting the structures of mature synapses at least within the hindbrain regions examined.

2.3.6 Spinal cord injury induces neurite sprouting in the brain

Next, we examined the ultrastructural features of the neurites within the same lamprey hindbrain regions. Compared to control brains, the brains of spinal transected lampreys appeared to have more neurites that were smaller in size (Fig. 2.8A-D). Indeed, by 11 weeks post-transection, there was a 43% increase in neurite density, which was significantly different from control values (Fig. 2.8E) (*Control*: 4.9 ± 0.2/μm^2, n = 21 images, 2 lampreys; *Transected*: 7.0 ± 0.2/μm^2, n = 20 images, 2 lampreys; $p < 0.05 \times 10^{-8}$; *T-test*). As well, the average size (circumference) of the neurites was significantly reduced by 13% in the brains of spinal-transected lampreys in comparison to controls (Fig. 2.8F) (*Control*: 1.1 ± 0.02 μm, n = 2521 neurites, 2 lampreys; *Transected*: 0.96 ± 0.01 μm, n = 3437 neurites, 2 lampreys; $p < 0.05 \times 10^{-6}$; *T-test*). After spinal cord transection, there were more neurites across a range of sizes in the lamprey hindbrain with the greatest increase being in a population of small neurites that were ~0.5-1.0 μm in size (circumference) (Fig. 2.8G). Thus, spinal cord transection induced neurite sprouting in the lamprey brain, a finding that is consistent with our observations of increased numbers of synapsin I- and phospho-synapsin (Ser 9) positive puncta (Fig. 2.4-2.5).

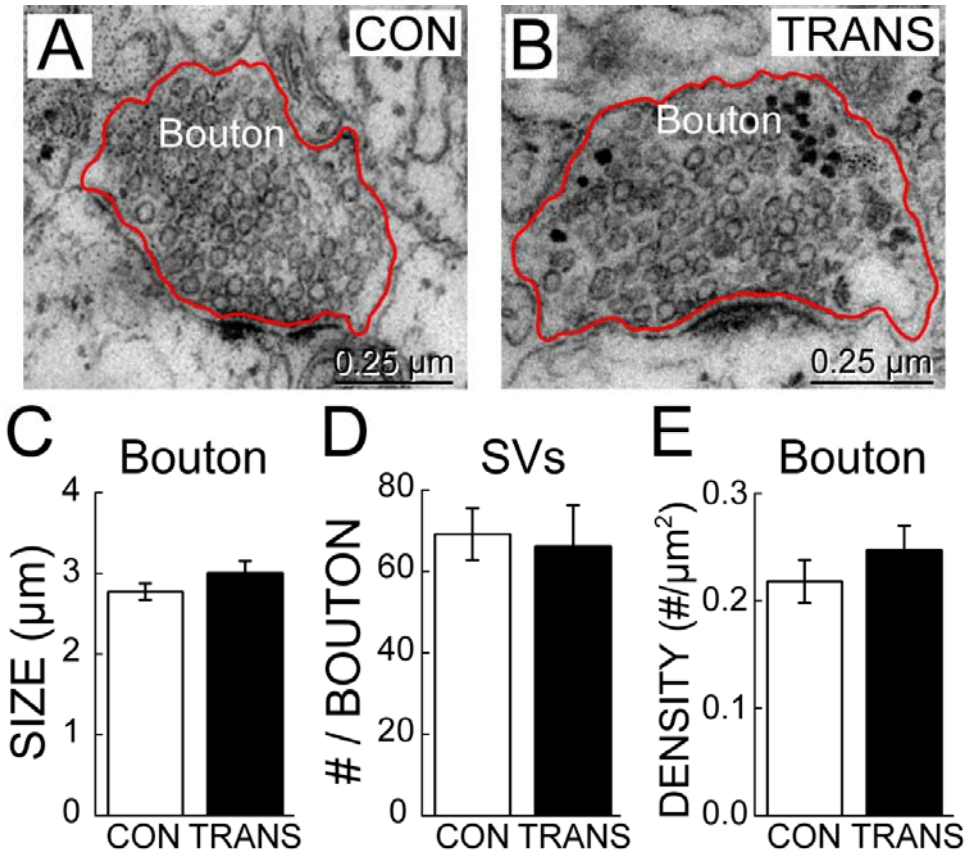

Figure 2.7. Synaptic structures are unchanged within the lamprey hindbrain regions examined after spinal cord injury.

(A, B) Electron micrographs showing representative synaptic boutons in hindbrain of control lampreys (A) and transected lampreys at 11 weeks post-transection (B). Red line indicates the boundary of the synaptic bouton. (C-E) Neither the bouton size (C) nor the number of synaptic vesicles (SVs) per bouton (D) nor the bouton density in the neuropil (E) were altered after transection. Bars indicate mean ± S.E.M.

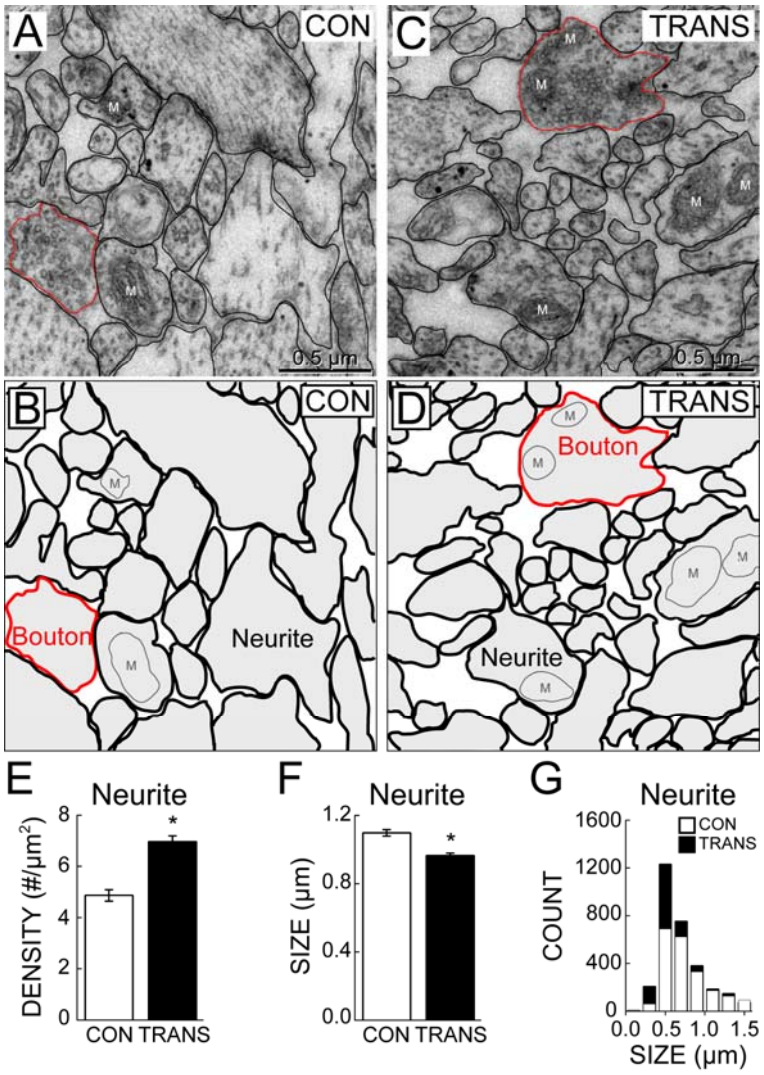

Figure 2.8. Neurite density is increased in lamprey hindbrain after spinal cord injury.

(A-D) Representative electron micrographs and schematic drawings showing the neuropil in the lamprey hindbrain regions examined. (A, B) In the control brain, many neurites (black lines) and a few boutons (red lines) are present. (C, D) In comparison, at 11 weeks post-transection, this hindbrain region contains more neurites, many of which are smaller in size. M = mitochondria. (E-G) At 11 weeks post-transection, neurite density increased (E), and the average neurite size decreased (F). Bars indicate mean ± S.E.M. Asterisks indicate statistical significance (*T-test; $p<0.05$*). Most of the additional small neurites were ~0.5-1.0 µm in circumference (size) (G).

2.4 DISCUSSION

These data are the first to demonstrate neurite sprouting in the lamprey brain following spinal cord injury. These findings are consistent with the anatomical and functional reorganization observed in mammalian brain after spinal cord injury (Jain et al., 1997; Jain et al., 2000; McKinley et al., 1987). Our data showing upregulation of

functional reorganization observed in mammalian brain after spinal cord injury (Jain et al., 1997; Jain et al., 2000; McKinley et al., 1987). Our data showing upregulation of synapsin I and increased phospho-synapsin (Ser 9) together with neurite sprouting strongly support a role for synapsin in this injury-induced anatomical plasticity (Fig. 2.9).

2.4.1 Injury-induced neurite sprouting in the brain and similarities with developmental processes

A well-accepted idea about the compensatory anatomical plasticity that occurs in response to spinal cord injury is that it likely shares some molecular mechanisms with developmental processes (Harel and Strittmatter, 2006). Here, we showed that spinal cord injury caused an increase in the expression of both synapsin I and phospho-synapsin (Ser 9) (Figs. 2.2-2.5), which was concomitant with enhanced neurite sprouting (Fig. 2.8). These findings are consistent with the known developmental roles for synapsin I and phospho-synapsin (Ser 9) in neurite formation, branching and extension. Thus, the data are consistent with the idea that injury-induced anatomical plasticity involving

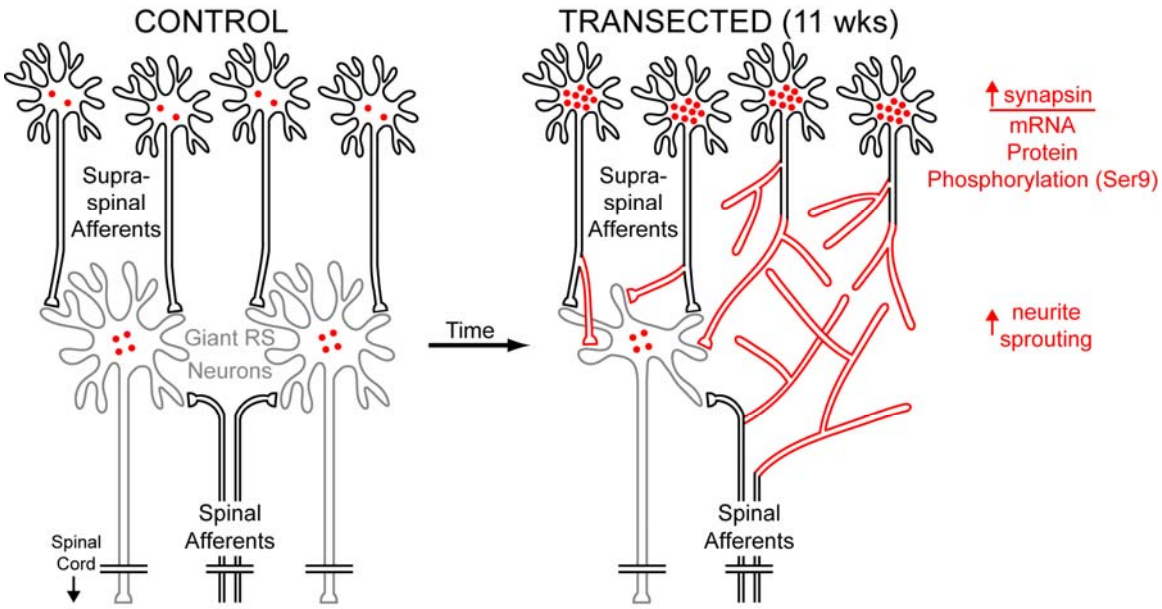

Figure 2.9. Current working model of injury-induced plasticity in lamprey brain.
In control brains, giant RS neurons receive inputs from both supraspinal and spinal afferents, and expression levels of synapsin are modest (red dots). In the brains of spinal transected lampreys (11 weeks post-transection), some giant RS neurons have died or retracted their dendritic branches. The current working model is that this loss of target neurons induces upregulation of synapsin (mRNA and protein), increased phospho-synapsin (Ser 9), and increased neurite sprouting by afferent neurons within the brain and spinal cord. One possible functional consequence includes strengthening of synaptic connections with the remaining giant RS neurons so that the output to spinal cord remains robust.

synapsins has recapitulated developmental processes at least to some degree. Although the precise mechanisms by which increased synapsin could cause such morphological changes in the number and sizes of neurites remain unknown, rearrangements of actin cytoskeleton and enhanced membrane addition have been implicated in other models of synapsin-mediated plasticity (Han and Greengard, 1994; Kao et al., 2002; Menegon et al., 2006).

Going forward, it will be valuable to identify the extent to which other developmental mechanisms are utilized during neurite sprouting in the brain after spinal cord injury. Using lamprey, one can attempt to identify other evolutionarily conserved pathways that may be involved. Several candidates include the axon guidance molecules such as semaphorins, netrins, RGM, and their receptors, whose mRNA expression levels have been shown to change in response to spinal cord injury in lampreys and mammals (Harel and Strittmatter, 2006; Pasterkamp et al., 1999; Shifman and Selzer, 2000, 2007; Shifman et al., 2009). Another conserved pathway with the potential to influence neurite sprouting includes generation of cAMP and subsequent PKA activation, which enhances axon regeneration across invertebrate and vertebrate species, including lampreys (Ghosh-Roy et al., 2010; Jin et al., 2009; Piper et al., 2007; Qiu et al., 2002). Indeed, in the current study, increased levels of phospho-synapsin (Ser 9), which was coincident with neurite sprouting, is suggestive of increased PKA activity since PKA-mediated phosphorylation of serine 9 on synapsin mediates neurite sprouting in other

developmental contexts (Kao et al., 2002). Although not yet examined in lampreys, adhesion molecules L1, integrin, and cadherins are other developmental molecules that contribute to axon regeneration in other vertebrates and therefore have the potential to influence neurite sprouting in the brain (Becker et al., 2004; Blackmore and Letourneau, 2006).

2.4.2 Anatomical pathways in brain that may be sprouting after spinal cord injury

At present, the origins of the additional synapsin-positive puncta and neurites that emerge in hindbrain after spinal cord injury remain unknown. However, the increased levels of synapsin I and phospho-synapsin (Ser 9) indicates that a good portion of the additional structures are axonal in nature, given the predominant localization of synapsins to axonal varicosities and synapses in both developing and mature neurons (De Camilli et al., 1983; Fletcher et al., 1991; Gitler et al., 2004). Since synapsins may also play additional roles in the structural development of dendrites (Ferreira et al., 1996), it is possible that some of the additional neurites observed could be dendritic in nature. Given the current information available, we cannot rule out the possibility that some of the additional "neurites" observed after injury may actually be glial elements. On one hand, the additional processes we observed contained microtubules and intermediate filaments with spacing that is more characteristic of axons and dendrites than of the tightly-packed spacing of intermediate filaments in glial cells described in lamprey spinal cord (Lurie et al., 1994; Merrick et al., 1995; Pijak et al., 1996). On the other hand, the ultrastructural features of glia in lamprey brain have not been described. Therefore, it is still possible

that glial cell sprouting or proliferation occurred, and this will need to be addressed directly in future experiments.

Though the anatomical pathways that are sprouting have yet to be identified, it is unlikely that the neurite sprouting originates from the axotomized giant RS neurons themselves. Previous studies reported that complete spinal cord transection at the level of the 5th gill (i.e. our injury model) did not cause dendritic sprouting in giant RS neurons, but instead caused a swelling, retraction, and elimination of the dendrites (Hall and Cohen, 1988; Shifman et al., 2008). Dendritic sprouting only occurred when the giant RS neurons were axotomized very close to the cell body (< 1 mm), and under these conditions the dendritic sprouts were relatively large and very sparse (Hall and Cohen, 1988). By our estimates, the dendrites of giant RS neurons range from ~1.5 μm in diameter (or ~4.7 μm in circumference) in control lamprey brains up to ~5-10 μm in diameter (or ~16-31 μm in circumference) in the brains of transected lampreys (Fig. 2.6, arrows). Thus, the dendrites of giant RS neurons, in particular after injury, tend to be quite a bit larger than the small neurite sprouts (~0.5-1.0 μm in circumference) that emerged abundantly in hindbrain after spinal cord injury (Fig. 2.8). As a consequence, we think it is highly unlikely that neurite sprouts emerging in hindbrain after spinal cord injury are originating from the giant RS neurons themselves.

Instead, we currently favor the possibility that afferents to the RS neurons are sprouting in response to spinal cord injury (Fig. 2.9). Synaptic contacts are assuredly disrupted when the dendrites of giant RS neurons undergo retraction or elimination (Hall and Cohen, 1988) or when the subpopulation of giant RS neurons dies in response to

axotomy induced by spinal cord transection (Shifman et al., 2008). Loss of giant RS neurons or their dendrites could therefore stimulate supraspinal afferent neurons in the brain to rearrange or re-route their axonal inputs to strengthen connections with remaining RS neurons (Fig. 2.9). Another possibility is that some of the additional neurites observed after injury originated from rostrally-projecting spinal afferents that regenerated back into the hindbrain and subsequently sprouted (Armstrong et al., 2003; Buchanan, 2001; Yin and Selzer, 1983). Given the robust increase in synapsin levels and neurite sprouting, it is somewhat surprising that we did not observe any changes in the density or other ultrastructural features of synapses (Fig. 2.7). However, it is possible that the additional neurites were still in transit to their final destinations or that additional synapses were formed in other locations that were not examined (Fig. 2.9). Identifying the precise anatomical pathways that give rise to the additional neurites, as well as their final destinations, remain open questions for future experiments.

2.4.3 Potential functional outcomes resulting from increased synapsin levels and neurite sprouting

The highly conserved nature of synapsin function at vertebrate and invertebrate synapses allows us to speculate about the possible outcomes of increased levels of synapsin I and phospho-synapsin (Ser 9) in the lamprey hindbrain (Fig. 2.4-2.5). We observed increased synapsin I immunofluorescence intensity at synapses after spinal cord transection (Fig. 2.4C-D). This was not due to a recruitment of more synaptic vesicles to synapses, because the number of vesicles per bouton did not change (Fig. 2.7A-B, D). Instead, there may be greater levels, or tighter packing, of synapsin protein within

individual synapses. At mature and developing synapses, synapsin I positively regulates the availability of synaptic vesicles (Gitler et al., 2008; Pieribone et al., 1995; Ryan et al., 1996), transmitter release kinetics (Hilfiker et al., 1998), and synaptic plasticity (Rosahl et al., 1993). PKA-mediated phosphorylation of synapsin at serine 9 enhances synaptic strength (Menegon et al., 2006) and vesicle recycling (Chi et al., 2001). Thus, although the functional consequences of increasing synapsin I and phospho-synapsin (ser 9) levels after injury remain unknown, synaptic strengthening through any of these mechanisms is possible.

The functional consequences of increased synapsin and neurite sprouting after injury may include strengthening of synaptic connections or synaptic rewiring, leading to enhanced behavioral recovery (Ballermann and Fouad, 2006; Blesch and Tuszynski, 2009; Rich and Wenner, 2007; Weidner et al., 2001). In lamprey, this compensatory plasticity could lead to sustained output or increased drive from the remaining giant RS neurons to the spinal cord (Fig. 2.9), potentially contributing to behavioral recovery of locomotion (Cohen et al., 1986; Cooke and Parker, 2009; Davis et al., 1993; Oliphint et al., 2010). Ultimately, the strong correlation between increased levels of synapsin and neurite sprouting suggests that synapsin may be a key positive regulator of injury-induced plasticity and that its potential could be harnessed for promoting maximal recovery from spinal cord injury.

Chapter 3: cAMP promotes cellular plasticity and survival in lampreys after spinal cord injury

3.1 INTRODUCTION

Damaged axons in the central nervous system typically exhibit a poor capacity to regenerate after spinal cord injury, as demonstrated in many vertebrate studies. In monkeys and rats, many axotomized corticospinal neurons grow axons toward the lesion site but do not regenerate across it (Brock et al., 2010; Liebscher et al., 2005; Pearse et al., 2004). In zebrafish, axotomized Mauthner neurons also do not regenerate across the lesion site (Becker et al., 1997; Bhatt et al., 2004). In addition to the lack of axon regeneration, spinal cord injury also induces death of the axotomized neurons. For example, axotomized corticospinal neurons of the rat primary motor cortex undergo apoptosis after spinal cord injury (Feringa et al., 1983; Hains et al., 2003). In addition, axotomy of the goldfish Mauthner axons induces death of these brain neurons (Zottoli et al., 1984). Furthermore, transection of the lamprey spinal cord induces cell death of a distinct subset of the axotomized giant reticulospinal (RS) neurons (Busch and Morgan, 2012; Shifman et al., 2008). Taken together, the lack of axon regeneration combined with the injury-induced cell death can impede functional recovery. Therefore, a major goal of spinal cord injury research is to identify novel ways to promote axon regeneration and prevent cell death.

Recent studies in both vertebrates and invertebrates have found that increasing cyclic adenosine monophosphate (cAMP) in axotomized neurons promotes their ability to regenerate. For example, in rats, elevation of cAMP by a combined application of a

cAMP analog, dibutyryl-cAMP (db-cAMP), and rolipram, an inhibitor of phosphodiesterase, increases axon regeneration into and beyond the Schwann cell grafts placed at the injury site (Pearse et al., 2004). Injection of db-cAMP into axotomized zebrafish Mauthner neurons promotes their axons to extend across the lesion site (Bhatt et al., 2004). In lampreys, bath application of db-cAMP onto the transected spinal cords prevents axon retraction and increases axon regeneration towards the lesion site at 2-4 weeks post-injury (Jin et al., 2009), when transected lampreys begin to recover some locomotion (Davis and McClellan, 1993; Oliphint et al., 2010; Selzer, 1978). Finally, axotomized sensory neurons of *C. elegans* re-grow their axons after treatment with forskolin (Ghosh-Roy et al., 2010), a compound that activates adenylyl cyclase, which converts ATP to cAMP. Thus, elevating cAMP is an evolutionarily conserved mechanism for enhancing axon growth after spinal cord injury. However, the cellular and molecular mechanisms by which cAMP enhances axon regeneration are not well understood.

In other context, specifically during development, cAMP is known to regulate biological pathways essential for normal physiological functions of neurons. For example, in mammalian culture studies, elevation of cAMP prolongs the survival time of the developing brain and spinal neurons (Hanson et al., 1998; Li et al., 2000; Meyer-Franke et al., 1995; Rydel and Greene, 1988). In addition, cAMP promotes axon branching of the developing mammalian cultured neurons (Rumajogee et al., 2006; Weeks et al., 1991). cAMP also regulates the expression of genes that promote axon growth during development (Sanna et al., 1991; Tartaglia et al., 2001). One of these

genes is synaptotagmin, a transmembrane synaptic vesicle protein that regulates both exo- and endocytosis at mature synapses (Sudhof and Rizo, 1996; Yao et al., 2012). During neuronal development, synaptotagmin is enriched on growth cone vesicles that fuse with the plasma membrane, and therefore may be responsible for membrane addition as axons elongate (Ide, 1996; Igarashi et al., 1997; Mikoshiba et al., 1999). Overexpression of synaptotagmin in PC12 cells lines enhances the length of growing neurites (Fukuda and Mikoshiba, 2000). cAMP positively modulates the protein level of synaptotagmin by interacting with the brain-derived neurotrophic factor (BDNF) pathway in hippocampal slice cultures (Tartaglia et al., 2001). Though cAMP is known to induce such positive effects on developing neurons, the extent to which cAMP promotes neuronal survival, axon branching and enhances expression of axon growth-promoting proteins during spinal cord regeneration is entirely unknown.

Here, we used the central nervous system of the lampreys to identify the cellular and molecular events regulated by cAMP that might underlie the cAMP-mediated enhancement of axon regeneration. Lampreys provide the ideal model for addressing these questions. First, spinal cord injury in lampreys induces spontaneous axon regeneration of both ascending and descending neurons, which includes ~50% of the descending projection giant RS neurons (Armstrong et al., 2003; Davis and McClellan, 1993; Oliphint et al., 2010; Rovainen, 1976; Selzer, 1978; Yin and Selzer, 1983). In addition, approximately 40% of the regenerated giant RS axons branch new axons, 30% of which branch in the rostral portion of the spinal cord (Oliphint et al., 2010). Moreover, about 25% of regenerated RS axons turn rostrally towards the brainstem

(Oliphint et al., 2010). Second, cAMP has been shown in lampreys to prevent RS axon retraction and promote RS axon growth towards the lesion site after spinal cord transection at 2-4 weeks post-injury (Jin et al., 2009), indicating that the cAMP-mediated enhancement of axonal growth occurs in lamprey. Third, the growing tips of the regenerating RS neurons are large and can be readily identified by 2 weeks post-transection (Jin et al., 2009). And fourth, while there are axotomized giant RS neurons that survive the injury at the time of behavioral recovery, there is a distinct subset of them that tends to undergo delayed cell death (Busch and Morgan, 2012; Shifman et al., 2008). Taken together, the robust anatomical and cellular changes induced by spinal cord injury and cAMP make lampreys an ideal model to identify the cellular and molecular mechanisms in the cAMP-mediated enhancement of axon regeneration.

Here, we report that cAMP increased the number of regenerated axons across the lesion site at 11 weeks post-injury. This increased axon regeneration was paralleled by a greater number of surviving giant RS neurons in the brain. Moreover, regenerating giant RS axons with elevated cAMP projected with fewer branches and rostral turns. The cAMP-enhanced axon regeneration also coincided with a significant increase of synaptotagmin protein at the growing axon tips. Taken together, our results indicate that the cAMP-enhanced axon regeneration beyond the lesion site is conserved in lampreys. In addition, cAMP activates at least some molecular pathways that are similar to neuronal development processes for enhancing axon regeneration.

3.2 METHODS

3.2.1 Spinal cord transections, db-cAMP application, and brain and spinal cord dissections

Late larval stage sea lampreys (Petromyzon marinus; 11-14 cm) housed at room temperature (RT, 25ºC) were used for all experiments. For spinal cord transection, lampreys were anaesthetized using MS-222 (0.1 g/L tank water; Argent Labs, Redmond, WA). Complete spinal transections were made at the level of 5th gill, as previously described (Jacobs et al., 1997; Lau et al, 2011; Oliphint et al., 2010). Dibutyryl-cAMP (db-cAMP; 100 mM; Enzo Life Sciences, Farmingdale, NY), dissolved in lamprey Ringer solution (in mM: 91 NaCl, 2.1 KCl, 2.6 CaCl2, 1.8 MgCl2, 4 glucose, 0.5 glutamine, 2 HEPES, pH 7.4), was applied to the spinal cord at the site and time of transection via a 1x1x1 mm piece of Gelfoam (Pharmacia & Upjohn Company, Kalamazoo, MI). Controls received a piece of Gelfoam soaked in Ringer solution. At the appropriate recovery time points, lampreys were re-anaesthetized, and the brains and spinal cords were dissected for further experimentation. All procedures were approved by the Institutional Animal Care and Used Committee at The University of Texas at Austin in accordance with standards set by the NIH.

3.2.2 Axon regeneration and image analysis

Axon regeneration studies were performed either at 2 weeks or 11 weeks post-transection to determine axon growth or regeneration across the lesion site, respectively. To assess axon growth at 2 weeks post-transection, dextran tetramethylrhodamine (DTMR; 10kDa; 5 mM; Molecular Probes, Eugene, OR) dissolved in lamprey Ringer

was also included in the Gelfoam applied at the time of transection. DTMR allowed for the examination of all cut axons. Ringer-treated controls (n = 16) and db-cAMP-treated lampreys (n = 16) were used for this experiment. For axon regeneration studies, spinal-transected lampreys at 11 weeks post-transection were re-anaesthetized and a second transection was made more rostrally at the level of the 2nd gill for the purpose of labeling regenerating axons. Gelfoam containing 10 mM dextran fluorescein (DF; 3kDa; Molecular Probes, Eugene, OR) dissolved in Ringer solution was applied at the newly transected site, and the lampreys were allowed to recover for an additional 3 days at RT. This method of labeling allowed for the examination of all axons that have the potential to regenerate across the original lesion site. These will include both ascending and descending regenerated axons. Ringer-treated controls (n = 5) and db-cAMP treated lampreys (n = 5) were used for this experiment.

To image labeled axons, dissected un-fixed spinal cords were pinned onto a sylgard dish containing Ringer solution. Z-stacks of confocal images were immediately obtained using a Zeiss Pascal Exciter attached to an Axioskop 2FS (10X, 0.3 NA EC Plan-NEOFLUAR; Carl Zeiss, Thornwood, NJ), using identical acquisition settings for lampreys treated with or without db-cAMP. For the 2-week experiment, three projection images were generated from the z-stacks: one contained the lesion site and two images contained the immediate proximal region of the spinal cord, where the retracting and regenerating giant RS axons were located. For the 11-week experiment, projection images were taken to include the lesion site and at least 10 mm of the distal spinal cord. All projections were stitched together using Adobe Photoshop 10.0 (Adobe Systems Inc.,

San Jose, CA) and represented as a single image (Fig. 3.1A, B, D). For the 11-week images, only the first 5 mm of the distal spinal cord is shown (Fig. 3.1D). Using ImageJ (NIH), the distance between axon tips and the lesion site was measured at 2 weeks post-transection. At 11 weeks post-transection, the number of regenerated axons was counted at 1 mm interval, up to 10 mm distal to the lesion site. To do this, the distance was measured distally away from the lesion site using ImageJ. At each marked distance, a line perpendicular to the spinal cord was drawn, and all the labeled axons were counted. The measurements would also include both small and giant axons of ascending and descending projecting neurons. All statistical analysis and graphs were generated using OriginPro 7.0 (OriginLab, Northampton, MA).

3.2.3 Immunofluorescence and image analysis

Immediately following axon imaging, spinal cords of 2 weeks post-transected lampreys were fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4) overnight at 4 ºC. Following a previously published immunostaining protocol (Lau et al., 2011), longitudinal sections of lamprey spinal cords (sectioned at 20 μm) were incubated with a synaptotagmin mouse monoclonal antibody (mAb 48; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IO) at 1:100 working concentration for 2 hours at RT, followed by incubation with AlexaFluor® 488-conjugated anti-mouse antibody (1:300; Invitrogen, Carlsbad, CA) for 1 hour at RT. To determine the level of synaptotagmin expression at axon tips, z-series of images were acquired for each axon tip using a Zeiss Pascal Exciter attached to an Axioskop 2FS (40X, 1.3 NA EC Plan-

NEOFLUAR, oil immersion lens; Carl Zeiss) and then merged into a 3D projection using Zeiss LSM Image Browser v4.0 software. The center image of each axon tip was identified. Then, a region of interest was drawn around the perimeter of the axon tip from which the average intensity measurement was obtained using ImageJ. All statistical analyses and graphing were performed using Origin Pro 7.0 (OriginLab).

3.2.4 Nissl staining and neuronal survival analysis

Nissl staining was performed using a previously published protocol (Lau et al., 2011). Briefly, fixed brains were stained with toluidine blue O (EM Sciences, Hatfield, PA), washed with Bodian's fixative, dehydrated in 95% and 100% ethanol, cleared in cedar wood oil and coverslipped onto slides with Permount (EM Sciences, Hatfield, PA). Images were acquired using a Leica DFC420C camera attached to a Leica MZ10F stereoscope (Leica Microsystems Inc., Bannockburn, IL). Assessment of neuronal survival of the giant reticulospinal (RS) neurons was performed as previously described (Busch and Morgan, 2012; Shifman et al., 2008). Briefly, ImageJ (NIH) was used to measure the average Nissl staining intensity of 34 giant RS neurons found in the lamprey midbrain and hindbrain. Background measurements were obtained from the surrounding neuropil immediately adjacent to the cell, and subtracted from the intensity measurements of the cells. Giant RS neurons that exhibited Nissl intensity greater than or less than the adjacent neuropil were considered as Nissl (+) cells or Nissl (-) cells, respectively. Ringer-treated control lampreys (n = 8) and db-cAMP treated lampreys (n = 7) were used

for this experiment. All statistical analyses and graphs were generated using Origin Pro 7.0 (OriginLab).

3.2.5 Axonal microinjection and image analysis

As previously described (Oliphint et al., 2010), giant RS axons of Ringer-treated and db-cAMP-treated spinal cords at 4 weeks post-injury were microinjected with 1 mM dextran fluorescein (Molecular Probes) dissolved in lamprey internal solution (180 mM KCl, 10 mM HEPES, pH 7.4). Immediately after microinjection, Z-stacks of confocal images were obtained using a Zeiss Pascal Exciter attached to an Axioskop 2FS with 10X and 40X objectives (40X, 0.8 NA Zeiss Achroplan water-dipping objective; Carl Zeiss) and then merged into a 3D projection using Zeiss LSM Image Browser v4.0 software. The numbers of axons that branched and turned rostrally were visually counted, and represented as the percentage of total injected axons exhibiting those properties. Graph was generated using Origin Pro 7.0 (OriginLab).

3.2.6 Western blotting

Western blotting experiments were performed following a previously published protocol (Lau et al., 2011). Briefly, 15 µg of lamprey and rat brain extracts were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. Membrane was incubated with synaptotagmin antibody at a concentration of 1:1000 at RT. The synapotagmin antibody recognizes a 65 kDa band in the lamprey brain extracts that is similar in molecular weight as that of rat brain extracts and is the predicted size for synaptotagmin (Fig. 3.5A).

3.3 RESULTS

3.3.1 db-cAMP increases axon regeneration across the lesion site after spinal cord injury

A previous study showed that elevating cAMP at the time of spinal cord transection in lampreys enhanced axon growth towards the lesion site at 2 weeks post-injury (Jin et al., 2009). However, it remained unknown whether this cAMP-enhanced axon growth is persistent and leads to axon regeneration across the lesion site at longer post-transection time points, for example at 11 weeks post-injury when locomotor behavior is fully restored (Cohen et al., 1986; Davis and McClellan, 1993; Oliphint et al., 2010). To test this, we applied 100 mM db-cAMP at the time and site of transection, along with a fluorescent dye (dextran tetramethyl-rhodamine or dextran fluorescein) to label severed axons, and assessed axon regeneration at both 2 and 11 weeks post-injury. Consistent with the previously published results at 2 weeks post-injury, the distance between the axon tips and the lesion site was significantly shorter in the db-cAMP-treated spinal cords, compared to Ringer-treated controls (Fig. 3.1A-C; *-dbcAMP*: 521.4 ± 35.5 μm, n = 101 axons; *+dbcAMP*: 418.0 ± 27.5 μm, n = 96 axons; $p = 0.023$; *T-test*). Thus, elevation of cAMP at the time of spinal cord transection either prevented axon retraction and/or promoted axon growth. Similarly, at 11 weeks post-injury, cAMP enhanced axon

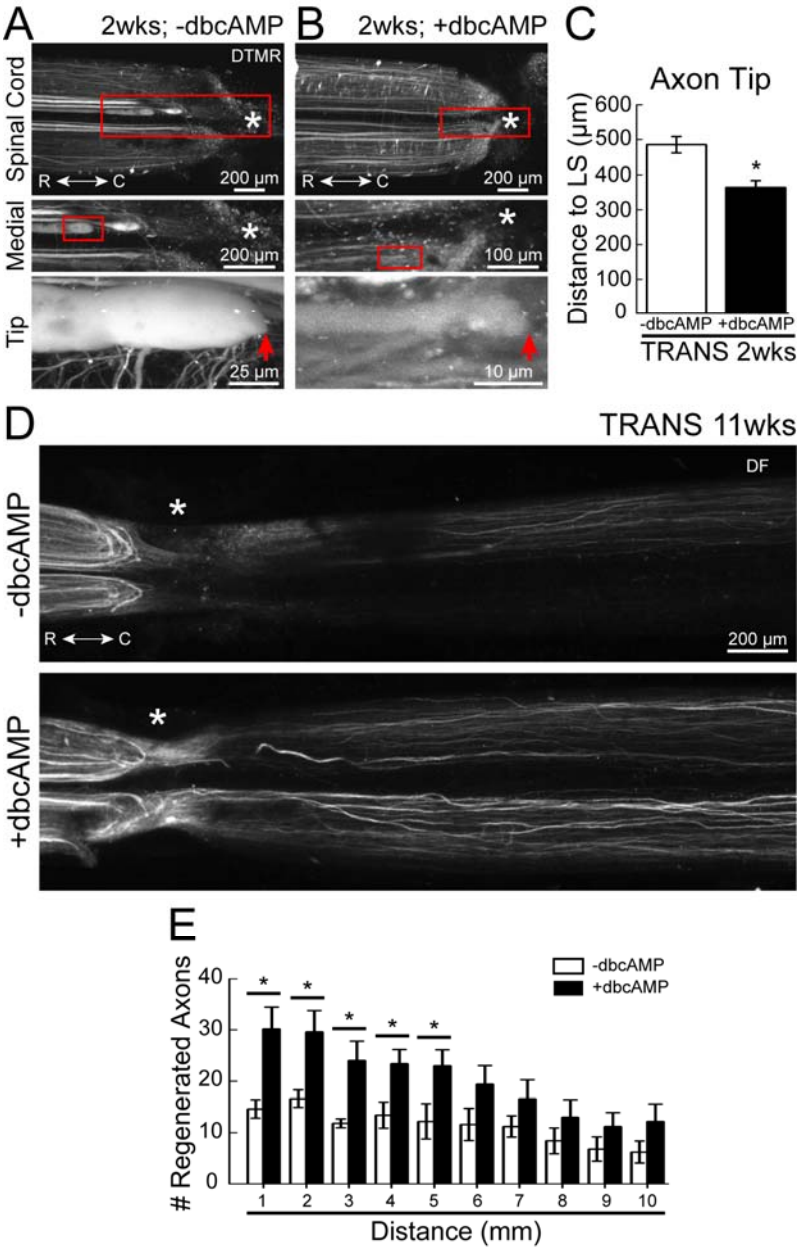

Figure 3.1. cAMP enhances axon regeneration in lampreys after spinal cord injury. (A-B) Confocal projections showing dextran-tetramethyl-rhodamine (DTMR)-labeled spinal axons of Ringer- (A; -dbcAMP) and db-cAMP-treated (B; +dbcAMP) spinal cords at 2 weeks post-injury (2wks). Top panel images show all labeled spinal axons rostral to the lesion site (asterisk). Regions boxed in red are enlarged in the middle panel images to show the axons of the medial tract (Medial) and the lesion site. Axon tips (red boxes) are further enlarged in the bottom panel images for better visualization. Red arrows point to the axon tip. R = rostral, C = caudal. (C) At 2 weeks post-injury (TRANS 2wks), the distance between the axon tips and the lesion site (LS) was significantly shorter after db-cAMP treatment, compared to Ringer-treated controls. (D) Confocal projections showing dextran-fluorescein (DF)-labeled regenerated axons distal to the lesion site (asterisk) of Ringer- treated and db-cAMP-treated spinal cords at 11 weeks post-injury (TRANS 11wks). Compared to control spinal cord, db-cAMP increases the number of labeled axons in the distal spinal cord. Scale bar applies to both images. (E) db-cAMP significantly increased the number of regenerated axons at least 5 mm beyond the lesion site. (C, E) Bars represent mean ± S.E.M. Asterisks indicate statistical significance (*ANOVA; $p<0.05$*).

regeneration across the lesion site, compared to Ringer-treated controls (Fig. 3.1D). This was apparent due to the presence of many more labeled axons that grew into the distal spinal cord. A statistical analysis revealed that db-cAMP significantly increased the number of regenerated axons as far as 5 mm beyond the lesion site (Fig. 3.1E) (n = 5 transected lampreys for each condition; $p < 0.05$; *ANOVA*). Compared to Ringer-treated controls, db-cAMP significantly increased the number of regenerated axons by 107% at 1 mm beyond the lesion site, and by 78% at 2 mm, 103% at 3 mm, 75% at 4 mm, and 89% at 5 mm. Moreover, small increases in the number of regenerated axons were also apparent up to 10 mm beyond the lesion site of the db-cAMP-treated spinal cords (Fig. 3.1E). Taken together, cAMP enhanced axon regeneration toward and across the lesion site in lamprey spinal cord.

There are several possibilities that might explain how cAMP enhances axon regeneration after spinal cord injury. One possibility is that cAMP might increase the number of surviving axotomized neurons, resulting in a larger pool of axons available for regeneration. A second possibility is that cAMP might alter projection patterns of regenerating giant RS axons. A third possibility is that cAMP might increase the levels of some molecular components that promote growth at the axon tip, such as synaptotagmin. Therefore, we set out to test these possibilities in order to gain a better understanding of the cellular and molecular mechanisms by which cAMP supports enhanced axon regeneration.

3.3.2 db-cAMP increases overall survival of axotomized giant RS neurons after spinal cord injury

First, we explored the possibility that cAMP increased the survival of the identified giant reticulospinal (RS) neurons after spinal cord injury. Within the lamprey brain, 17 pairs of identified giant RS neurons (total of 34 cells) are found in the midbrain and hindbrain (Fig. 3.2A-B). These cells include the Müller neurons of the mesencephalic (M), isthmic (I) and bulbar (B) brain regions, as well as the Mauthner (Mth) and auxillary Mauthner (mth') neurons. Some of the giant RS neurons undergo delayed cell death after a complete spinal cord transection, while others reproducibly survive and regenerate their axons (Busch and Morgan, 2012; Jacobs et al., 1997; Shifman et al., 2008). To determine the effect of cAMP on neuronal survival or death after spinal cord injury, we applied 100 mM db-cAMP at the time of transection and allowed the transected lampreys to recover for 11 weeks. At 11 weeks post-injury, brains of Ringer- and db-cAMP-treated lampreys were Nissl stained, a method for reliably determining whether giant RS neurons are healthy or degenerated (Busch and Morgan, 2012; Shifman et al., 2008). Healthy giant RS neurons exhibit a dark blue, uniform Nissl stain and are classified as "Nissl (+) cells". RS neurons that are undergoing delayed cell death have a swollen appearance and exhibit a reduced or complete loss of Nissl substance, and are classified as "Nissl (-) cells". At 11 weeks post-injury, brains of Ringer-treated control lampreys contained giant RS neurons that were Nissl (-), indicating neurodegeneration (Fig. 3.2C). The Nissl (-) neurons were located throughout the different brain regions (Fig. 3.2C), including the hindbrain (Fig. 3.2E). In contrast,

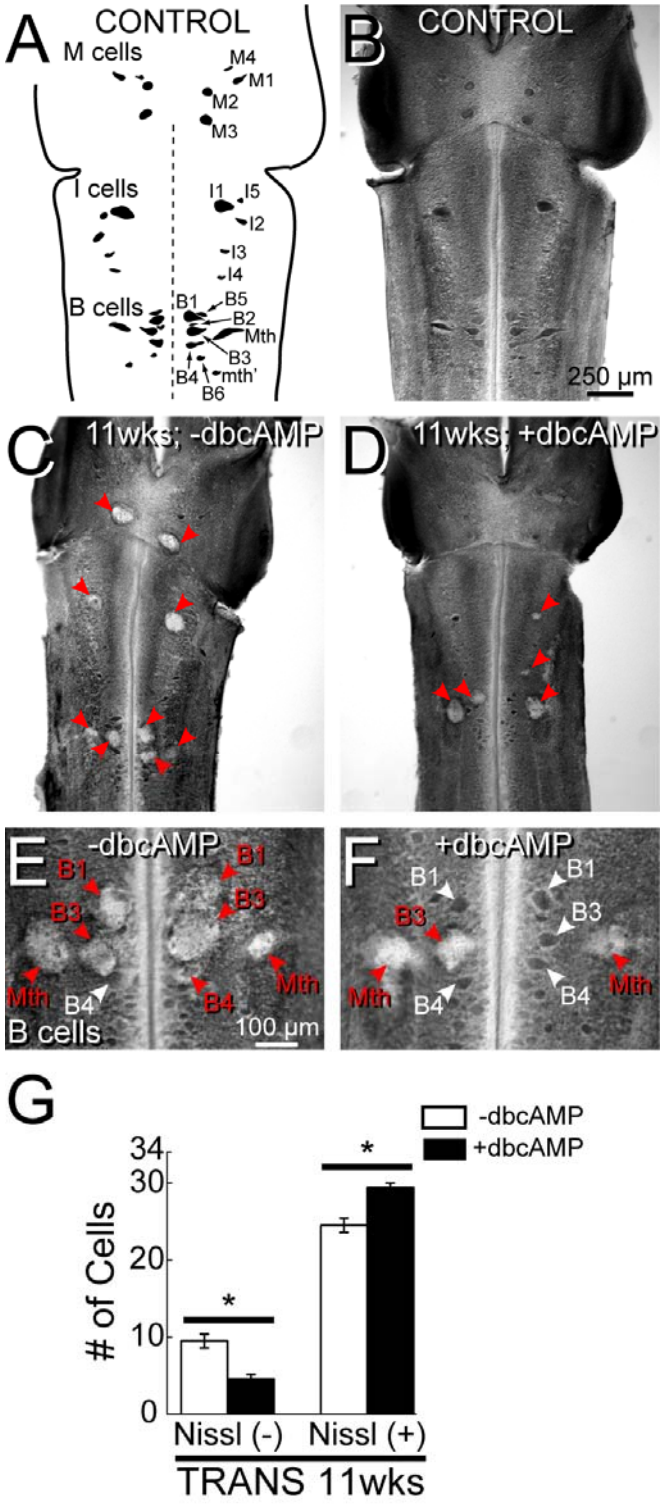

Figure 3.2. cAMP promotes overall cell survival in lamprey brain after spinal cord injury. (A) Diagram showing the individual giant RS neurons located in the mesencephalic (M), isthmic (I) and bulbar (B) brain regions. (B) Nissl-stained brain of an uninjured control lamprey. Dark staining represents Nissl substance. Scale bar also applies to C and D. (C-F) At 11 weeks post-injury (11wks), brains of Ringer-treated lampreys (-dbcAMP) have Nissl (-) cells (red arrowheads) and Nissl (+) cells (white arrowhead) (C, E). In contrast, brains of db-cAMP-treated lampreys (+dbcAMP) have fewer Nissl (-) and more Nissl (+) cells (D, F). Scale bar in E also applies to F. (G) At 11 weeks post-injury (TRANS 11wks), db-cAMP significantly decreased the number of Nissl (-) cells (left), and significantly increased the number of Nissl (+) cells (right). Bars represent mean ± S.E.M. Asterisks indicate statistical significance (*T-test; $p<0.05$*).

brains of db-cAMP-treated lampreys had fewer Nissl (-) RS neurons and more Nissl (+) RS neurons (Fig. 3.2D, F). The treatment of db-cAMP significantly reduced the number of Nissl (-) neurons per brain by 48% and concomitantly increased the number of Nissl (+) neurons by 20% (Fig. 3.2G, Nissl (-): *-dbcAMP*: 9.5 ± 0.9 cells, n = 8 animals; *+dbcAMP*: 4.6 ± 0.6, n = 7 animals; $p = 6.6 \times 10^{-4}$; *T-test*; Nissl (+): *-dbcAMP*: 24.5 ± 0.9 cells, n = 8 animals; *+dbcAMP*: 29.4 ± 0.6 cells, n = 7 animals; $p = 6.6 \times 10^{-4}$; *T-test*). Thus, elevating cAMP level at the time of spinal cord transection enhanced the number of giant RS neurons that survived up to 11 weeks post-injury.

After injury, there are giant RS neurons that have a high tendency to survive the injury and regenerate their axons (Busch and Morgan, 2012; Shifman et al., 2008). These "good survivors" retain strong Nissl staining [Nissl (+)] > 80% of the time at 11 weeks post-injury under control (-dbcAMP) conditions. In contrast, there is a distinct subset of giant RS neurons that has a high tendency to undergo delayed cell death after the injury. These "poor survivors" are defined as those cells that retain strong Nissl staining < 40% of the time at 11 weeks post-injury under control (-dbcAMP) conditions. Next, we determined whether cAMP selectively improved the survival of the good survivors, poor survivors, neither or both at 11 weeks post-injury. In the brains of Ringer-treated control lampreys, many giant RS neurons tended to be Nissl (+) throughout the midbrain and hindbrain (Fig. 3.3A-B) (Fig. 3B; *-dbcAMP*: n = 12 cells per cell type). In this dataset, these "good survivors" were the M1, M2 and M4 cells in the midbrain, as well as I2-I5, B2, B4-B6 and auxillary Mauthner cells in the hindbrain. In addition, there were some giant RS neurons that tended to lose Nissl staining (Fig. 3.3A-B). In this dataset, these

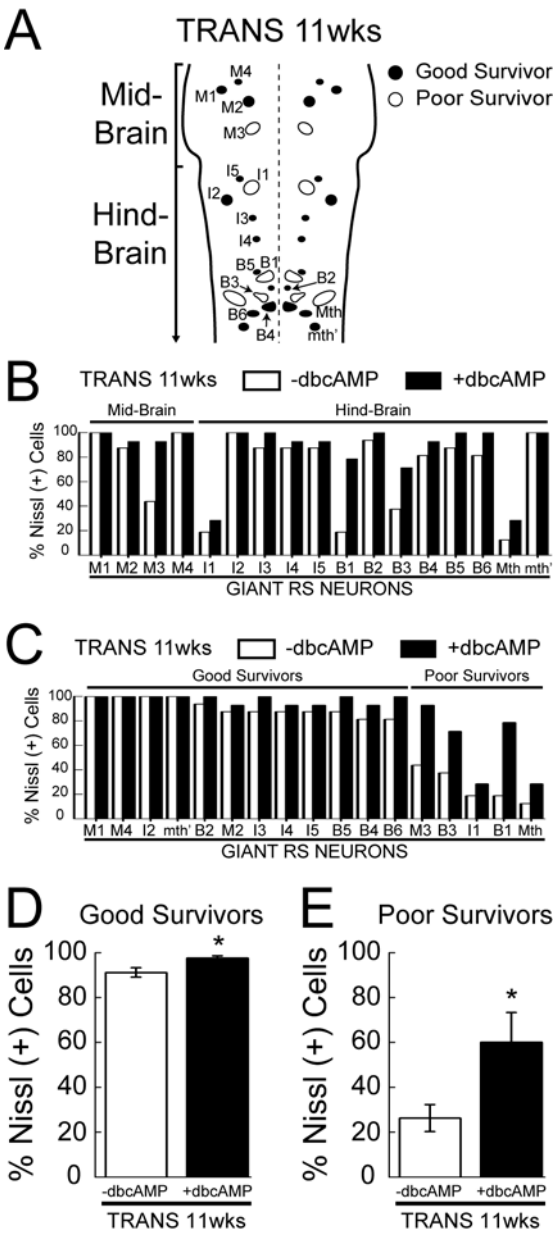

Figure 3.3. cAMP promotes survival of both "good survivors" and "poor survivors" after spinal cord injury.
(A) Diagram showing the "good survivors" (filled circles) and "poor survivors" (opened circles) located in midbrain and hindbrain at 11 weeks post-injury (TRANS 11wks). (B) At 11 weeks post-injury, throughout the midbrain and hindbrain of Ringer-treated controls (-dbcAMP), there are individual cell types that, as a population, have a high tendency to retain Nissl substance, and those that have low tendency to retain Nissl substance. db-cAMP increases the propensity for many cell types to retain Nissl substance (+dbcAMP). (C) Cell types are ranked order based on their propensity to lose Nissl substance in brains of Ringer-treated lampreys, and are categorized as "good survivors" and "poor survivors." (B-C) Bars represent % of population for each cell type with Nissl substance. (D-E) Compared to the brains of Ringer-treated controls, db-cAMP significantly increased the number of Nissl (+) cells of both "good survivors" (D) and "poor survivors" (E). Bars represent mean ± S.E.M. Asterisks indicate statistical significance (*T-test; $p<0.05$*).

"poor survivors" were the M3 cells in the midbrain as well as I1, B1, B3 and Mauthner cells in the hindbrain. Strikingly, db-cAMP increased the tendency for most of the "good survivors" and "poor survivors" to retain Nissl substance (Fig. 3.3B; *+dbcAMP*: n = 10 cells per cell type). To statistically compare the survival effect of db-cAMP have on the good and poor surviving giant RS neurons, all 17 giant RS cell types were rank ordered by their propensity to lose Nissl substance in the brains of Ringer-treated lampreys (Fig. 3.3C). Compared to Ringer-treated controls, db-cAMP significantly increased the survival of "good survivors" by 7% (Fig. 3.3D; *-dbcAMP*: 91.1 ± 2%, n = 12 cell types; *+dbcAMP*: 97.6 ± 1%, n = 12 cell types; $p = 0.011$; *T-test*). Moreover, db-cAMP also increased the survival of the "poor survivors" by 128% (Fig. 3.3E; *-dbcAMP*: 26.3 ± 6%, n = 5 cell types; *+dbcAMP*: 60.0 ± 13%, n = 6 cell types; $p = 0.050$; *T-test*). Taken together, these data indicated that elevating cAMP at the time of spinal cord transection promoted neuronal survival up to 11 weeks after spinal cord injury, thereby increasing the pool of neurons available to regenerate their axons.

3.3.3 db-cAMP alters growth patterns of regenerating giant RS axons after spinal cord injury

A previous study reported that injured giant RS axons regenerated with abnormal pattern of projections after spinal cord injury (Oliphint et al., 2010). Specifically, about 40% of the regenerated RS axons branched new axons, 30% of which branched in the rostral portion of the spinal cord. Moreover, about 25% of the regenerated RS axons turned rostrally and grew towards the brain stem. However, it remained unknown whether cAMP alters the projection patterns of regenerating giant RS axons. To test this,

at 4 weeks post-injury, I microinjected giant RS axons of Ringer-treated and db-cAMP-treated spinal cords with 1 mM dextran fluorescein (DF) and 4% horse radish peroxidase (HRP) in order to visualize the projection patterns of the regenerating giant RS axons. I chose the 4-week time point because it reflects mid-recovery of swimming behavior (Oliphint et al., 2010) and the injured giant RS axons are in the process of regeneration (Zhang et al., 2005). In Ringer-treated controls, some regenerating giant RS axons exhibited branching and rostral turning (Figure 3.4A, B). In contrast, regenerating giant RS axons of db-cAMP-treated spinal cords projected in a straighter path, without exhibiting branching and rostral turning (Figure 3.4C, D; *-dbcAMP*: *Branch*: 20%; *R-turn*: 10%; 10 axons, 4 animals; *+dbcAMP*: *Branch*: 0%; *R-turn*: 0%; 13 axons, 5 animals). Thus, elevating cAMP at the time of spinal cord transection promoted injured giant RS axons to grow in a straighter path, thereby, providing a larger pool of axons to regenerate across the lesion site.

3.3.4 db-cAMP increases synaptotagmin expression at axon tips after spinal cord injury

As shown here and by others (Jin et al., 2009), elevating cAMP decreased the distance between regenerating axon tips and the lesion site at 2 weeks post-injury, either by promoting axon growth and/or preventing axon retraction. This suggested that cAMP increased the levels of axon-growth proteins that were necessary for membrane addition as axons elongated. To test this, I examined synaptotagmin protein level at the regenerating axon tips at 2 weeks post-injury. I chose synaptotagmin because it has been

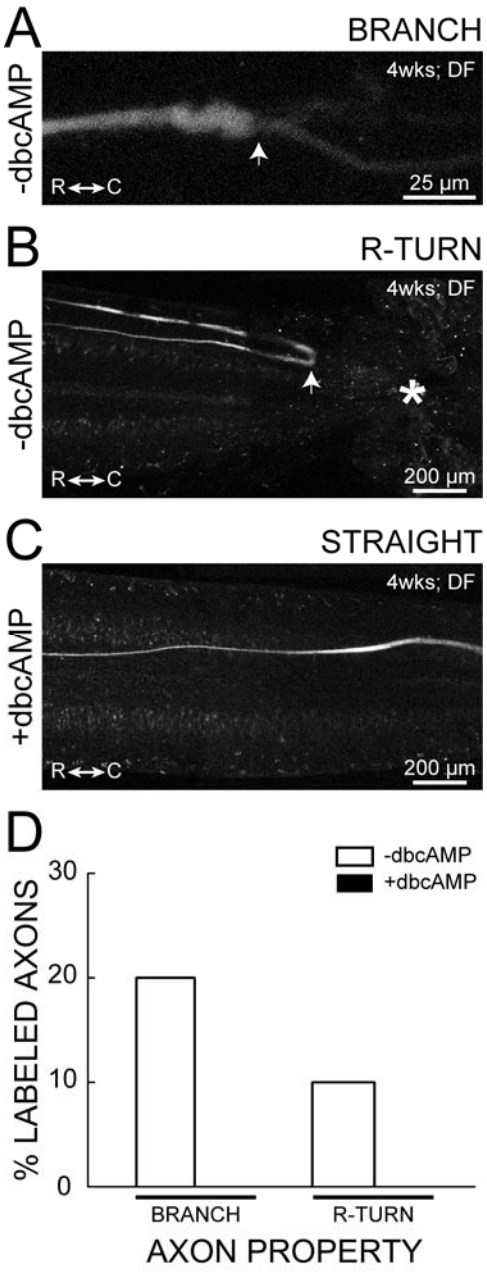

Figure 3.4. cAMP alters projection path of regenerating giant RS axons in lampreys after spinal cord injury.

(A-C) Confocal projections showing dextran-fluorescein (DF) labeled regenerating giant RS axons of Ringer-treated (A, B, -dbcAMP) and db-cAMP-treated (C, +dbcAMP) spinal cords at 4 weeks post-injury (4wks). Some regenerating giant RS axons of Ringer-treated controls branch new axons (A, arrow) or turn rostrally (B, arrow) away from the lesion site (asterisk). In contrast, regenerating giant RS axons of db-cAMP-treated spinal cords project in a straighter path (C). R = rostral, c = caudal. (D) Compared to Ringer-treated spinal cords, where some regenerating giant RS axons exhibited branching and rostral turning, regenerating giant RS axons of db-cAMP-treated spinal cords did not exhibit these properties. Bars represent % of total injected axons exhibiting each property for each treatment group.

shown to promote developmental neurite growth (Fukuda and Mikoshiba, 2000), and its protein expression is positively modulated by cAMP (Tartaglia et al., 2001). In addition, I have shown that the density of synaptotagmin-positive puncta was increased in the lamprey hindbrain at 11 weeks post-transection (Appendix Figure A1.4).[4] I chose the 2-week time point because regenerating axon tips can be reliably identify based on their distinct morphology. Unlike other vertebrate growth cones where many filopodia extrude from the growing axon ends, the regenerating axon tips of the lamprey have a sharply contoured morphology without apparent filopodia (Jin et al., 2009). The synaptotagmin antibody, which was made against the cytoplasmic domain of rat synaptotagmin I, detected a single band in lamprey brain extracts that was similar in molecular weight as that of rat brain extracts (Fig. 3.5A). Immunostaining of Ringer-treated transected spinal cords revealed a low fluorescent signal for synaptotagmin at the axon tips (Fig. 3.5B). In contrast, semi-quantitative analysis revealed that db-cAMP significantly increased the fluorescence associated with synaptotagmin at the axon tips by 2 folds, compared to Ringer-treated controls (Fig. 3.5C, D) (*-dbcAMP*: 19.4 ± 1.2 AU; n = 15 tips, 9 animals; *+dbcAMP*: 39.4 ± 6.8 AU, n = 17 tips, 8 animals; $p = 0.011$; *T-test*). Thus, elevating cAMP at the time of spinal cord transection increased synaptotagmin at the growing axon tips during axon regeneration.

[4] This was not part of the original Lau et al., 2011 publication.

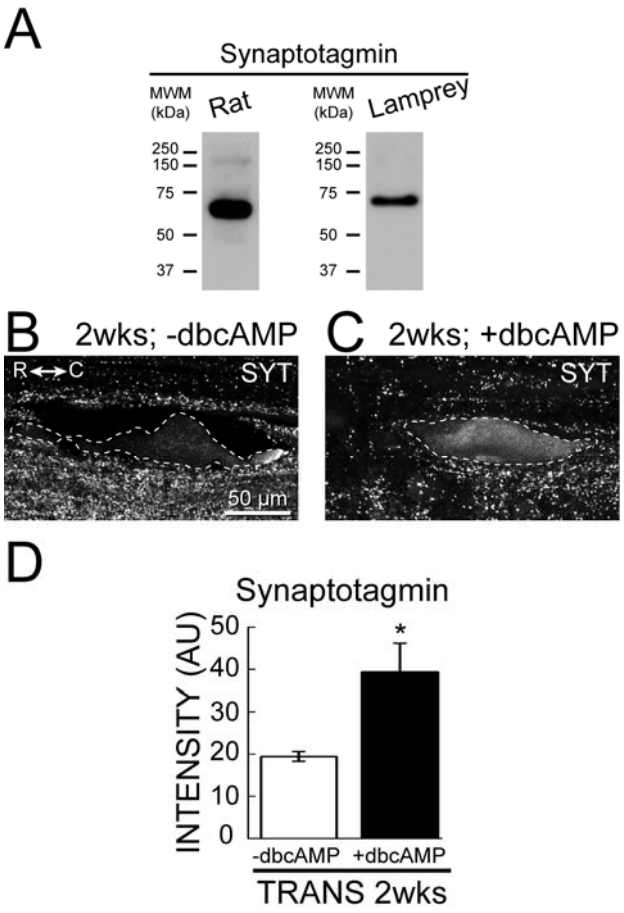

Figure 3.5. cAMP increases synaptotagmin protein level at regenerating axon tips in lampreys after spinal cord injury.

(A) Western blots for synaptotagmin in rat and lamprey brain extracts. Synaptotagmin antibody recognized a single protein band that was similar between rat and lamprey. Molecular weight markers (MWM), in kilodalton (kDa) are shown. (B-C) Confocal projections showing synaptotagmin protein (SYT) at regenerating axon tips of Ringer-treated (B; -dbcAMP) and db-cAMP-treated (C; +dbcAMP) spinal cords at 2 weeks post-injury (2wks). Fluorescent signal of synaptotagmin is detected at the axon tips (outlines) and the surrounding neuropil of the spinal cord. Treatment of db-cAMP increases synaptotagmin protein at the axon tips. R = rostral, C= caudal. Scale bar also applies to C. (D) Treatment of db-cAMP significantly increased the intensity of synaptotagmin at regenerating axon tips at 2 weeks post-transection (TRANS 2wks). Bars represent mean ± S.E.M. Asterisk indicates statistical significance (*T-test; $p<0.05$*).

3.4 DISCUSSION

The data presented here are the first to demonstrate that cAMP prevents cell death of axotomized neurons, promotes regenerating axons to elongate in a straighter path and increases synaptotagmin levels at the growing axon tips, all of which strongly support for the role of cAMP in enhancing axon regeneration after spinal cord injury. The data also show that cAMP enhances axon regeneration beyond the lesion site in lampreys after spinal cord injury.

3.4.1 cAMP enhances axon regeneration by regulating biological pathways similar to neuronal developmental pathways

The positive effects of cAMP represent a well conserved mechanism for enhancing axon growth and regeneration, which has been demonstrated in many species of developmental models (Aglah et al., 2008; Cai et al., 2001; Rumajogee et al., 2006; Weeks et al., 1991; Wong et al., 2002), as well as in spinal cord injury models (Bhatt et al., 2004; Ghosh-Roy et al., 2010; Pearse et al., 2004). Here, I showed that elevation of cAMP increased the number of regenerated axons across the lesion site in lamprey after spinal cord injury. This finding further highlights the conserved role of cAMP in enhancing axon regeneration after spinal cord injury.

Although the precise mechanism of how cAMP promotes axon regeneration after injury is unclear, findings from studies of neuronal development may provide several explanations. In mammalian studies using cultured neurons, elevation of cAMP promotes the survival of many types of neurons (Hanson et al., 1998; Li et al., 2000;

Meyer-Franke et al., 1995; Rydel and Greene, 1988), possibly by activating the anti-apoptic pathways such as the mitogen-activated protein kinase (MAPK; ERK1/2) (Troadec et al., 2002). These data suggest that elevating cAMP after spinal cord injury might activate anti-apoptic pathways to promote the survival of the axotomized neurons. One can test this hypothesis by determining the level of phosphorylated ERK1/2, the activated form of ERK1/2 (Troadec et al., 2002), in axotomized neurons that were treated with or without db-cAMP after spinal cord injury. One would expect a higher expression level of phospho-ERK1/2 in the surviving axotomized neurons treated with db-cAMP, compared to those that are dying/dead and without db-cAMP treatment. Our finding of cAMP-enhanced cell survival of a subset of axotomized neurons that have a high tendency to die is in agreement with the survival-promoting effect of cAMP.

During neuronal development, cAMP also positively modulates the protein levels of axon-growth genes, including synaptotagmin (Tartaglia et al., 2001). Synaptotagmin is enriched on growth cone vesicles (Ide, 1996; Igarashi et al., 1997; Mikoshiba et al., 1999), and its overexpression in cultured cells enhances the length of growing neurites (Fukuda and Mikoshiba, 2000). Our finding that cAMP-enhanced synaptotagmin protein at the regenerating axon tips is in agreement with the developmental role of synaptotagmin in axon elongation. The potential outcome of having more synaptotagmin at the growing axon tips is discussed in the next two sections. Going forward, identifying other cAMP-activated target pathways and genes that promote cell survival and axon elongation after spinal cord injury will provide insight for improving functional recovery.

3.4.2 Potential mechanisms for cAMP in promoting survival of axotomized neurons after spinal cord injury

At present, it is not clear as to how elevating cAMP at the time of spinal cord injury promotes survival of axotomized neurons. In addition to the cAMP-activated anti-apoptic pathways mentioned above as a possible mechanism, others also exist. Injury to the axon plasma membrane provides an immediate opening for extracellular molecules to enter the damaged cell. One of these molecules is calcium, which can become toxic and lethal to the injured neurons when overloaded within the damaged cell (Nguyen et al., 2005; Schlaepfer and Bunge, 1973). To prevent the overload of these molecules, the damaged axons repair the damaged site by recruiting vesicles towards the cut end to form a plasmalemmal seal (Bloom and Morgan, 2011). Such mechanism of repair has been demonstrated in cultured neurons of invertebrate (Eddleman et al., 1997; Krause et al., 1994) and vertebrate (Chakrabarti et al., 2003). Proper sealing is positively mediated by the cAMP pathway (Spaeth et al., 2010) and dependent on exocytic synaptic vesicle proteins, including synaptotagmin (Alderton et al., 1994; Detrait et al., 2000a; Detrait et al., 2000b). In addition, it has been suggested previously that faster plasmalemmal sealing of the damaged axons may prevent the death of the axotomized neurons (Nguyen et al., 2005; Spaeth et al., 2010). Taken together, our *in vivo* findings suggest that cAMP prevents the death of axotomized neurons by increasing synaptotagmin-mediated vesicle fusion at the cut end of the axon, thereby enhancing plasmalemmal sealing. These ideas should be tested to further understand the mechanism of how cAMP promotes cell survival after injury.

3.4.3 Potential mechanisms for cAMP-enhanced synaptotagmin expression at growing axon tips after spinal cord injury

At present, the mechanisms of how cAMP promotes axon regeneration are not clear. However, our data suggest at least one possible mechanism. The synaptic vesicle proteins that mediate plasmalemmal repair after injury are also important for axon elongation during normal neuronal development (Bloom and Morgan, 2011). In neuronal culture studies, all three major SNARE proteins (SNAP-25, synaptobrevin/VAMP and synataxin) and synaptotagmin positively mediate neurite extension by promoting fusion of vesicles with the growing end of the axon plasma membrane (Fukuda and Mikoshiba, 2000; Igarashi et al., 1996; Kimura et al., 2003; Morihara et al., 1999). In our study, the increased synaptotagmin expression at the regenerating axon tips suggests that vesicle fusion events are increased to promote axon growth, which is consistent with our findings that more regenerated axons are found distal to the lesion site. However, having more synaptotagmin at the regenerating axon tips could also reflect an increase in the number of vesicles available for fusion, an idea that has not been tested. A previous study has shown that normal regenerating axon tips contain vesicle-like inclusions (Jin et al., 2009). Whether elevating cAMP will increase these inclusions at the regenerating axon tips has not been explored. Assuredly, either an increase in vesicle fusion events or an increase in the number of available vesicles for fusion or both by cAMP application will result in more axon growth and regeneration.

Chapter 4: General Discussion and Future Directions

Studies from mammalian models of spinal cord injury have suggested that spontaneous recovery may be supported by anatomical plasticity, such as axon sprouting and regeneration of both injured and uninjured neurons located in the spinal cord and the brain (Fig. 1.1). Although the anatomical pathways that undergo injury-induced anatomical plasticity have been identified, the molecular mechanisms mediating such plasticity remain elusive. With my studies, I have now identified synapsin and phospho-synapsin (Ser 9) as well as synaptotagmin as potential molecular targets for mediating neurite sprouting after spinal cord injury (Fig. 4.1). In addition, I have determined for the first time that cAMP prevents the death of axotomized neurons and increases synaptotagmin levels at the regenerating axon tips, which are potential mechanisms for enhancing axon regeneration after spinal cord injury (Fig. 4.1). Taken together, my findings strongly suggest at least some genes that regulate neuronal development are activated to mediate anatomical plasticity after injury. In addition, my findings suggest that cAMP also activates some molecular pathways to prevent cell death after the injury. Further understanding of these injury-induced and cAMP-enhanced molecular pathways associated with anatomical plasticity will provide insight for improving recovery after spinal cord injury.

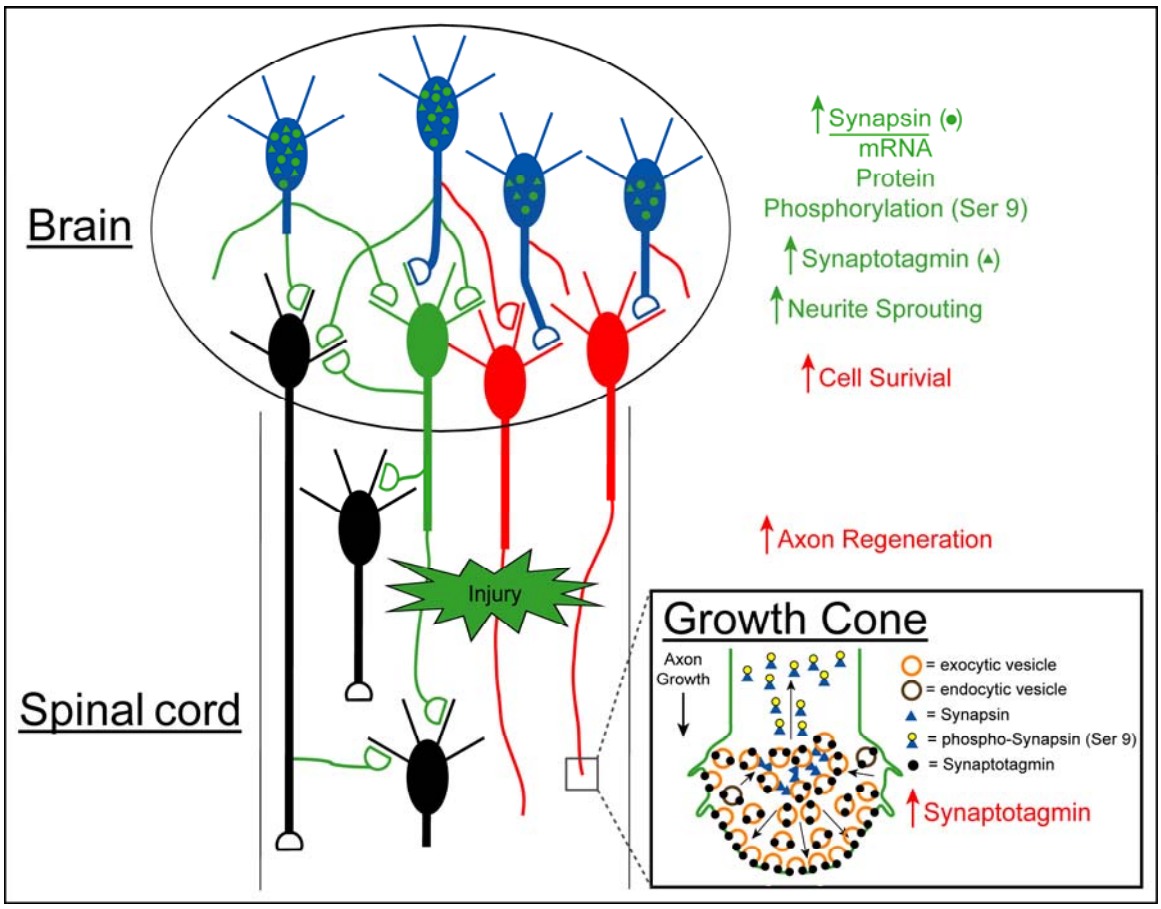

Figure 4.1. Model of injury-induced and cAMP-enhanced anatomical plasticity after spinal cord injury.

(Green) Spinal cord injury induces the upregulation of synapsin (mRNA and protein) as well as phospho-synapsin (Ser 9) and synaptotagmin protein levels in some of brain afferent neurons (blue), which results in neurite sprouting of these afferent neurons. Some of the sprouts can form functional synapses with injured (green) and uninjured (black) brain neurons, which may provide support for axon regeneration across the lesion site (Injury) and functional recovery. (Red) cAMP enhances axon regeneration across the lesion site. Current model of cAMP-enhanced axon regeneration is that cAMP promotes survival of brain neurons that have high tendency to die after injury (red), thereby, providing a larger pool of axons for regeneration. Cell survival may also prevent the loss of synaptic connections with afferent neurons, thereby, afferent neurons may produce fewer synapsin, synaptotagmin, and sprouting neurites and synapses. Preventing cell death may prevent maladaptive neurite sprouting as seen in primate model of spinal cord injury (Jain et al., 2000). cAMP also enhances synaptotagmin level at the growth cone, which may increase vesicle fusion events and axon elongation.

4.1 ANATOMICAL PATHWAYS IN THE BRAIN THAT MAY BE SPROUTING AND POSSIBLE MECHANISM FOR TRIGGERING SPROUTING AFTER SPINAL CORD INJURY

At present, it is not clear which neuronal populations are sprouting neurites in the lamprey hindbrain after spinal cord injury. As I have put forth a possible scenario in Chapter 2, it is unlikely that the new neurites are coming from the dendrites of giant RS neurons themselves. Instead, the potential candidates for neurite sprouting are the afferent neurons that make direct synaptic connections with the giant RS neurons (Fig. 2.9; 4.1). Going forward, the identity of the afferent neurons that are sprouting can be delineated by several methods. Based on my model, the afferent neurons that have sprouted axons will make more synapses with the surviving giant RS neurons. This idea can be tested by using electrophysiological techniques. Many of the biophysical properties of the synaptic connections between afferent neurons and the giant RS neurons of the uninjured lamprey have been well defined (Smetana et al., 2010; Viana Di Prisco et al., 2005; Zompa and Dubuc, 1996). Using these same stimulation protocols to stimulate the different groups of afferent neurons, a larger response will be expected in the surviving giant RS neurons if any of the brain nuclei has sprouted new axons.

Another method for identifying afferent neurons that are sprouting is the use of retrograde trans-synaptic labeling technique. One such technique is the use of pseudorabies virus (PRV; Barth strain), which has been successfully used for studying neuronal connectivity of the mammalian CNS (Aston-Jones and Card, 2000). The PRV virus can cross from the postsynaptic cell into the presynaptic cell at the synapse and retrogradely transported to the cell bodies. PRV particles can be detected using

immunohistochemistry. To identify the afferent neurons that are sprouting after spinal cord transection in lampreys, PRV can be injected into the surviving giant RS neurons after injury. Using immunohistochemistry, more PRV particles will be expected in the afferent neurons that have sprouted and made connections with the surviving giant RS neurons, compared to their counterparts in uninjured lampreys. By identifying which brain nuclei are sprouting, and further validating in higher vertebrate models of spinal cord injury will provide new insight for improving recovery.

It has been suggested that in some incidences, injury-induced neurite sprouting within the brain can be maladaptive. In monkeys, the face afferents sprout and make new connections with the axotomized brain neurons that normally respond to the hand and arm (Jain et al., 2000). This maladaptation will lead to malfunction of the neural circuits that regulate the hand and arm. This raises the question of how to prevent maladaptive neurite sprouting after spinal cord injury. To answer this question, one will need to identify the events that trigger afferent neurons (i.e. face neurons) to sprout neurites even though they are not axotomized after spinal cord injury. In this dissertation, I have provided one possible explanation in Chapter 2. One possible trigger for neurite sprouting is the loss of synaptic connections between the afferent neurons and the axotomized giant RS neurons that have undergone cell death. If the death of axotomized neurons is the trigger for the afferent neurons to sprout, then preventing them from dying after injury should reduce neurite sprouting (Fig. 4.1). Going forward, this idea can now be tested in lampreys. In this dissertation, I have identified for the first time in a spinal cord injury model that cAMP can prevent death of the axotomized giant RS neurons that

have a high tendency to die after injury. Many of these poor survivors are located in the hindbrain where neurite sprouting occurs (Fig. 3.3). If neurite sprouting is induced by these dying giant RS neurons, then preventing them from dying by elevating cAMP with an analog, db-cAMP, will result in fewer sprouts after spinal cord transection. These studies will provide a better understanding of how sprouting occurs in the brain, and provide insight for improving therapy to prevent maladaptations after spinal cord injury.

4.2 ANATOMICAL PLASTICITY AFTER INJURY UTILIZES SIMILAR MOLECULAR PATHWAYS THAT REGULATE NEURONAL DEVELOPMENT

It has been proposed that anatomical plasticity after spinal cord injury is likely to utilize similar molecular mechanisms that mediate neuronal development (Harel and Strittmatter, 2006). In this dissertation, I have provided evidence for the upregulation of two synaptic/developmental genes, synapsin and synaptotagmin, which coincided with neurite sprouting in the lamprey brain after spinal cord injury (Fig. 4.1). Synapsin (Chin et al., 1995; Ferreira et al., 1998; Ferreira et al., 2000; Han and Greengard, 1994) and synaptotagmin (Fukuda and Mikoshiba, 2000) are positive regulators of neurite growth during neuronal development. Interestingly, both of these genes are targets for the cAMP/PKA pathway (Bonanomi et al., 2005; Chi et al., 2001; Menegon et al., 2006; Tartaglia et al., 2001). I have also provided evidence that synaptotagmin is increased at the regenerating axon tips upon application of db-cAMP, and this increase in synaptotagmin level parallels the cAMP-enhanced axon regeneration. Since the cAMP/PKA pathway as well as synapsin, phospho-synapsin (Ser 9) and synaptotagmin are known to promote developmental neurite growth, my studies further support the idea

that genes regulating neuronal development are utilized for mediating anatomical plasticity after spinal cord injury (Fig. 4.1).

Going forward, it will be valuable to identify other developmental pathways as well as additional targets of cAMP that mediate anatomical plasticity after spinal cord injury. The lamprey provides a great model for identifying other evolutionary conserved developmental pathways in mediating anatomical plasticity. Using a gene candidate approach, other lamprey studies have successfully identified changes in mRNA levels of the conserved axon guidance molecules, including semaphorins and netrins, after spinal cord injury (Shifman and Selzer, 2000; Shifman and Selzer, 2007; Shifman et al., 2009). These findings are consistent with rodent studies, where spinal cord injury increases mRNA levels of semaphorins (De Winter et al., 2002) and netrins (Wehrle et al., 2005) at the lesion site. In this dissertation, I have identified the evolutionary conserved cAMP/PKA pathway, as well as its conserved targets [synapsin, phospho-synapsin (Ser 9) and synaptotagmin] as potential molecules for mediating anatomical plasticity. In addition to axon guidance molecules and the cAMP pathway, there is new evidence to suggest that the notch signaling pathway is involved in injury-induced plasticity and cell vitality, but the mechanisms mediating such plasticity and cell vitality remain unclear (Ables et al., 2011; Bejjani and Hammarlund, 2012; Dias et al., 2012; Hayes et al., 2007). As more sequence information becomes available for the lamprey genome, one can perform microarray analysis and/or transcriptome analysis to further elucidate other conserved developmental and cAMP-activated pathways that mediate anatomical plasticity after spinal cord injury (Smith et al., 2011).

4.3 POSSIBLE ROLES OF SYNAPSIN, PHOSPHO-SYNAPSIN (SER 9) AND SYNAPTOTAGMIN IN MEDIATING NEURITE SPROUTING

The roles for synapsin, phospho-synapsin (Ser 9) and synaptotagmin in mediating the injury-induced anatomical plasticity are not clear. However, one can speculate about their functions in the injury-context by evaluating their functional roles in neuronal development. During development, synapsins promote axon formation and elongation (Chin et al., 1995; Ferreira et al., 1998; Ferreira et al., 2000; Han and Greengard, 1994), which are dependent on the phosphorylation of serine 9 (Kao et al., 2002). Synaptotagmin mediates neurite elongation but not neurite formation (Fukuda and Mikoshiba, 2000). Taken together, these results suggest a differential role for synapsin/phospho-synapsin (Ser 9) and synaptotagmin in mediating neurite sprouting after spinal cord injury. After injury, synapsin and its phosphorylated form (Ser 9) might regulate the formation of axon sprouts and the elongation of the growing neurites. Synaptotagmin might specifically regulate the elongation of neurites, which is consistent with my findings that cAMP increased synaptotagmin level at the growing axon tips.

Going forward, the functional roles of synapsin and synaptotagmin in neurite sprouting and axon regeneration should be further examined. While the lamprey synapsins have been fully sequenced (Kao et al., 1999), the full length lamprey synaptotagmin genes have yet to be determined. However, this hurdle can be overcome as more sequence information becomes available for the lamprey genome. In addition, full length sequence of synaptotagmin can be cloned by performing Rapid Amplification of cDNA Ends (RACE) PCR, a method that has been used successfully in lamprey

studies (Busch and Morgan, 2012). Using the full length sequence information, one can design expression constructs and morpholinos to manipulate the expression levels of synapsin and synaptotagmin after spinal cord injury, and examine the consequences of anatomical plasticity. Using the giant RS axons as a model, expression constructs and morpholinos can be loaded into the newly axotomized giant RS axons via Gelfoam to overexpress and to knockdown protein levels, respectively, and assess the outcome of axon regeneration at the time of recovery. The loading of compounds into the lamprey giant RS axons has been demonstrated successfully in my studies (Chapter 3) as well as others (Davis and McClellan, 1993; Jin et al., 2009; Shifman et al., 2008; Zhang and McClellan, 1999). An expression construct for synapsin I can be loaded into axotomized RS axons to address whether increasing synapsin I protein level will further enhance regeneration and neurite sprouting, and improve recovery. In addition, expression constructs of synapsin with mutated serine 9 [serine to alanine to prevent phosphorylation (S9A) or serine to glutamate to mimic constitutive phosphorylation (S9E)] can be used to assess the significance of synapsin (Ser 9) in axon regeneration. If phospho-synapsin (Ser 9) is essential for injury-induced axon regeneration and sprouting, expressing synapsin (S9A) will be expected to reduce or prevent regeneration and sprouting, and expressing synapsin (S9E) will be expected to enhance regeneration and sprouting.

The overexpression and knockdown technique can also be used to address the significant roles of the different structural domains of synapsin and synaptotagmin in injury-induced anatomical plasticity. Previous studies have shown that the different domains of synapsin regulate different aspects of synaptic transmission: phosphorylation

of serine 9 in domain A results in enhanced synaptic activity (Chi et al., 2001; Menegon et al., 2006) and domain E modulates the kinetics of vesicle fusion (exocytosis) (Hilfiker et al., 1998). However, whether these domains differentially regulate injury-induced anatomical plasticity, such as the formation of new axon sprouts versus elongation of axons, is unknown. By determining the exact roles for each synapsin domain during axon regeneration and sprouting will provide a better understanding of the molecular mechanism of how synapsin mediates injury-induced anatomical plasticity. Deciphering the roles of synapsin/phospho-synapsin (Ser 9) and synaptotagmin in mediating anatomical plasticity after spinal cord injury will provide insight for improving recovery.

4.4 SUMMARY

In this dissertation, I have identified several key events and molecules in lampreys that coincide with anatomical plasticity after spinal cord injury. The lamprey brain undergoes neurite sprouting after spinal cord injury, which is consistent with findings in mammals (Jain et al., 2000). Parallel with neurite sprouting is the increased expressions of synapsin, phospho-synapsin and synaptotagmin, which are consistent with their evolutionary roles in mediating developmental neurite growth. In addition, elevating cAMP enhances axon growth and regeneration across the lesion site in lampreys, a mechanism that is consistently found in other species. Moreover, the cAMP-enhanced axon growth and regeneration correlate with the increased expression of synaptotagmin. This is also consistent with the conserved role of cAMP in modulating synaptotagmin expression level. Finally, elevating cAMP can promote survival of the axotomized giant

RS neurons after spinal cord injury, and this is consistent with the role of cAMP in promoting cell-survival in mammalian cell cultures. Taken together, lampreys provide a great tool for studying the molecular mechanisms mediating anatomical plasticity after spinal cord injury. The valuable information from these and future studies can be further validated in higher vertebrates, such as rodents, as a way to gain insight for improving recovery and developing new therapies for people suffering from spinal cord injury.

Appendix

A1 SUPPLEMENTAL DATA

A1.1 PKA phosphorylates lamprey synapsin at serine 9

PKA phosphorylates synapsin at serine 9 in other species (Czernik et al., 1987; Hosaka et al., 1999; Kao et al., 2002), but whether PKA phosphorylates synapsin in lampreys is unknown. To test this, dissected uninjured control lamprey brains were treated with either 1 mM H89 (PKA inhibitor; Enzo Life Sciences, Farmingdale, NY) or 10 mM db-cAMP (PKA activator) for 1 hour at RT. Separate sets of brains were treated with either DMSO or Ringer to serve as controls for H89 or db-cAMP, respectively. Western blotting experiment was performed using 2 μg of brain extracts along with antibodies against phospho-synapsin (Ser 9), total synapsin and tubulin (tubulin: working concentration = 1:2000; mouse monoclonal; Sigma-Aldrich, St. Louis, MO). Averaged intensity values were measured for each protein band using ImageJ. Inactivation of PKA with H89 significantly reduced the level of phospho-synapsin (Ser 9), compared to DMSO-treated controls (Fig. A1.1A, B) (*CON*: 113.1 ± 7.9 AU; n = 7 animals; *H89*: 64.0 ± 10.5 AU; n = 7 animals; $p = 0.003$; *T-test*). H89 did not significantly altered total synapsin level (Fig. A1.1A, C) (*CON*: 86.0 ± 8.9 AU; n = 6 animals; *H89*: 71.0 ± 18.0 AU; n = 6 animals; $p = 0.47$; *T-test*) or tubulin level (Fig. A1.1A, D) (*CON*: 108.2 ± 4.9 AU; n = 7 animals; *H89*: 104.8 ± 6.6 AU; n = 7 animals; $p = 0.69$; *T-test*). Activation of PKA with db-cAMP increased the level of phospho-synapsin (Ser 9), although a significant difference was not observed compared to Ringer-treated controls (Fig. A1.1E,

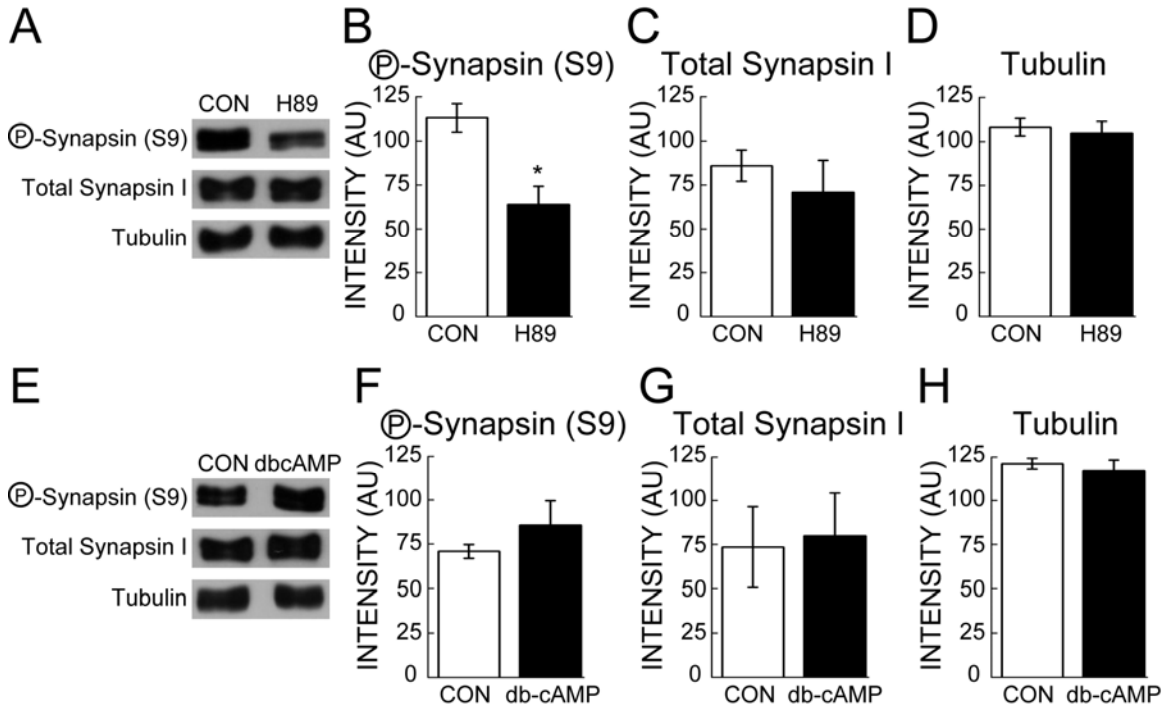

Figure A1.1. PKA phosphorylates lamprey synapsin at serine 9.
(A) Representative western blots for phospho-synapsin (Ser 9; S9), total synapsin I and tubulin in lamprey brain lysates treated with Ringer (CON) or H89. (B-D) H89 treatment significantly reduced phospho-synapsin (Ser 9) level (B) without significantly affecting the levels of total synapsin I (C) and tubulin (D). (E) Representative western blots for phospho-synapsin (Ser 9; S9), total synapsin I and tubulin in lamprey brain lysates treated with DMSO (CON) or db-cAMP. (F-H) db-cAMP increased phospho-synapsin (Ser 9) level (F) without affecting the levels of total synapsin I (G) and tubulin (H). Bars represent mean ± S.E.M. for 6-7 animals (H89) and 4 animals (db-cAMP). Asterisk indicates significant difference (*T-test*; $p<0.05$).

F) (*CON*: 70.9 ± 3.9 AU; n = 4 animals; *dbcAMP*: 85.7 ± 13.8 AU; n = 4 animals; p = 0.34; *T-test*). db-cAMP did not significantly altered total synapsin level (Fig. A1.1E, G) (*CON*: 73.5 ± 22.9 AU; n = 4 animals; *dbcAMP*: 79.9 ± 24.2 AU; n = 4 animals; p = 0.85; *T-test*) or tubulin level (Fig. A1.1E, H) (*CON*: 121.2 ± 3.0 AU; n = 4 animals; *dbcAMP*: 117.2 ± 6.1 AU; n = 4 animals; p = 0.58; *T-test*). Thus, PKA phosphorylated the lamprey synapsin at serine 9, an evolutionary conserved mechanism of PKA.

Elevating cAMP did not significantly increase the level of phospho-synapsin (Ser 9) by western blotting can be explained by the activation of cAMP-dependent phosphatase(s) that de-phosphorylates phospho-synapsin (Ser 9). It has been shown previously that protein phosphatase 2A (PP2A) de-phosphorylates phospho-synapsin (Ser 9) (Jovanovic et al., 2001), and the activity of PP2A is activated by cAMP (Feschenko et al., 2002). These findings suggest that co-incubation of control lamprey brains with db-cAMP and inhibitors of PP2A, such as okadaic acid (Feschenko et al., 2002), will enhance the level of phospho-synapsin (Ser 9).

A1.2 Phospho-synapsin (Ser 9) level begins to increase at 6 weeks post-transection in lamprey hindbrain after spinal cord injury

Chapter 2 showed that the phospho-synapsin (Ser 9) level was increased in the lamprey hindbrain at 11 weeks post-transection. However, it remained unknown when phospho-synapsin (Ser 9) level begins to increase after spinal cord transection. To determine this, hindbrain sections obtained from control as well as spinal transected lampreys at 1, 3, 6-7 (collectively called "6 wks"), 11 and 15-17 weeks (collectively called "16 wks") post-transection were immunostained with phospho-synapsin (Ser 9) antibody. These time points reflect paralysis (1 week), mid-recovery (3 weeks) and late recovery (6 weeks and beyond) after spinal cord transection. In uninjured control hindbrain, very few phospho-synapsin (Ser 9)-positive puncta were observed (Fig. A1.2A). After injury, no significant difference was detected in the intensity of phospho-synapsin (Ser 9)-positive puncta between all time points (Fig. A1.2A, B) (*CON*: 34.8 ± 2.5 AU, n = 950 puncta, 8 animals; *1wk*: 44.6 ± 6.5 AU, n = 593 puncta, 4 animals; *3wks*: 34.6 ± 4.8 AU, n = 607 puncta, 8 animals; *6wks*: 48.6 ± 4.1 AU, n = 1828 puncta, 5 animals; *11wks*: 34.0 ± 2.7 AU, n = 829 puncta, 4 animals; *16wks*: 55.1 ± 16.8, n = 701 puncta, 3 animals; $p > 0.05$; *T-test*). In contrast, semi-quantitative analysis revealed significant increases in the density of phospho-synapsin (Ser 9)-positive puncta beginning at 6 weeks and persisted at least up to 17 weeks after injury, compared to controls (Fig. A1.2A, C) (*CON*: 0.048 ± 0.004/μm^2, n = 32 ROIs, 8 animals; *1wk*: 0.059 ± 0.008/μm^2, n = 16 ROIs, 4 animals; *3wks*: 0.065 ± 0.004/μm^2, n = 32 ROIs, 8 animals; *6wks*: 0.091 ± 0.006/μm^2, n = 20 ROIs, 5 animals; *11wks*: 0.083 ± 0.009/μm^2, n = 16 ROIs, 4 animals;

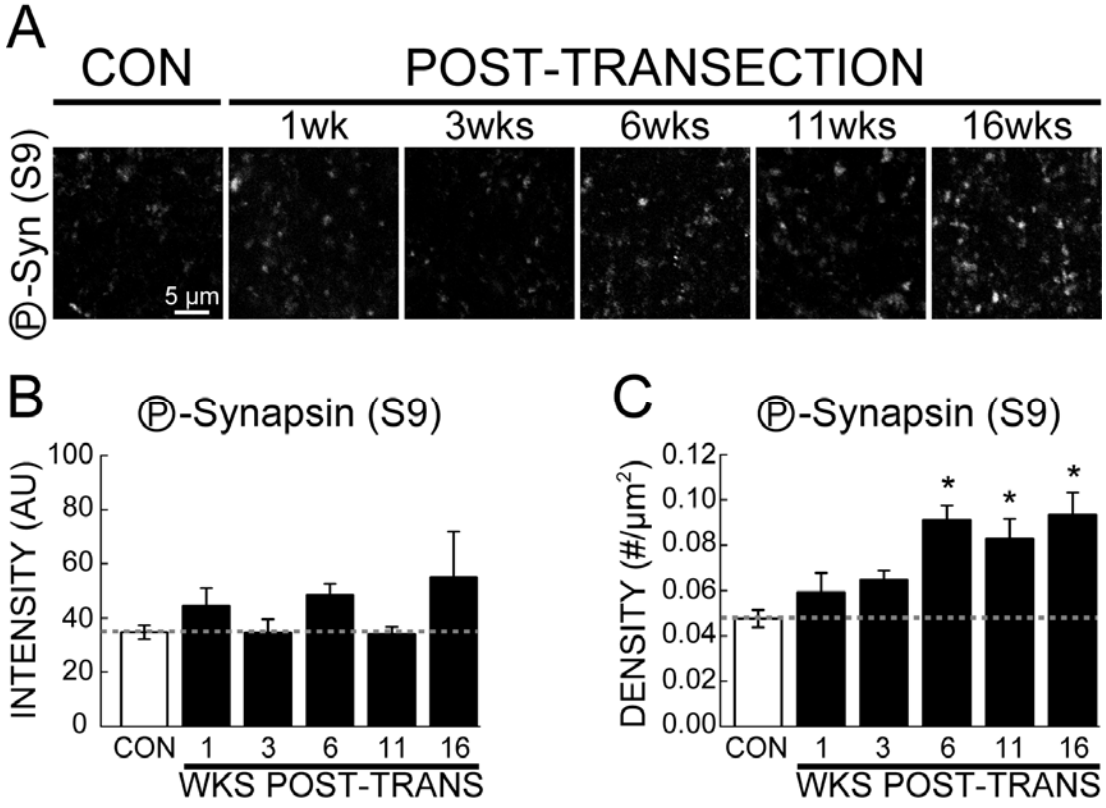

Figure A1.2. Phospho-synapsin (Ser 9) level begins to increase at 6 weeks after spinal cord injury.

(A) Representative confocal images showing phospho-synapsin (Serine 9; S9) in lamprey hindbrain. Brains of control (CON), 1 week (1wk) and 3 weeks (3wks) post-transected animals exhibited very few phospho-synapsin (S9)-positive puncta. Starting at 6 weeks post-transection (6wks), more phospho-synapsin (S9)-positive puncta were observed, and persisted to 11 weeks (11wks) and 16-17 weeks (16wks) post-transection. Scale bar applies to all images. (B, C) While the average intensity of phospho-synapsin (S9)-positive puncta was not significantly altered between all time points (B), the density of phospho-synapsin (S9)-positive puncta was significantly increased at 6 weeks post-transection and beyond (C). Bars indicate mean ± S.E.M. for 593-1829 puncta (intensity) or 12-32 regions of interest (density) measured from 3-8 animals. Asterisks indicate significant difference compared to controls as well as between 3 and 6 weeks post-transection (*T-test*; $p<0.05$).

16wks: 0.093 ± 0.01/μm^2, n = 12 ROIs, 3 animals; $p < 0.05$; *T-test*). A significant difference was also observed between 3 weeks and 6 weeks post-transection ($p = 0.022$; *T-test*). Thus, spinal cord injury induced a delayed upregulation of phospho-synapsin (Ser 9) that began at 6 weeks after the injury, an effect that persisted for at least a few more months.

Take together, these results suggest that neurite sprouting in the lamprey hindbrain began at 6 weeks after the injury, a time point that correlates with more recovery of swimming behavior than at 3 weeks post-transection (Oliphint et al., 2010). Going forward, at the ultrastructural level, one should confirm that the density of neurites is increased in the lamprey hindbrain at 6 weeks after the injury using the same protocol described in Chapter 2. In addition, one should determine whether the increased phospho-synapsin (Ser 9) in lamprey hindbrain mediates behavior recovery. To test this, one can activate or inactivate PKA activity by delivering db-cAMP or H89, respectively, via poly(lactic-co-glycolic acid) (PLGA) microspheres, which allows for a longer duration of drug delivery (Rooney et al., 2011), in the lamprey brain at the time of transection and/or at 3 weeks post-transection, and assess behavior recovery as well as phospho-synapsin (Ser 9) levels and neurite density in the lamprey hindbrain at 6 weeks and 11 weeks post-transection. It is expected that db-cAMP will increase the rate of recovery as well as the levels of phospho-synapsin (Ser 9) and density of neurites in the lamprey hindbrain, and H89 will decrease these properties.

A1.3 Examining phospho-synapsin (Ser 9) level at regenerated RS synapses

Previous studies identified large excitatory postsynaptic potentials (ESPSs), as well as increased frequency and amplitude of miniature EPSPs at regenerated RS synapses in lamprey after recovering from a complete spinal cord transection (Cooke and Parker, 2009; Mackler and Selzer, 1987). However, the molecular mechanisms mediating such enhancement were not identified. As discussed in Chapters 1 and 2, phospho-synapsin (Ser 9) positively regulates synaptic transmission at presynaptic termini. Whether its expression is increased and mediated by PKA at regenerated RS synapse after spinal cord transection are unknown. These questions will be addressed in the next two sections.

A1.3.1 Phospho-synapsin (Ser 9) levels are increased at regenerated RS synapses of spontaneously recovered lampreys

To examine phospho-synapsin (Ser 9) levels at spontaneously regenerated RS synapses, spinal cords were dissected from uninjured control as well as spontaneously recovered lampreys at 11 weeks post-transection. Cryosections at 30 μm thick were immunostained with antibodies against SV2 and phospho-synapsin (Ser 9). Regenerated RS synapses were identified by SV2-positive puncta localized within the giants RS axons (Fig. A1.3.1A, B). Twenty RS synapses positive for SV2 were imaged within 1 mm of the spinal cord distal to the lesion site or the 5th gill for control. Using ImageJ, ROIs were drawn to measure SV2 intensity. The same ROIs were then used to measure phospho-synapsin (Ser 9) intensity. Percentage of SV2-positive puncta co-localized with

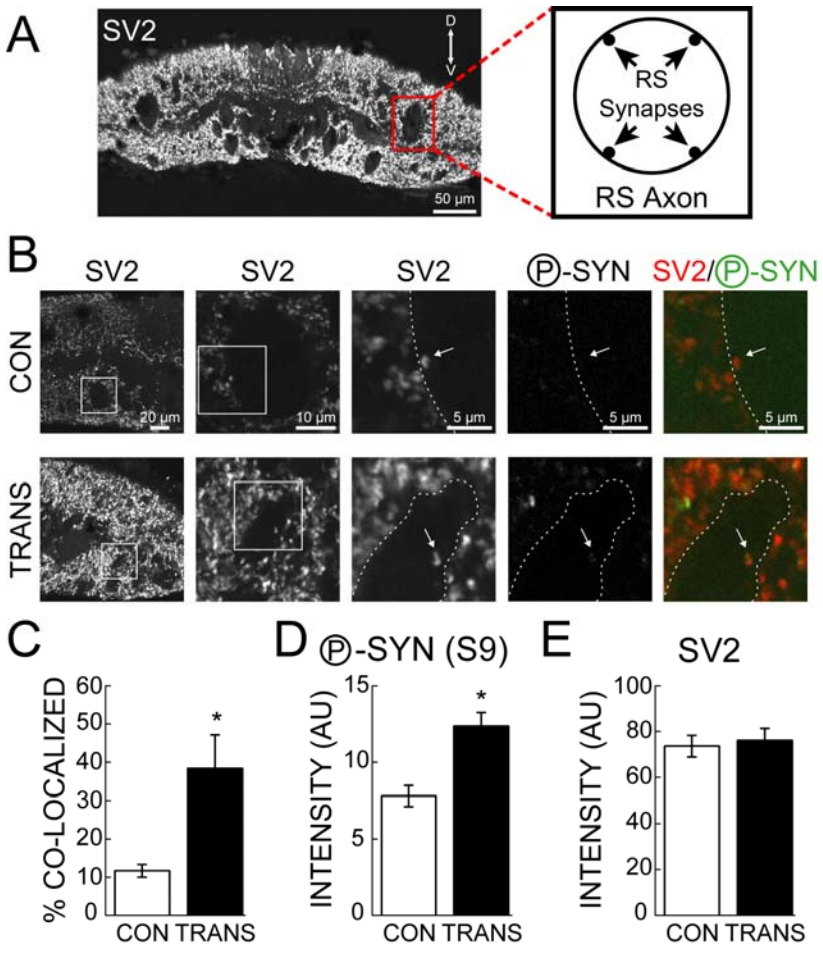

Figure A1.3.1. Phospho-synapsin (Ser 9) levels are increased at spontaneously regenerated RS synapses after spinal cord injury.

(A, left) Confocal image of an uninjured control spinal cord immunostained for SV2. D = dorsal, v = ventral. (A, right) Schematic drawing of RS axon and its presynaptic termini (RS synapses). (B) Representative confocal images showing SV2- and phospho-synapsin (Ser 9; P-SYN)-positive puncta in spinal RS axons of control (CON) and 11 weeks post-transected (TRANS) lampreys. While control RS synapses lacked phospho-synapsin (Ser 9), regenerated RS synapses contained more phospho-synapsin (Ser 9). Borders of RS axons are outlined. Arrows are inside the RS axons and they indicate SV2-positive puncta. Scale bars also apply to lower panel of images. (C-E) A significantly larger percentage of regenerated RS synapses contained phospho-synapsin (Ser 9) compared to control (C). The intensity of phospho-synapsin (Ser 9)-positive puncta was significantly brighter at regenerated RS synapses compared to control (D), while SV2 intensity was not altered (E). Bars represent mean ± S.E.M for 3 animals (% co-localization) as well as 60 puncta (intensities) for 3 animals (*T-test*; $p<0.05$).

phospho-synapsin (Ser 9) was calculated. Phospho-synapsin (Ser 9)-positive puncta with intensity values > 1.35X brighter than background were considered to co-localize with SV2-positive puncta.

While most of the control RS synapses lacked phospho-synapsin (Ser 9), a significantly larger percentage of the regenerated RS synapses exhibited co-localization with phospho-synapsin (Ser 9) (Fig. A1.3.1B, C) (*CON*: 12 ± 2%, n = 3 animals; *TRANS*: 38 ± 8%, n = 3 animals; p = 0.041; *T-test*). Moreover, individual regenerated RS synapses contained significantly more fluorescent intensity for phospho-synapsin (Ser 9), compared to controls (Fig. A1.3.1D) (*CON*: 7.8 ± 0.7 AU, n = 60 synapses, 3 animals; *TRANS*: 12.4 ± 0.9 AU, n = 60 synapses, 3 animals; p = 8.3E-5; *T-test*). The fluorescent intensity for SV2 was not significantly different between control and regenerated RS synapses (Fig. A1.3.1E) (*CON*: 73.6 ± 4.7 AU, n = 60 synapses, 3 animals; *TRANS*: 76.2 ± 5.2 AU, n = 60 synapses, 3 animals; p = 0.71; *T-test*). Thus, spinal cord injury increased phospho-synapsin (Ser 9) at regenerated RS synapses, a possible molecular mechanism to support enhanced synaptic transmission after spinal cord injury.

A1.3.2 Activation of PKA does not increase phospho-synapsin (Ser 9) at regenerated RS synapses after spinal cord injury

PKA phosphorylates synapsin at serine 9 (Czernik et al., 1987). Whether the increased phospho-synapsin (Ser 9) levels at regenerated RS synapses were mediated by PKA after spinal cord injury was unknown. To test this, newly transected lampreys were treated with 100 mM db-cAMP using the same procedures described in Chapter 3. Lampreys treated with Ringer solution were used as control. At 11 weeks post-transection, phospho-synapsin (Ser 9) levels were compared between Ringer-treated and db-cAMP-treated lampreys using the same procedures described in section A1.3.1 above.

The percentage of RS synapses that were co-localized with phospho-synapsin (Ser 9) was not significantly different in db-cAMP treated lampreys, compared to Ringer-treated controls (Fig. A1.3.2A) (*-dbcAMP*: 43.3 ± 3%, n = 3 animals; *+dbcAMP*: 51.7 ± 3%, n = 3 animals; $p = 0.15$; *T-test*). No Significant difference was observed in the intensities of phospho-synapsin (Ser 9)- and SV2-positive puncta (Fig. A1.3.2B, C, respectively) (*P-syn (S9)*: *-dbcAMP*: 9.5 ± 1.4 AU, n = 60 puncta, 3 animals; *+dbcAMP*: 8.6 ± 1.0 AU, n = 60 puncta, 3 animals; $p = 0.57$; *T-test; SV2*: *-dbcAMP*: 72.5 ± 4.1 AU, n = 60 puncta, 3 animals; *+dbcAMP*: 79.3 ± 5.2 AU, n = 60 puncta, 3 animals; $p = 0.31$; *T-test*). Thus, elevating cAMP at the time of spinal cord transection did not alter phospho-synapsin (Ser 9) levels at regenerated RS synapses at 11 weeks post-transection.

There are several possibilities to explain why elevating cAMP did not alter the level of phospho-synapsin (Ser 9) at regenerated RS synapses 11 weeks after the injury.

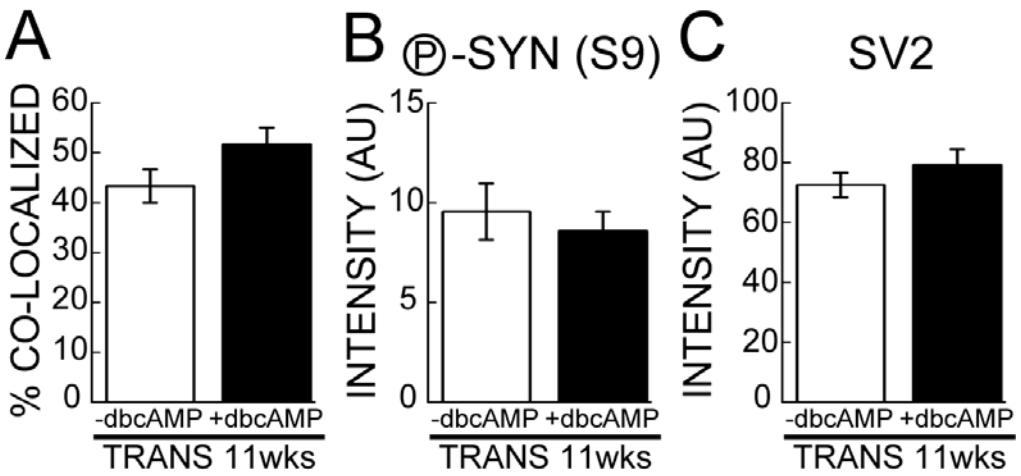

Figure A1.3.2. Activated PKA does not increase phospho-synapsin (Ser 9) levels at regenerated RS synapses after spinal cord injury.

(A-C) At 11 weeks post transection (TRANS 11wks), the percentage of RS synapses co-localized with phospho-synapsin (Ser 9) was not significantly different between Ringer-treated (-dbcAMP) and db-cAMP-treated (+dbcAMP) lampreys (A). No significant difference was observed in the intensities of phospho-synapsin (Ser 9)- (B) and SV2- (C) positive puncta. Bars represent mean ± S.E.M for 3 animals (% co-localized) as well as 60 puncta (intensities) for 3 animals.

One possibility is that the db-cAMP applied at the time of transection may have washed away too early before it can enhance PKA activity to phosphorylate more synapsin at 11 weeks post-transection. A chronic application of db-cAMP may be needed to determine whether it can increase the levels of phospho-synapsin (Ser 9) at 11 weeks post-transection. A second possibility is that db-cAMP may have also enhanced the activity of protein phosphatase 2A (PP2A) (Feschenko et al., 2002), a known enzyme that de-phosphorylates phospho-synapsin (Ser 9) (Jovanovic et al., 2001). Therefore, the enhanced PP2A activity by db-cAMP may have counteracted the effect of the enhanced PKA activity on phospho-synapsin (Ser 9) levels. To test for this possibility, one can co-apply db-cAMP and okadaic acid, an inhibitor of PP2A (Feschenko et al., 2002), at the time and site of transection, and assess phospho-synapsin (Ser 9) levels at regenerated RS synapses 11 weeks after the injury. It is expected that this treatment will increase phospho-synapsin (Ser 9) at the regenerated RS synapses at 11 weeks post-transection. A third possibility is that the levels of phospho-synapsin (Ser 9) at the spontaneously regenerated RS synapses are already at its maximum at 11 weeks post-transection. Therefore, increasing PKA activity by db-cAMP will not further enhance the levels of phospho-synapsin (Ser 9). An alternative method to determine whether PKA mediates the increased phospho-synapsin (Ser 9) levels at regenerated RS synapses is to inhibit PKA activity. As I have shown in section A1.1 that H89 can decrease phospho-synapsin (Ser 9) level *in vitro*, H89 can be applied at the time and site of transection, and assess phospho-synapsin (Ser 9) levels at regenerated RS synapses at 11 weeks post-transection.

A1.4 Synaptotagmin protein level increases in the lamprey hindbrain after spinal cord injury

In Chapter 3, I have shown that synaptotagmin is another potential molecule for promoting anatomical plasticity. This finding has prompted the question of whether synaptotagmin is also increased in the lamprey hindbrain when neurite sprouting occurs after spinal cord injury. To test this, cryosections containing the hindbrain of uninjured control and 11 weeks post-transected lampreys were immunostained with the synaptotagmin antibody. In the uninjured control lamprey hindbrain, many synaptotagmin-positive puncta were detected (Fig. A1.4A). At 11 weeks post-transection, there was significantly more synaptotagmin-positive puncta, relative to controls (Fig. A1.4A, C) (*Control*: 0.22 ± 0.02/μm^2, n = 16 ROIs, 4 animals; *Transected*: 0.29 ± 0.007/μm^2, n = 16 ROIs, 4 animals; $p = 0.041$; *T-test*). No significant difference in the intensity of synaptotagmin-positive puncta was detected at 11 weeks post-transection (Fig. A1.4B) (*Control*: 43.3 ± 6.1 AU, n = 1999 puncta, 4 animals; *Transected*: 60.6 ± 11.0 AU, n = 2348 puncta, 4 animals; $p = 0.22$; *T-test*). Thus, spinal cord transection increased synaptotagmin expression that paralleled with the increased synapsin, phospho-synapsin (Ser 9) and neurite density in lamprey brain, suggesting for the role of synaptotagmin in mediating neurite growth (Fukuda and Mikoshiba, 2000).

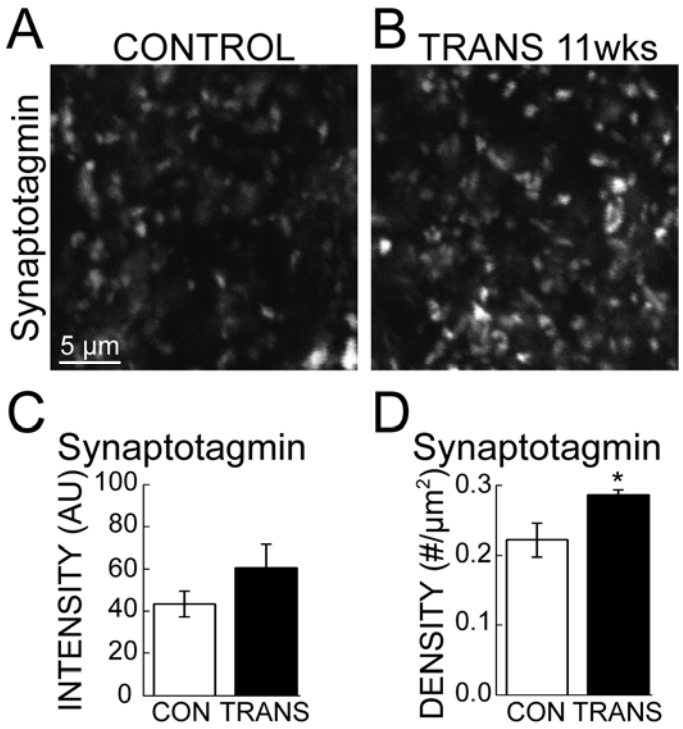

Figure A1.4. Synaptotagmin protein level is increased in lamprey hindbrain after spinal cord injury.

(A-B) Confocal images showing synaptotagmin-positive puncta in the lamprey hindbrain. Compared to the uninjured controls (A), more synaptotagmin-positive puncta were observed at 11 weeks post-transection (TRANS 11wks) (B). Scale bar applies to both images. (C-D) While the intensity of synaptotagmin-positive puncta did not significantly change at 11 weeks post-transection (TRANS), compared to controls (C), the density of synaptotagmin-positive puncta significantly increased after injury (D). Bars indicate mean ± S.E.M. for 1999-2348 puncta (intensity) and 16 ROIs (density) measured from 4 animals. Asterisk indicates statistical significance (*T-test; $p<0.05$*).

A2 PARAPLEGIA NEWS ARTICLE: "FISHING" FOR NEW STRATEGIES TO IMPROVE RECOVERY

The following published news article in the *Paraplegia News* magazine (June, 2012) highlights the significant findings described in Chapter 2.

"Fishing" for New Strategies to Improve Recovery

According to the National Spinal Cord Injury Association (NSCIA), more than 250,000 Americans are living with spinal-cord injury (NSCISC), and at least 7,000 new injuries occur annually. SCI often leads to devastating and irreversible damage within the nervous system, with the unfortunate consequence of limiting the recovery process.

However, certain people do spontaneously recover some degree of movements and sensations after SCI, suggesting the nervous system has at least a limited capacity to rewire and repair itself even in the absence of treatment (Fawcett et al., 2007). Indeed, spontaneous recovery has been the subject of many recent studies using animal models of SCI. To date, these studies suggest spontaneous recovery occurs because injured and uninjured nerve cells (neurons) can sprout new connections (Blesch and Tuszynski, 2009; Fouad et al., 2011). Collectively, these animal studies indicate some spinal tracts undergo a fair amount of spontaneous sprouting, providing new neuronal pathways that could be targeted for increasing growth, regeneration, and functional recovery of movements and sensations.

But the mechanisms that promote injury-induced neuron sprouting are unknown, thereby limiting the development of therapeutic strategies designed to improve the recovery process. This prompted Dr. Jennifer Morgan, an assistant professor at The

University of Texas at Austin, and Billy Lau, a graduate student in her lab, to investigate the molecular mechanisms that underlie neuron sprouting after SCI.

For their experiments, Lau and Morgan used as a model organism the sea lamprey, which is a jawless fish. Unlike most other vertebrates, including humans, lampreys spontaneously recover to an extraordinary degree after SCI. As evidence for this, they consistently recover from paralysis to making nearly normal movements (e.g., swimming) by 10-12 weeks after a complete spinal-cord transection (Fig. A2, top) (Cohen et al., 1988; Oliphint et al., 2010).

Coupled with the recovery of movements is extensive neuron sprouting and regeneration in the lamprey nervous system, highlighting that these creatures arc an ideal animal model in which to study the mechanisms of sprouting that support functional recovery. To add to this, the neuronal circuits that control swimming in lampreys are wired up similarly to the circuits that control walking in humans, lending additional relevance to the findings.

Taking advantage of these features of lampreys, Lau measured the expression levels of a panel of ~ 10 molecules before and after SCI. The idea underlying this experiment is that molecules whose expression levels change in response to injury are those that are most likely to support the recovery process. Therefore, these molecules need to be identified.

While all the other molecules tested were unchanged after injury, conversely, there was increased expression of synapsin (Lau et al., 2011). Synapsin is a protein known to play a positive role during nervous system development by increasing neuron

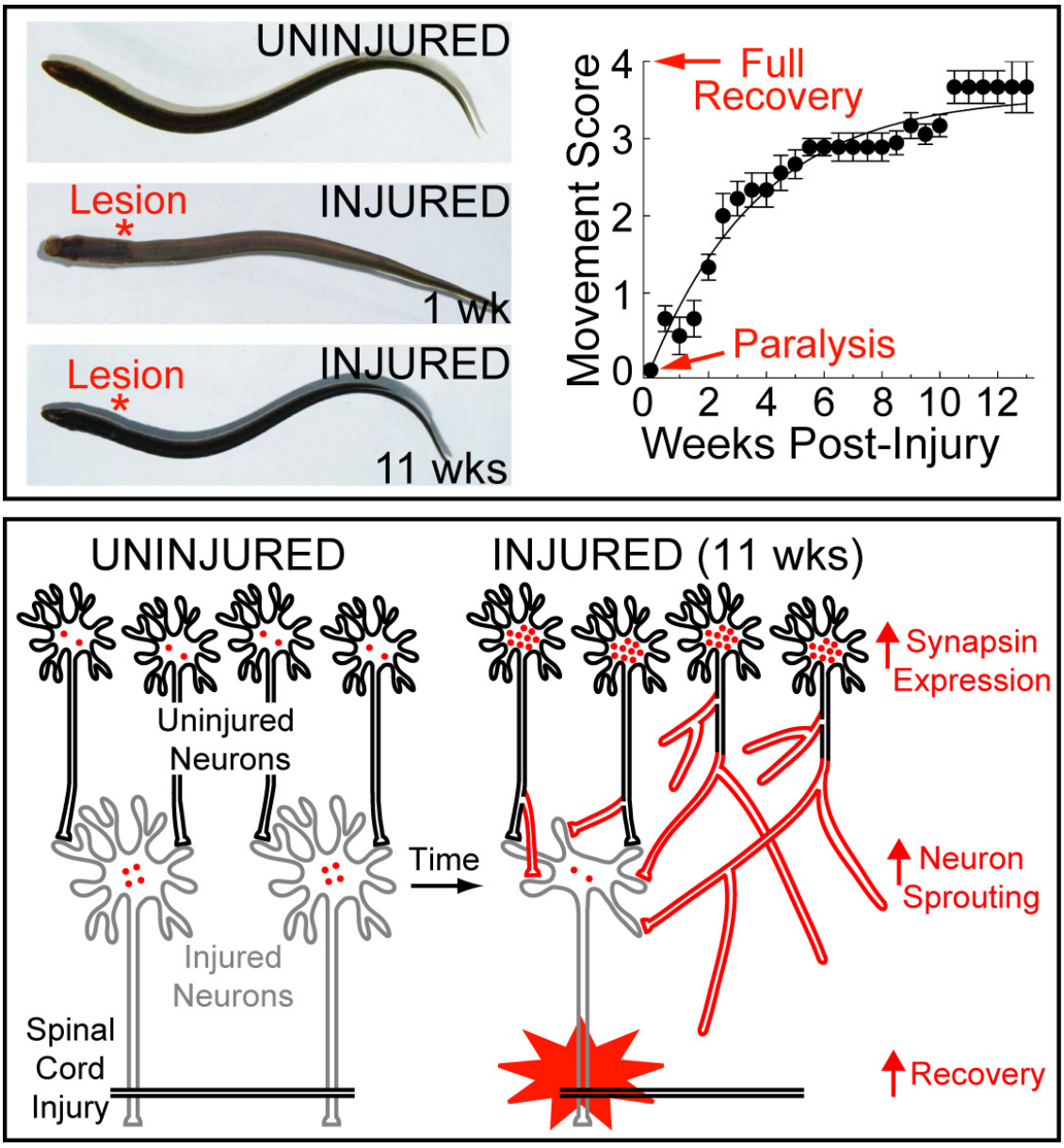

Figure A2. Recovery after spinal-cord injury in lampreys.
(Top) Uninjured lampreys produce wavelike movements during swimming. At one week post-injury, lampreys are still paralyzed. However, swimming movements return to nearly normal levels by 10-12 weeks post-injury. (Bottom) Hypothetical model summarizing how increased synapsin and neuron sprouting might assist in the recovery process. After spinal-cord injury, some injured neurons will die. Therefore, synapsin expression is increased, leading to enhanced neuron sprouting and strengthening of the connections with remaining neurons. This is predicted to improve the overall recovery. Model adapted from Lau et al., 2011.

growth and enhancing the formation of synapses, the functional connections between neurons (Cesca et al., 2010). Therefore, synapsin is a great candidate molecule with the potential to enhance neuron growth and regeneration after injury.

Lau and Morgan discovered increased numbers of sprouting neurons within the same lamprey brain regions that had exhibited the increased levels of synapsin (Fig. A2, bottom) (Lau et al., 2011). Taken together, their data suggest spontaneous neuron sprouting after injury is mediated by increasing the amount of synapsin in the nervous system. Their data also imply that the sprouting mechanism reuses some of the same molecules that support neuronal growth during development, an idea that will be tested in future studies.

How might increased synapsin expression and neuron sprouting contribute to recovery of movements after SCI? Lau and Morgan developed a working model to answer this question.

After SCI, some injured neurons die, thereby decreasing the population of neurons available to support recovery of movements. Lau and Morgan suggest increasing synapsin expression after injury can induce neuron sprouting and, therefore, increase the numbers of new connections with the surviving neurons. The net effect would be to increase the strength of these connections so the surviving neurons can send stronger signals to their targets in the spinal cord, and in doing so promote greater recovery (Fig. A2, bottom).

Lau and Morgan plan to test the predictions of this model in future experiments in lampreys and rodent models. What implications does their work have for human health?

Interestingly, synapsin is also highly expressed in the nervous system of humans (Kao et al., 1999). Therefore, it is tempting to speculate that any strategies designed to increase synapsin expression or neuron sprouting after SCI could potentially be used to develop therapies to improve recovery in humans.

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Vita

Billy Lau was born in Canton, China, and moved to San Francisco, CA at the age of 8. While in San Francisco, he attended Lawton Alternative Middle School and Abraham Lincoln High School, and pursued his undergraduate studies at University of California, Davis. After graduation, he worked as a lab technician with Dr. Su Guo at University of California, San Francisco, where he was fortunate to have independently studied several human-related behaviors using zebrafish as the model system. With his newly found interest in neurobiology, Billy began his graduate work at The University of Texas at Austin in 2007. He has taken a post-doctoral position in the lab of Dr. Stephen Shea at Cold Spring Harbor Laboratory in New York to continue his strong interest in neurobiology.

Email address: billylau12@gmail.com

This dissertation was typed by the author.