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PACSIN1, an intracellular neuronal protein, activates LRP1 dependent cell signaling in Schwann Cells

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Mark Carter

Committee in charge:

Professor Wendy Campana, Chair
Professor Douglass Forbes, Co-Chair
Professor Sonya Neal

2020

The Thesis of Mark Carter is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2020

Dedication

To my family,

None of this would be possible without your unconditional love and support. For this, I shall forever be grateful. Through you, we accomplished this milestone, and will accomplish many more.

Epigraph

Fairy tales are more than true: not because they tell us that dragons exist, but because they tell us dragons can be beaten

C.K. Chesterton

Everybody is a genius. But if you judge a fish by its ability to climb a tree, it will live its whole life believing that it is stupid

Albert Einstein

Challenges are what make life interesting and overcoming them is what makes life meaningful.

Joshua J. Marine

You must be the change you wish to see in the world

Gandhi

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Acknowledgments

I would like to acknowledge Dr. Wendy Campana for her support as the chair of my committee. I thank her for giving me the opportunity to be a part of the Campana lab and learning many valuable skills along the way. With her guidance, and many edits, I was able to make it through this.

I would like to thank Dr. Sonya Neal for her support and showing me that you can overcome all obstacles with hard work.

I would like to thank Dr. Douglass Forbes for her support throughout graduate school. I will be forever grateful for her mentorship and opening doors for many opportunities that were vital to my success as a student.

ABSTRACT OF THE THESIS

PACSIN1, an intracellular neuronal protein, activates LRP1 dependent cell signaling in Schwann Cells

by

Mark Carter

Master of Science in Biology

University of California San Diego, 2020

x

Professor Wendy Campana, Chair
Professor Douglass Forbes, Co-Chair

Peripheral nerves have the unique ability to regenerate following injury, as opposed to nerves in the central nervous system (CNS) which do not spontaneously regenerate. The ability of peripheral nerves to regenerate is due to the remarkable and versatile principle glia cell of the peripheral nervous system (PNS), the Schwann cell (SC). SCs have the ability to detect injury in peripheral nerves, such as the dorsal root ganglion (DRG), and transform phenotypically to facilitate nerve repair. Downstream cell-signaling pathways and changes in gene expression that drive SC phenotypic transformation in injury have been previously described as well as SC receptors that detect injury. LDL receptor-related protein (LRP1) is a receptor for numerous ligands, including protein components of degenerated myelin. After injury, LRP1 is dramatically upregulated in SCs and functions with the N-methyl-D-aspartate receptor (NMDA-R) to trigger cell signaling in response to ligands. Using a capture technology and LC-MS/MS to identify novel LRP1 ligands in the injured nerve, we identified Protein kinase C and casein kinase substrate in neurons protein 1, PACSIN1, as a LRP1 ligand. Not much is known of PACSIN1 as it pertains to the PNS injury. Herein, I demonstrate, for the first time, that PACSIN 1 is specifically localized in the cell bodies and axons of both large and small sensory neurons in the dorsal root ganglia (DRGs), confirming neuronal specificity. I hypothesized that after injury PACSIN1 is released by dorsal root ganglia (DRG) neurons and binds to Schwann cell LRP1 receptor. Indeed, when rhPACSIN was added to primary SC cultures, LRP1-dependent cell signaling was activated. PACSIN1 robustly activated c-Jun, the SC repair protein, and ERK1/2 in SCs within 10 minutes. This response was blocked when LRP1 was genetically silenced or when the NMDA-Receptor was pharmacologically antagonized with MK801. These data further confirm that the NMDAR is an active co-receptor for LRP1 in SCs. Finally, I demonstrate, for the first time, that expression of PACSIN1 in DRGs is significantly decreased by 5 days after sciatic nerve injury, suggesting that PACSIN1 may play a role in SC signaling in early stages after injury. We conclude that the Schwann cell LRP1 receptor is indeed responsive to intracellular

neuronal proteins released at injury. Our results support a model in which SC LRP1 serves as a major injury-induced receptor in the peripheral nervous system.

Chapter 1

Introduction

1.1 Schwann Cells in development and maturity

The Schwann cell (SC) is the principal glia cell of the peripheral nervous system (PNS) and serves as a "first responder" to injury (Jessen and Mirsky, 2005). Injury to the peripheral nervous system triggers a transformation in Schwann cell gene expression and physiology, which is essential for Wallerian degeneration and activation of the peripheral nervous system or PNS Repair Program (Arthur-Farraj et al., 2012; Lehman and Hoke, 2010; Viader et al., 2013). Distal to the site of injury, SCs de-differentiate, down-regulating myelin gene expression and substantially altering their mRNA transcriptome. Molecular signals originating from activated SCs at the PNS injury site up(?)regulate expression of regeneration-associated genes (RAGs) in the damaged sensory neurons in the dorsal root ganglion (DRG) neurons, promoting regeneration of these nerve fibers (Abe and Cavalli, 2008; Poplowski et al., Glia, 2018). The process in which SC's undergo molecular changes upon injury involves: 1) up-regulation of pathways that support survival in the challenging microenvironment of the injured PNS; 2) activation of the transcription factor c-Jun; 3) orchestration of the inflammatory response; and 4) secretion of growth factors and structural proteins that guide and support axonal regeneration(Jessen and Mirsky, 2005) This process is known as activation of the Schwann cell repair program. Notably, in rodents, aged SCs fail to appropriately activate the SC repair program in response to injury, which reduces the growth capacity of regenerating peripheral axons (Painter et al., 2014). Identifying novel molecular triggers for the PNS Repair Program is thus an important goal both for identifying molecular mechanism in rodents and how this alters with age (YES?).

Schwann Cells are either myelinating or non-myelinating in function. The two main cell types that reside in adult peripheral nerves are myelinating Schwann cells, which surround large axons, and non-myelinating Schwann cells, which enclose smaller axons in invaginations of their surfaces (Mirsky et al., 2008). Myelinating Schwann cells wrap around the axons of sensory and motor neurons forming the

myelin sheath, increasing the speed of action potentials along the axon (Bhatheja et al, 2006). Both the myelinating and non-myelinating Schwann cells in peripheral nerves are derived from the neural crest, which is a transient and multipotent embryonic structure that also generates the other main glial subtypes of the peripheral nervous system (PNS) (Woodhod et al., 2008). In spinal nerves, Schwann cell generation is a protracted process that involves two embryonic transitional stages; first is the process of gliogenesis, whereby the neural crest cells are specified to form Schwann cell precursors (SCPs), followed by a maturation of these cells into immature Schwann cells (Dong et al., 1995, 1999; Jessen et al., 1994). Cells will then begin the process of differentiation to form mature myelinating and non-myelinating Schwann cells. In the rat, SCPs are present in the spinal nerves at around embryonic day 14/15 (E14/15) and they give rise to immature Schwann cells, which are present in the nerves at around E17/18 (Jessen et al., 1994). In the mouse, however, this stage of Schwann cell development proceeds 2 days earlier; SCPs are present in the nerves at around E12/13, and they give rise to Schwann cells, present at around E15/16 (Dong et al., 1999). Next, SCPs transform into immature Schwann cells, which occupy nerves from E15/E16. This stage continues until around birth, at which these immature Schwann cells start to generate first the myelinating Schwann cells and subsequently the non-myelinating Schwann cells found in adult nerves. Immature Schwann cells become either myelinating or non-myelinating cells depending on the axons with which they associate (Mirsky et al, 2005, 2008).

Extracellular signals that induce specific transcription factors are involved in the determination of the fate of Schwann cell lineage. Three cell-cell signals or ligands that regulate Schwann cell development have been identified through in vivo and in vitro studies. These are neuregulin-1, which carries out several major functions in developing and myelinating nerves, endothelin, which is involved in the SCP/Schwann cell transition, and Notch signaling (Brennan et al., 2000; Garratt et al., 2000b; Wakamatsu et al., 2000; Kubu et al., 2002; Nave and Salzer, 2006). The importance of neuregulin-1 signaling at all stages of Schwann cell development from the neural crest stage to fully differentiated Schwann cells cannot be overemphasized (Garratt et al., 2000b; Lemke, 2006; Nave and Salzer, 2006)

Transcription factors play a pivotal role in SC development. Sox10 is essential for the generation of the earliest cells in the Schwann cell lineage and has been shown to be required for myelination (Britsch et al., 2001; Schreiner et al., 2007). Krox-20 (Egr-2) is essential for myelination (Topilko et al., 1994; Topilko and Meijer, 2001).
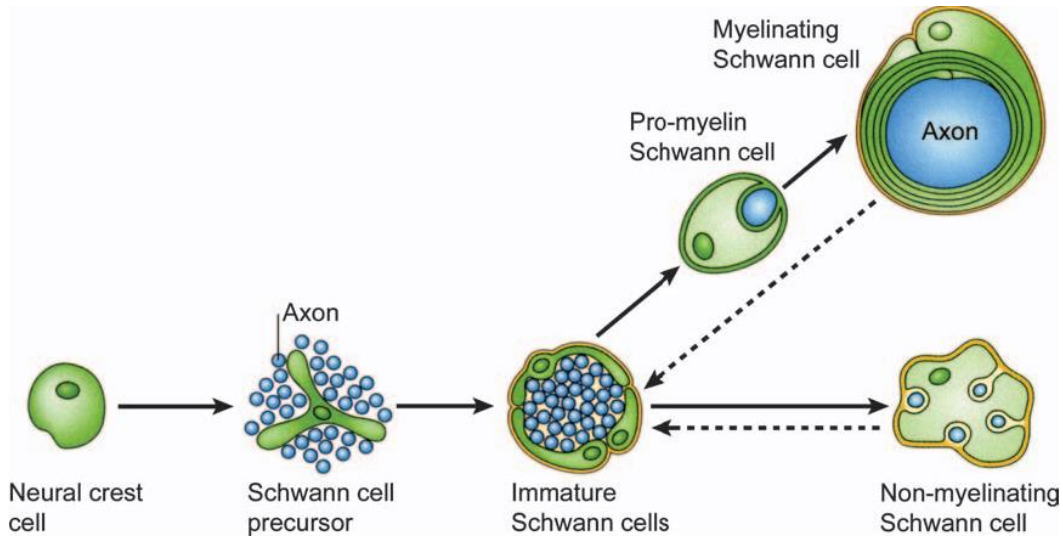

Figure 1.1: The Schwann cell lineage. Schematic illustration of the main cell types and developmental transitions in Schwann cell development. Stippled arrows indicate the reversibility of the final, and in rodents largely post-natal, transition that generates mature myelinating and non-myelinating cells. The embryonic phase of Schwann cell development involves three transient cell populations. First, migrating neural crest cells. Second, Schwann cell precursors. These cells express several differentiation markers not found on migrating crest cells including BFABP, P0, desert hedgehog (DHH) and others. Third, immature Schwann cells. All these cells are considered to have the same developmental potential, and their fate is dictated by the axons with which they associate. Only those Schwann cells that by chance envelop the large-diameter axons will be induced to myelinate, while those cells that sheath small-diameter axons progress to become mature non-myelinating cells. Reproduced from Nature Reviews Neuroscience with minor modifications (Jessen and Mirsky, 2005).

1.1.2 The role of Schwann cells after Injury

A feature of SC's is their plasticity which has been observed in the dedifferentiation of both myelinating and non-myelinating Schwann cells that occurs after nerve cut or crush injury, but is also seen when myelinating Schwann cells dedifferentiate in neuron-free cultures (Mirsky et al., 2008). Distal to the injury site, SCs de-differentiate, down-regulating myelin gene expression and substantially altering their mRNA transcriptome (Arthur-Farraj et al., 2012) Furthermore, Schwann cells are known to be

important in nerve regeneration. Upon injury of a nerve, Schwann cells will act as first responders. They are immediately involved in the digestion of the damaged axons via phagocytosis and start the process of axonal regeneration (Bhatheja et al, 2006). The activation of this cellular repair mechanism is aided by various downstream proteins activated by various binding ligands to cell surface proteins on the Schwann Cell. The Low-density lipoprotein receptor-related protein 1 (LRP1) is a receptor with various ligands, many of which are present in the injured nerve (Campana et al, 2007). LRP1 is upregulated after injury in SC (Campana et al., 2006).

1.2 Nerve Injury, Wallerian degeneration and neuronal regeneration

Nerve injury can occur in many ways and is injury to the nervous tissue. Nerve injury differs in the central nervous system (CNS) and the peripheral nervous system (PNS) in that in the PNS neurodegeneration is possible (Fenrich K et al, 2004). In the PNS, the process of nerve injury to neurodegeneration can be broken down to the following: Wallerian degeneration, axon regeneration/growth, and nerve reinnervation, and occurs with respect to the axis of the nerve injury. The proximal end refers to the area of injury closes to the cell body, which will regenerate, while the distal end refers to the area closer to the end of axon that will degenerate in the process prior to regeneration (Fenrich K et al, 2004).

During peripheral regeneration, nerves undergo Wallerian degeneration in order to remove debris and start the regeneration process. Wallerian degeneration is the active process of degeneration that results when a nerve fiber is cut or crushed resulting in cytoskeletal disassembly and granular degeneration of the axon distal to the injury site (Wang et al, 2012). Following traumatic nerve injury in the PNS, axon will first begin to swell followed by retracting from the target tissue resulting in complete degeneration. This axonal degeneration event is mediated by extracellular Ca2+ influx and activation of the intracellular Ca2+-dependent protease calpain, occurring 5-30 minutes post nerve injury (Wang et al, 2012). The Schwann cell and macrophages interact and began to phagocytose degenerated myelin and damaged axon distal to the injury site in a rapid manner. Schwann cells also play a role in the recruitment

of neurotrophic factors such as nerve growth factor (NGF) and ciliary neurotrophic factor (CNTF), which promote growth of Schwann cell and axon and guide the growing axon. Numerous signaling pathways are activated and transcription factors aid the regenerative capacity of the peripheral neurons (Mahar et al, 2018). Bands of Büngner will form from un-innervated proliferating Schwann cells and aid in guiding the regenerating axon (Campbell et al, 2008). Proximal to the injury site, retrograde degeneration occurs. The cell body will swell and undergoes chromatolysis in which the nucleus migrates to the periphery of the cell body and the endoplasmic reticulum breaks up and disperses. Nerve damage causes a metabolic switch from production of molecules that promote synaptic transmission to factors that that promote growth and repair, such as GAP-43, tubulin and actin (Campbell et al, 2008). Somatic chromatolysis is reversed once the neuron is prepared for axonal regeneration. At the axonal stump, proximal to site of injury, sprouting occurs forming a growth cone. The remaining Schwann cells will proliferate and align along the axon to facilitate the sprouting of the axon. The Schwann cells will secret the myelin sheath (Alvites et al, 2017).
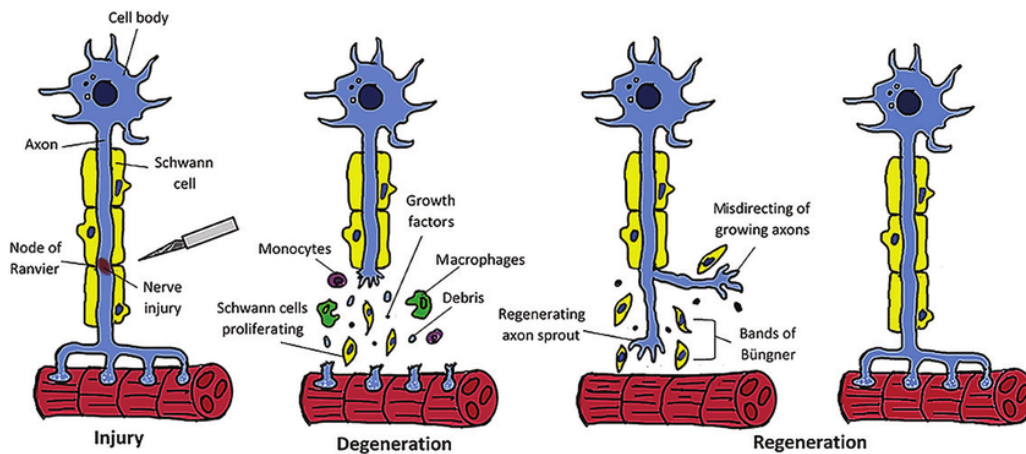

Figure 1.2: Schematic representation of the Wallerian degeneration (Alvites et al, 2017)

1.3 Schwann Cell Repair Program: The Remarkable ability the PNS repairs itself

Injury to the PNS is detected by Schwann cells and triggers extensive changes in the differentiation state of both injured neurons and of the Schwann cells distal to the injury. Neurons change expression of numerous transcription factors, which allow the neuron to shift from cell-cell signaling to axon regeneration (Blesch et al. 2012). Similarly, the myelin and non-myelin (Remak) Schwann cells distal to nerve injury undergo a large-scale change in gene expression, probably involving some thousands of genes, and change function from maintenance of axonal ensheathment and myelin to that of supporting regeneration (Nagarajan et al. 2002). Following sciatic nerve crush, axons readily grow back to their targets, redundant myelin is removed, and new myelin is formed around regenerated axons, with the result that nerve tissue that is broadly normal in structure and function is restored in a surprisingly short time, 3–4 weeks (Jessen et al, 2016). The process of nerve repair involves: 1) up-regulation of pathways that support survival in the challenging microenvironment of the injured PNS; 2) activation of the transcription factor c-Jun; 3) orchestration of the inflammatory response; and 4) secretion of growth factors and structural proteins that guide and support axonal regeneration. Activated SCs also participate in the first phase of myelin degradation. Collectively, these changes are referred to as "Activation of the SC Repair Program" (Flütsch et al, 2016).

1.4 N-methyl-D-aspartate receptor (NMDA-R): Glutamate receptor that aids in nerve repair.

The N-methyl-D-aspartate receptor (NMDA-R) is an extensively studied neuronal ionotropic glutamate receptor, composed of GluN1 (NR1, encoded by GRIN1) and GluN2 (NR2, encoded by GRIN2A–GRIN2D) subunits (Furukawa et al., 2005). In post-synaptic densities in neurons, the NMDA-R is physically and functionally associated with receptors in the low-density lipoprotein (LDL) receptor family, including LRP1 and ApoER2 (also known as LRP8) (Strickland et al., 2002; May et al., 2004; Hoe et al., 2006; Martin et al., 2008). LRP1 interacts with NMDA-R, essentially functioning as a co-receptor for LRP1 ligands, including α2- macroglobulin (α2M), tissue-type plasminogen activator (tPA) and apolipoprotein E, to facilitate downstream cell-signaling pathways E (Bacskai et al., 2000; Qiu et al., 2002; Samson et al., 2008; Martin et al., 2008; Sheng et al., 2008; Mantuano et al., 2013). The functional

consequences of LRP1-activated cell signaling in SCs include cell survival, cell migration, and expression of cytokines that are chemoattractant for monocytes (Campana et al., 2006; Shi et al., 2011; Orita et al., 2013). Conversely, when LRP1 is genetically deleted in SCs in scLRP1$^{-/-}$ mice or antagonized with receptor-associated protein (RAP), SC survival, together with other functions that are critical in the response to PNS injury, are compromised (Campana et al., 2006a; Orita et al., 2013).

The N-methyl-D-aspartate receptor (NMDA-R), is a glutamate receptor and ion channel protein found in nerve cells. The NMDA receptor is one of three types of ionotropic glutamate receptors, which are subdivided into three groups based on their pharmacology structural similarities and differences. Furthermore, ionotropic glutamate receptor subunits possess four hydrophobic regions within the central portion of the sequence. The other receptors are the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA, AMPAR) and Kainite receptors. AMPA receptors consist of four types of subunits which are GluA1 (GRIA1), GluA2 (GRIA2), GluA3 (GRIA3), and GluA4, alternatively called GluRA-D2 (GRIA4). These subunits combine to from tetramers (Chen et al, 2007). Dorsal root ganglion neurons express functional AMPA and kainite receptors near their central terminals (what are these?). Upon activation, these receptors cause a decrease in glutamate release during action potential evoked synaptic transmission. Due to differences in kinetic properties and expression patterns of these two families of glutamate receptors in subpopulations of sensory neurons, AMPA and kainite receptors are expected to function differently (Lee et al, 2004). AMPA has roles in mediating basal synaptic transmission, synapse stabilization, and synaptic plasticity in the central nervous system (CNS) (Chen et al, 2007). AMPA channels are mainly permeable to sodium and gating therefore leads to membrane depolarization (Johns et al, 2014). Similar to AMPA and NMDA receptors, kainite receptors form tetramers comprised of subunits GluR5 (GRIK1), GluR6 (GRIK2), GluR7 (GRIK3), KA1 (GRIK4) and KA2 (GRIK5) (Fritsch et al, 2014). Kainite receptors play a role in presynaptic and post synaptic transmissions but, however, are more limited in their expression compared to NMDA and AMPA receptors and are not well defined. Kainite receptors containing the GluK1 subunit have an impact on excitatory and inhibitory

neurotransmission in brain regions, such as the amygdala and hippocampus, which are relevant to seizures and epilepsy (Fritsch et al, 2014).

The NMDA-R receptor is activated when glutamate and glycine (or D-serine) bind to it and, when activated, allows positively charged ions to flow through the cell membrane. (Furukawa et al, 2005). NMDAR is composed of GluN1 (NR1, encoded by GRIN1) and GluN2 (NR2, encoded by GRIN2A–GRIN2D) subunits (Furukawa et al., 2005). NMDAR is very important for controlling synaptic plasticity and memory function (Li F et al, 2009) and is now recognized to be an important receptor of Schwann cells. NMDA-R plays a role in neuronal development, including effects on cell migration, differentiation of progenitors and dendrite development (Nacher and McEwen, 2006; Rajan and Cline, 1998). In mature neurons, the NMDA-R localizes to post-synaptic densities, where it plays an important role in long-term potentiation based on its mechanism of channel activation that requires both glutamate and membrane depolarization (Bliss and Collingridge, 1993; Lynch, 2004). Ca2+ flux through the NMDA-R regulates cell signaling and, in turn, activated signaling factors regulate the function of the NMDA-R (Wang and Salter, 1994).

In post-synaptic densities in neurons, the NMDA-R is physically and functionally associated with receptors in the low-density lipoprotein (LDL) receptor family, including the LDL-receptor-related protein-1 (LRP1) and ApoER2 (also known as LRP8) (Strickland et al., 2002; May et al., 2004; Hoe et al., 2006; Martin et al., 2008). NMDA-R acts as an essential signaling co-receptor with LRP1 ligands to facilitate activation of many neuronal pathways (Mantuano et al., 2013), and is required for cell signaling in response to diverse LRP1 ligands, including α2M, tPA and matrix metalloproteinase 9 (MMP9) (Mantuano et al., 2013). LRP1 is expressed by Schwann cells mainly after nerve injury (Campana et al., 2006) and functions as a robust cell signaling receptor (Mantuano et al., 2008a, b, 2010, 2011). NMDA-R is expressed by rat

Recently, the Campana lab showed that NMDA-R is expressed in cultured SCs isolated from postnatal pups (Campana et al., 2017 FASEB J). SCs with different phenotypes are glutamate

responsive, including non-myelinating SCs that form Remak bundles and SCs that myelinate axons before crush injury. Schwann cells are known to release glutamate that interacts with neuronal NMDA-R (Wu et al., 2005). The NMDA receptor is involved in activation of the phosphorylated ERK1/2 pathway which is important for Schwann cell survival (Campana et al, 2017). The NMDA-R also functions as an independent Schwann cell receptor for tPA, activating ERK1/2 (also known as MAPK3 and MAPK1, respectively) and promoting cell migration in response to tPA even when LRP1 is unavailable. NMDA-R has also been identified as an important independent receptor for the protein ligand tPA and as a co-receptor for LRP1 in the Schwann cell following peripheral nerve injury (Mantuano et al., 2013).

1.5 LDL receptor-related protein 1 (LRP1): A key receptor in activation of the SC repair program

LDL Receptor-related Protein-1 (LRP1) is an endocytic and cell-signaling receptor that is substantially up-regulated in SCs in response to PNS injury, promoting many of the changes that are observed in the activation of the SC repair program (Herz and Strickland, 2001; Gonias and Campana, 2014; Campana et al., 2006). When presented with specific ligands, LRP1 activates cell-signaling pathways in SCs, which support SC survival, even in the absence of axonal contact. LRP1 differs from most receptors in its ability to bind to over 60 ligands that are both structurally and functionally diverse (Strickland et al, 2002; Fernandex-Casteneda et al., 2013,). When SCs are treated with various extracellular proteins that are known LRP1 ligands, such as activated α_2-macroglobulin (α_2M*), matrix metalloproteinase-9 (MMP9), and tissue-type plasminogen activator (tPA), cell-signaling factors are activated that are vital for the response to PNS injury and aid in supporting PNS repair, including c-Jun, ERK1/2, Akt, and Rac1 (Mantuano et al., 2010; Mantuano et al, 2008; Fluetsch et al., 2016). LRP1 activated cell-signaling is not fully understood, however LRP1 functions favorably as a key receptor in activation of the SC repair program.

LRP1 is a type 1 transmembrane receptor protein that consist of 839 amino acids that mediates endocytosis and cell-signaling activity. LRP1 is one of six members [LRP, LRP1B, LRP2/gp330, LDL receptor, very low-density lipo-protein receptor, and LRP8/apolipoprotein E (apoE) receptor2] of the LDL receptor superfamily that are closely related structurally (Lillis et al.,2005). Members of the LDL receptor family are composed of modular domains that include cysteine-rich complement-type repeats, EGF repeats, □-propeller domains, a transmembrane domain and a cytoplasmic domain. LRP1 extracellular complement-type, or ligand-binding repeats, are found in four clusters of ligand binding repeats and are responsible for recognizing ligands (Lillis et al.,2005). LRP1 ligand-binding domain folds back and interacts with amino acid residues in the EGF repeats and β-propeller domain. The alpha-chain of the LRP1 extracellular domain consists of the four ligand-binding repeats (numbered I-IV) to which extracellular matrix proteins like PACSIN1, growth factors, proteases, protease inhibitor complexes, and other proteins involved in lipoprotein metabolism would bind. This interaction is a mechanism for ligand release in which the β-propeller domain functions as an alternate substrate for the ligand-binding domain resulting in displacement of the ligand (Lillis et al.,2005).
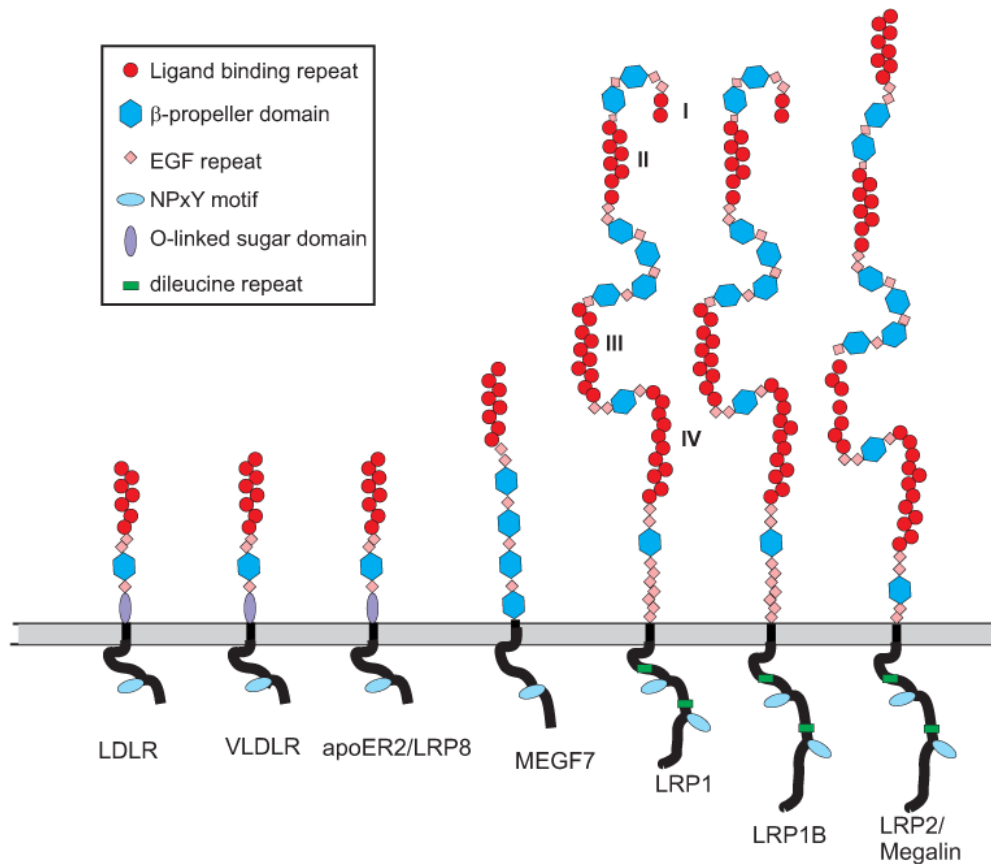
Figure 1.3: Modular domain organization of LDL receptor family members. In LRP1, the four clusters of complement-type repeats are numbered I – IV. (Lillis et al.,2008)

1.5.2 LRP1 in peripheral nerve injury

Upon peripheral nerve injury, SC express LRP1 within 24 hours, and once LRP1 is expressed it promotes many of the changes observed in the SC repair program (Campana et al, 2006). When presented with specific ligands, LRP1 activates cell-signaling pathways in SCs, which support SC survival, even in the absence of axonal contact (Flütsch et al, 2016). Importantly, when the LRP1 gene is deleted in Schwann cells in mice, numerous abnormalities are observed following sciatic nerve injury, including

accelerated demyelination, increased infiltration of the nerve by inflammatory cells, and abnormal regeneration (Orita et al., 2013). Activation of the ERK1/2 pathway via LRP1 ligands, induces expression of MCP-1/CCL2, a potent chemoattractant for inflammatory cells in PNS injury. These factors position LRP1 as a key player in the activation of the Schwann cell repair program upon PNS injury.

LRP1 is also a known activator of the global transcription factor, c-Jun, which plays a central role in activation of the SC Repair Program (Flütsch et al, 2016). Furthermore, LRP1 is expressed by human SCs in culture and initiates c-Jun activation, making the function of LRP1 as a cell-signaling receptor conserved in human and rat SCs. Identifying novel intracellular proteins that bind to LRP1 and cause the activation or augment the SC Repair Program, was a major focus for this study.

1.6 Protein kinase C and casein kinase substrate in neurons protein 1 (PACSIN1)

Protein kinase C and casein kinase substrate in neurons protein 1 (PACSIN1) is a part of a family of 3 PACSIN cytoplasmic phosphoproteins that a play a role in vesicle formation and transport. PACSIN 2, and 3 are mainly detected in lung and muscle tissue in contrast to the neuron specific PACSIN1 (J. Modregger et al, 2000). PACSIN1 also plays a role in the reorganization of the actin cytoskeleton and in neuron morphogenesis and the reorganization of the microtubule cytoskeleton. PACSIN1 is required and plays dual roles in controlling the endocytosis and recycling of AMPARs following NMDA stimulation (Widagdo et al., 2016).

Pacsin proteins are members of the Pacsin/Syndapin subfamily, one of six subfamilies of F-BAR proteins, and are involved in Clathrin-mediated endocytosis, actin polymerization and neuronal development (Mahmood et al, 2019). F-BAR proteins are a sub-family of the BAR superfamily of membrane curvature binding/bending proteins. The BAR superfamily can be subdivided into BAR/N-BAR modules that bind to membranes of high positive curvature, F-BAR modules that bind to a different range of positive membrane curvatures and I-BAR modules that binding to negatively curved membranes. BAR domains are generally known to be very rigid in nature based on simulation and theoretical studies.

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However, Pacsin1 has the ability adapt itself to a wide range of membrane curvatures and stabilize tubules with different diameters, merely from its internal structural flexibility (Mahmood et al,2019).

PACSIN1 was first identified in murine brain (Plomann et al, 1998) and contains a C-terminal SH3 domain that promotes protein-protein interactions (Plomann et al, 1998). The SH3 domain of PACSIN1 is a well-known protein-protein interaction module that binds to the large GTPase dynamin and to the actin cytoskeleton regulator N-WASP (neural Wiskott-Aldrich syndrome protein), both of which play crucial roles in endocytosis (Widagdo et al., 2016). PACSIN1 exists in dimers and oligomers, the other PACSIN1 SH3 domain can interact with the actin nucleator, N-WASP, to provide mechanical forces to propel vesicles away from the plasma membrane through active actin remodeling. Furthermore, PACSIN1 has been shown to potentially play a role as a scaffold protein linking dynamin and actin polymerization to promote vesicle fission and provide mechanical forces to propel these vesicles away from the plasma membrane (Widagdo et al., 2016). During AMPAR endocytosis, PACSIN1 is recruited to the Clathrin-coated pit at the plasma membrane through a SH3-dependent interaction with a component of the endocytic machinery, such as dynamin (Widagdo et al., 2016). The binding of dynamin to the SH3 domain forces PACSIN1 into an open conformation and releases the intramolecular inhibition of the PACSIN1 F-BAR domain, which is essential for its membrane deformation activity and facilitates vesicle endocytosis and fission (Widagdo et al., 2016). PACSIN1's role in peripheral nerve injury is not well known. We hypothesis that following peripheral nerve injury, intracellular PACSIN1 is released from sensory neuron into extracellular space allowing PACSIN1 to bind to LRP1 facilitating the activation of SC repair programs.

1.7 P-ERK1/2 activation

Protein kinases are crucial components of the signaling network that allows cells to function as an integral part of an organism (Mebratu et al, 2009). The molecular events triggered by extracellular signals that activate tyrosine phosphorylation have been the subject of intense study since Ushiro and Cohen (1980) discovered that the epidermal growth factor receptor had, in common with the transforming protein of Rous sarcoma virus, pp60v-src, protein-tyrosine kinase activity (Collett et al, 1980; Hunter and Sefton, 1980; Levinson et al, 1980). These protein kinases are known as extracellular signal–regulated kinases (ERKs) or classical MAP kinases. ERKs are widely expressed protein kinase intracellular signaling molecules that are involved in functions including the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells. Many different stimuli, including growth factors, cytokines, virus infection, ligands for heterotrimeric G protein-coupled receptors, transforming agents, and carcinogens, activate the ERK pathway.

Extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) are members of the mitogen-activated protein kinase (MAPK) super family that can mediate cell proliferation and apoptosis (Mebratu et al, 2009). This family of kinases is regulated by a phosphorylation cascade and requires an extracellular stimulus for activation, such as growth factors, cytokines, mitogens, hormones, and oxidative or heat stress (Cervelliniet al, 2018; Mebratu et al, 2009). Abnormal regulation of the MAPK pathways has been reported for a wide range of diseases including many cancers, obesity, diabetes, polycystic kidney diseases, cardiovascular diseases, Alzheimer's diseases, and pulmonary diseases, such as asthma, emphysema, and COPD10. As a result, drugs targeting the MAPKs are being tested for a variety of disease conditions (Mebratu et al, 2009). In the PNS, the inhibition of ERK1/2 in SCs arrested their differentiation, resulting in dramatic hypomyelination and suggesting a positive role of ERK1/2 in the regulation of myelination (Newbern et al., 2011; Ishii et al., 2014). Once activated, MAPKs primarily phosphorylate a multitude of target substrates on serine or threonine residues followed by a proline residue, and regulate cellular activities ranging from gene expression, mitosis, embryogenesis, cell differentiation, movement, metabolism, and programmed death. At least four members of the MAPK

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family have been identified: extracellular-signal-regulated kinase 1/2 (ERK1/2), c-Jun-amino-terminal kinase (JNK), p38, and ERK5 (Mebratu et al, 2009).

It is known that after nerve injury ERK1/2 signaling is rapidly activated and contributes to SC dedifferentiation phenotype of Wallerian degeneration (Sheu et al., 2000; Harrisingh et al., 2004). Sustained activation of MAPK/ERK in adult SCs is therefore deleterious to successful nerve repair, emphasizing the differences in the signaling processes coordinating nerve development and repair (Cervelliniet al, 2018).

1.8 c-Jun

c-Jun is a leucine-zipper zinc-finger transcription factor, a key component of the AP-1 transcription (Mirsky et al., 2008).In injured and diseased nerves, the transcription factor c-Jun in Schwann cells is elevated and variously implicated in controlling beneficial or adverse functions, including trophic Schwann cell support for neurons, promotion of regeneration, tumorigenesis, and suppression of myelination (Fazal et al, 2017). The transcription factor c-Jun is required for Schwann cell proliferation and death and is down-regulated by Krox-20 on myelination and is a negative regulator of myelination (Mirsky et al., 2008).Deletion of c-Jun in neonatal mice has been shown to delayed loss of myelin proteins and mRNA after nerve injury (Parkinson et al., 2008) suggesting Schwann cell c-Jun might play an important role in specifying the phenotype of denervated Schwann cells (Peter et al, 2012). Furthermore, c-Jun has been shown to be a cell-intrinsic determinant of Schwann cell morphology that controls the structure of the essential regeneration tracks that guide growing axons back to correct targets (Peter et al, 2012). The transcription factor, c-Jun, is a global regulator of the Schwann cell repair program. In c-Jun deficient mutant mice, the regeneration defects are substantially more severe than those reported for other mouse mutants, although the genetic defect is restricted to Schwann cells due to the number of diverse molecules controlled by c-Jun alone. The transcription factor, c-Jun, is vital in the

activation of a repair program in Schwann cells and the creation of a cell specialized to support

regeneration (Peter et al, 2012) following nerve injury.

1.9 Objectives

Following nerve injury in the PNS, Schwann cells transdifferentiate and upregulate LRP1 and

NMDAR expression. These receptors activate LRP1 dependent cell signaling that is essential for

Schwann cell survival, migration and repair of the injured nerve. However, when I arrive at the lab novel

LRP1 ligands produced in the injured nerve were not known. The Campana Lab utilized LRP1 capture

technology and mass spec discovery profiling to identify novel LRP1 ligands in the injured nerve. One

of the most specific, abundant and reproduceable novel ligand identified was PACSIN1. PACSIN1 is a

neuro-specific intracellular ligand, highly abundant in uninjured DRGs that binds to LRP1 and facilitates

LRP1 dependent cell signaling in primary cultured SCs. For my Masters' thesis project, I identified

PACSIN1 in the mouse PNS, measured Pacsin 1 levels in the DRG after injury *in vivo* and determined

whether, PACSIN1 activates LRP1 dependent cell signaling in Schwann cells.

Chapter 2

Methods

2.1 Primary Schwann cell cultures.

Sciatic nerves were isolated from 1-dold Sprague Dawley rats, and SCs were further selected from

fibroblasts using fibronectin-specific antibody and rabbit complement cytolysis, as described previously

(Campana et al., 1998, 2006). The final preparations consisted of 98% SCs, as determined by

immunofluorescence (IF) microscopy for S100, which is a specific SC marker. Primary cultures of SCs

were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, 100 g/ml streptomycin, 21 g/ml

bovine pituitary extract, and 4 M forskolin [complete medium (CM)] at 37°C under humidified 5% CO_2.

SC cultures were passaged no more than six times before conducting experiments.

Briefly, nerves were dissected and digested with trypsin and collagenase for 30 min at 37°C. Cell suspensions were washed three times and resuspended in DMEM with 10% heat-inactivated horse serum (HS), 0.5 M forskolin (Calbiochem), 100 U penicillin, and 100 g/ml streptomycin. Cells were plated on Poly-d-Lysine coated T-75 flask with standard growth medium (DMEM, 10% HS, 20 g/ml bovine pituitary extract, 0.5 M forskolin, 100 U/ml penicillin, and 100 g/ml streptomycin), and allowed to adhere overnight. Medium was changed every other day. Once cells were confluent, cells were then removed from flask using Trypsin/EDTA (for T-75, add 1000ul).

2.2 Phosphorylation assay

Primary cultures of rat SC were then plated on poly-d-lysine coated 6-well, flat bottom tissue culture plate with 2ml standard growth media and allowed to adhere overnight. 200k rat Schwann cells were placed in each well. The medium was changed every other day. After 48 hours, cells reached 85% confluency and placed in 1ml of serum free medium (no DMEM low glucose) per well for 45 minutes at 37°C under humidified 5% CO_2. After 45 minutes 2 wells would be treated with NMDA-R inhibitor MK801 (100ng/ml). After 60 minutes well then received individual treatment of either Neuregulin 1 (100ng/ml), or 50nM rhPacsin1 simultaneously. Previous studies examined whether the NMDA-R is required for LRP1 signaling in Schwann cells by first pre-treating Schwann cells with MK801, an uncompetitive antagonist of the NMDA-R, for 15 minutes (Mantuano et al 2015). At the conclusion of the experiments, cells were washed twice with ice-cold PBS. Cell extracts were prepared in RIPA buffer (PBS with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, proteinase inhibitor mixture, and sodium orthovanadate)

2.3 Gene Silencing

Rat NR1-specific siRNA ON-TARGET plus SMARTpool, which targets the NR1 subunit of the NMDA-R, and pooled non-targeting control (NTC) siRNA were from Dharmacon. Schwann cells (2×106) were transfected with NR1-specific siRNA (50 nM) or with NTC siRNA (50 nM) by electroporation using the Cell Line Nucleofector Kit V (Amaxa). The degree of gene silencing was determined at the mRNA level

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by qRT-PCR and at the protein level by immunoblot analysis. Cell signaling and migration experiments were performed 24–36 h after introducing the siRNAs

2.5 Immunoblotting

Rat Schwann cell were extracted in RIPA buffer. The protein concentration in cell and nerve extracts was determined by bicinchoninic acid assay (BCA). An equivalent amount of cellular protein (50 ug) was subjected to SDS-PAGE and electro transferred to nitrocellulose membranes. The membranes were blocked with 5% Bovine serum albumin (BSA) in Tris-HCl-buffered saline, pH 7.4, with Tween 20 and incubated with the primary antibodies to detect phospho-c-Jun, phospho- ERK, Total ERK, LRP1, GAPDH. The membranes were washed and treated with 5% non-fat milk and anti-rabbit Horseradish peroxidase (HRP) secondary antibodies for 1 h. Immunoblots were then washed and developed. Immunoblots were developed using enhanced chemiluminescence (GE Healthcare).

2.4 Nerve injury model systems and dorsal root ganglia collection.

For nerve crush injury studies, C57BL/6J mice were purchased from Charles Rivers Laboratories and housed with a 12 h light/dark cycle and ad libitum access to food and water. For surgeries, mice were initially anesthetized with 2-5% isoflurane (IsoSol; VedCo) in 1.5 liter per minute oxygen and maintained under 2% isoflurane. An incision was made through the skin below the femur. The muscle and fascia were blunt dissected using fine surgical scissors and forceps to expose the sciatic nerve. The sciatic nerve was crushed twice with flat forceps (15 seconds). The site of crush injury was then marked with an epineural suture. Mice were killed 0, 1- and 5-days post sciatic nerve crush injury. Mouse dorsal root ganglia were collected 0, 1- and 5-day post crush injury. A small dorsal incision is made at the tail to remove the fur and expose muscle and spinal column. Spinal column is isolated peeled in a rostral to caudal direction exposing the spinal cord. L3, L4, and L5 ipsilateral and contralateral DRG's are then collected.
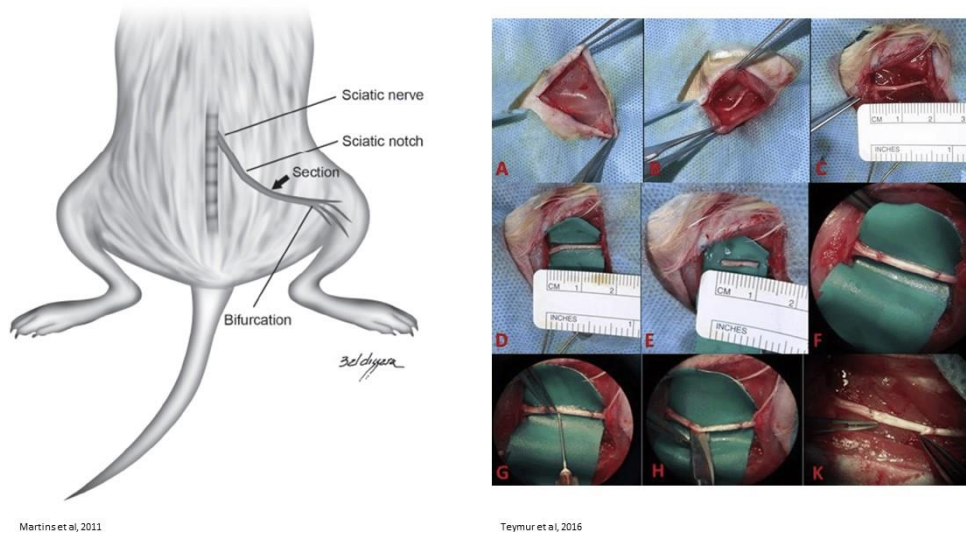
Figure 2.1 Exploration and dissection of rat sciatic nerve to release it from surrounding tissue **and illustration of rodent sciatic nerve location.** (A) Arising muscles after skin incision. (B) Exploration of nerve after separating semitendinosus and semimembranosus muscles. (C) Releasing of nerve from surrounding soft tissue. (D) In order to release sciatic nerve completely the thin motor branch to semitendinosus and semimembranosus muscles was sacrificed. (E) A 1-cm long nerve segment was measured and resected from 1–2 mm proximal to bifurcation point. (F) Reconstruction of defect with resected segment. (G) Resection of epineural layer from graft under magnification of microscope 25-gauge needle is used to incise epineurium. (H) Dissection and resection of epineurium from the central part of the graft. (K) Appearance after epineural resection.

2.6 qRT-PCR

Total RNA was isolated using the RNeasy Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized

with the iScript cDNA Synthesis Kit (BioRad, Hercules, CA, USA). qRT-PCR was performed using a

System 7300 instrument (Applied Biosystems, Carlsbad, CA) and a one-step program: 95°C, 10 min;

95°C, 30 s, 60°C, 1 min for 40 cycles (primer sequences are available from the corresponding author

upon request). GAPDH mRNA levels were determined in each sample as a normalizing gene. We and

others (Macdonald et al., 2001; Campana et al., 2006) have shown that GAPDH is an appropriate and

relatively stable housekeeping gene in peripheral nerve injury. mRNA levels were calculated by the

relative quantity ($\Delta\Delta$Ct) method. Experiments were performed in triplicate, with internal duplicate

determinations.

2.7 Statistics

Data are represented as mean ± SEMs Densitometry was performed with ImageJ (version 1.50i) and

statistical comparisons were done by one-way ANOVA and Tukey's post-hoc analysis in Prism

(GraphPad)

Chapter 3

Results

Specific recovery of extracellular proteins from sciatic nerve without cell damage

Our goal was to identify LRP1 ligands present in the extracellular spaces of the sciatic nerve,

before and after nerve injury. It was thus imperative to develop a method for isolating extracellular

proteins that did not damage intact cells and cause leakage of intracellular proteins. To test our method,

rats were subjected to sciatic nerve crush injury or sham operation. Crush injury irreversibly injures all

the axons at the site of the procedure. Injured and uninjured sciatic nerves were harvested and desheathed

by surgical dissection 24 h later. The endoneurium was then treated with 1.0% (w/v) collagenase type I

for 45 min at 37° C. After gently pelleting the cells by centrifugation ($250 \times g$), the supernatants (nerve

digest) were subjected to immunoblot analysis. Control nerves were extracted in RIPA buffer (nerve ex-

tract) after desheathing and subjected to immunoblot analysis. Fig. 3.1A shows that β-actin was present

exclusively in the RIPA nerve extracts and not in the collagenase nerve digests in uninjured nerves and in

crush-injured nerves 24 h after surgery.

Because the collagenase digests were β-actin free, we subjected the same extracts to immunoblot

analysis, probing for proteins known to be present in nerve extracellular spaces and known to bind to

LRP1. Decorin is an extracellular matrix protein present in peripheral nerves before and after nerve

injury (Gillen et al., 1995), which binds to LRP1 (Brandan et al., 2006). Fig. 3.1B shows that Decorin was present in nearly equal amounts in collagenase digests of sciatic nerves before and after crush injury (Fig. 3.1B). Rat α_1M is a constitutively expressed homologue of human α_2M present in plasma and an LRP1 ligand (Gonias et al., 1983). Rat α_1M also was detected in collagenase digests of rat sciatic nerve before and after crush injury. The level of rat α_1M was increased following crush injury.
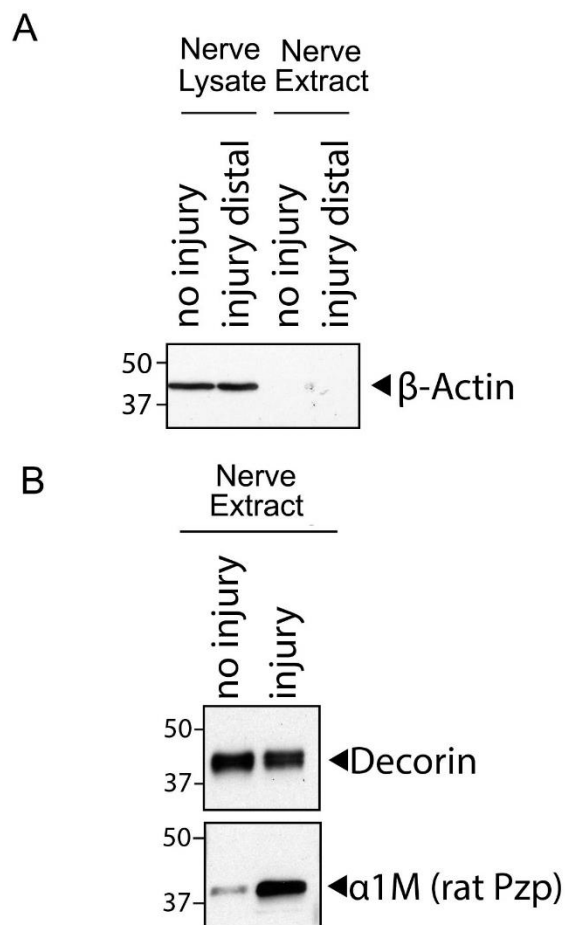
 Figure 3.1: Specific recovery of extracellular proteins Decorin and ☐M1 from sciatic nerve without cell damage. (A) Immunoblot analysis of rat nerve lysate and extracts following 1-day sciatic nerve crush injury. The endoneurium was treated with 1.0% (w/v) collagenase type I for 45 min at 37° C. Control nerve extract were extracted in RIPA buffer. (B) Immunoblot analysis of Decorin and Rat α_1M protein before sciatic nerve crush and 1 day following sciatic nerve crush of nerve extract.

Identification of LRP1 ligands in the extracellular spaces of sciatic nerves

Most of the ligand-binding activity of LRP1 reflects the activity of two of the four clusters of complement-like repeats (CCR2 and CCR4) in the structure of the LRP1 α-chain (Strickland et al., 2002). The ligand-binding specificness of CCR2 and CCR4 are highly overlapping. We expressed CCR2 as an Fc fusion protein and free Fc as a control (Stiles et al., 2013). Collagenase digests of rat sciatic nerve, isolated 24 h after crush-injury or sham operation, were incubated with CCR2 or Fc (30 μg) for 12 h at 4° C. The Fc fusion proteins were then captured with Protein A agarose, washed extensively, and subjected to trypsin digestion in the presence of ProteaseMax surfactant for LC-MS/MS. Studies were performed in triplicate.

To compare the reliability of identified CCR2-binding proteins, we generated Mass spectrometry interaction Statistics (MiST) scores, which are based on the abundance of the protein captured, reproducibility of capture, and specificity relative to the control bait (Free Fc) (Verschueren et al., 2016). CCR2-binding proteins with MiST scores higher than 0.75 are shown in Fig 3.2. Proteins sorted into two groups, those identified in the extracellular spaces of rat sciatic nerves before and after crush injury and those detected only after injury. Two known LRP1 ligands, α_1M and Decorin, were identified in the extracellular spaces of sciatic nerves subjected to crush injury or sham operation. Amongst the twenty CCR2-binding proteins with the highest MiST scores, many were intracellular proteins (Fig 3.2). Intracellular proteins identified as CCR2 ligands, which are selectively expressed in the nervous system, as reported in the Protein Atlas included NF-L, neurofilament medium peptide, Growth-arrest specific protein 7, PACSIN1, and synaptotagmin 1. Each of these proteins was detected only after sciatic nerve crush injury, apart from PACSIN1, which also was isolated from digests of uninjured nerve. PACSIN1 was previously identified as an LRP1 ligand based on CCR-2/4-binding experiments and LC-MS/MS using preparations of myelin vesicles isolated from rodent brain (Fernandez-Castaneda et al., 2013).
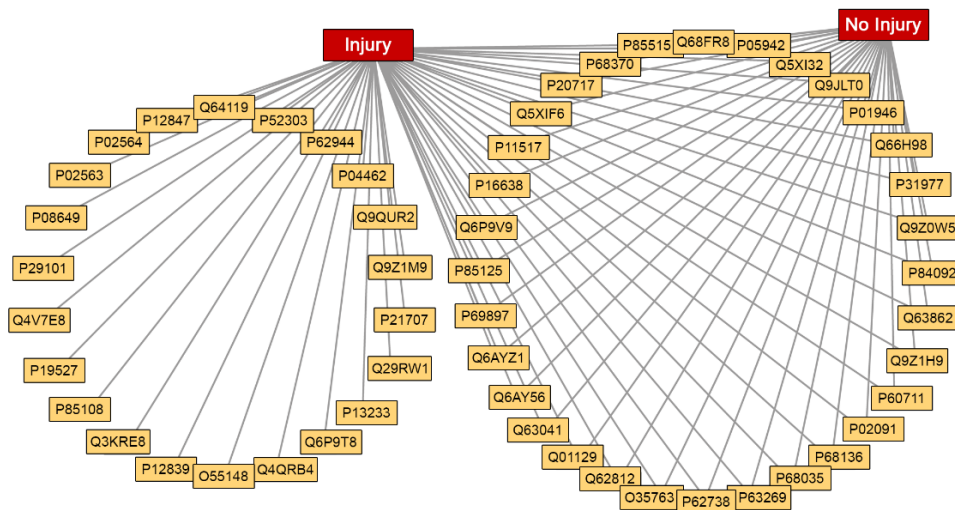
MiST	Entry	Protein name
0.99354	P08649	Complement C4
0.99127	P85515	Alpha-centractin (Centractin)
0.99060	P68370	Tubulin alpha-1A chain
0.98884	P16638	ATP-citrate synthase
0.99884	Q4V7E8	Leucine-rich repeat flightless-interacting protein 2
0.98884	P19527	Neurofilament light polypeptide (NF-L)
0.98605	Q63041	Alpha-1-macroglobulin (Alpha-1-M)
0.98554	Q01129	Decorin
0.98463	P12839	Neurofilament medium polypetide (NF-M)
0.98392	O35763	Moesin (Membrane-organizing extension spike protein)
0.98241	O55148	Growth arrest-specific protein 7 (GAS-7)
0.87977	Q9Z0W5	Protein kinase C and casein kinase substrate in neurons protein 1
0.87961	P21707	Synaptotagmin-1 (Synaptotagmin I) (SytI) (p65)
0.87809	P31977	Ezrin (Cytovillin) (Villin-2) (p81)
0.87539	Q9QUR2	Dynactin subunit 4 (Dynactin subunit p62)
0.87539	Q66H98	Serum deprivation-response protein (Cavin-2)
0.87332	Q5XI32	F-actin-capping protein subunit beta (CapZ beta)
0.85840	P62944	AP-2 complex subunit beta
0.85679	Q64119	Myosin light polypeptide
0.84537	P05942	Protein S100-A4 (Metastasin)

Figure 3.2: LRP1 ccr2 domain ligand-binding affinity: MiST scores of protein binding to the ccr2 binding domain of LRP1 from rat nerve extracts. Rat sciatic nerves were subjected to crush injuries and proteins sorted into two groups, pre and post sciatic nerve crush injury in the extracellular space with ccr2 binding ligands with a MiST score > 0.75. Notably, α_1M, Decorin, known LRP1 ligands, and PACSIN1 were identified.

PACSIN1 binds to LRP1 to facilitate downstream activation of transcription factor ERK ½

Binding of ligands to LRP1 regulates diverse cell signaling pathways in Schwann cells

(Mantuano et al., 2008a, b, 2010). When LRP1 is deleted conditionally in Schwann cells in mice,

abnormalities in the response to PNS injury are observed, including accelerated demyelination, abnormal

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regeneration and neuropathic pain (Orita et al., 2013). In this study, we examine PACSIN1 role as a ligand that binds to LRP1, and the downstream phosphorylation of ERK1/2 and c-Jun. To test this, we silenced LRP1 in rat SC's. In Schwann cells, that were transfected with non-targeting control (NTC) siRNA and treated with PACSIN1 or NRG1 for 10 min, ERK1/2 was activated. In cells transfected with LRP1- specific siRNA, the response to PACSIN1 was completely blocked and the response to NRG1 was decreased (Fig.3.2). Once the cultured rat Schwann cells had LRP1 knockdown, phosphorylated ERK1/2 expression was downregulated. To ensure LRP1 was being blocked, we probed with anti-LRP1 antibody. In SCs that were transfected with non-targeting control (NTC) siRNA and treated with PACSIN1 or NRG1 for 10 min, LRP1 was activated (Fig. X). In cells transfected with LRP-specific siRNA, the response to PACSIN1 and NRG1 was completely blocked. We conclude that activation of ERK1/2 requires PACSIN1 binding to LRP1.
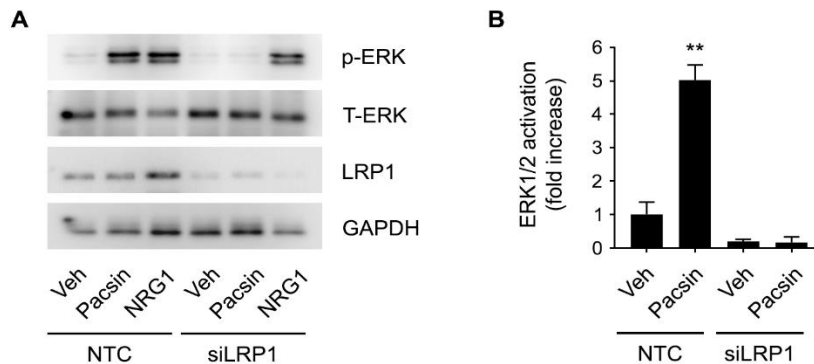

Figure 3.3: LRP1 gene silencing in rat Schwann cells blocks phosphorylation of ERK1/2 in response to LRP1 ligand PACSIN1. (A) Schwann cells transfected with NTC or LRP1-specific siRNA. PACSIN1 (50nM) and NRG1 (1nM) added after 45 mins for 15 mins. (B) Quantification of LRP1 silencing and ERK1/2 activation results. Data are expressed as the mean ± s.e.m. (n=4, ** denotes $p < 0.01$ compared with the vehicle control).

PACSIN1 binds to NMDA-R to facilitate downstream activation of ERK1/2 and c-Jun

In neurons, LRP1 signaling requires the NMDA-R as an essential co-receptor (Bacskai et al., 2000; Qiu et al., 2002; Samson et al., 2008; Martin et al., 2008; Sheng et al., 2008; Mantuano et al., 2013). PACSIN1 binding to LRP1/ NMDA co-receptor is not well known. In this study we examined the role NMDA-R has in relation to PACSIN1 binding. To test whether PACSIN1 binding to NMDA-R is required for LRP1 signaling in Schwann cells, first we pre-treated Schwann cells with MK801 (1 µM), an uncompetitive antagonist of the NMDA-R. We then treated Schwann cells with 50 nM PACSIN1. Fig 3.3 shows that MK801 blocked c-Jun and ERK1/2 phosphorylation in response to PACSIN1 and NRG1. These findings suggest that NMDA-R is a necessary co-receptor for activation of ERK1/2 and c-Jun in response to Pacsin1 binding to LRP1.
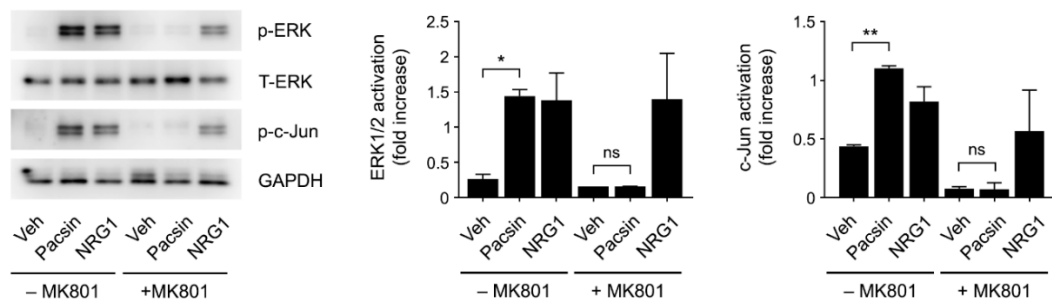
Figure 3.4: Neutralizing NMDA-R blocks ERK1/2 activation in response to LRP1 binding ligand PACSIN1 in rat Schwann cells. SCs were pre-treated with 1 µM MK801 (+) or with vehicle (−) for 45 min and then with PACSIN1 (50 nM), NRG1 (1 nM) or vehicle (PBS) for 15 min. Quantification of ERK1/2 and c-Jun activation is one -tailed paired t-test. Data are expressed as the mean ± STD. (n=3, *P<0.01 compared with the vehicle, PACSIN1, NRG1).

Sciatic nerve crush injury decreases mRNA expression of PACSIN1 in DRGs after crush injury

PACSIN1 mRNA expression has not been previously studied in the dorsal root ganglion cells (DRG). PACSIN1 was identified in small and large diameter cell bodies. PACSIN1 was not expressed in surrounding satellite cells or other cell types in the DRG. PACSIN1 was specifically expressed in neurons. These findings are consistent with studies of PACSIN1 in the CNS (Polmann et al, 2008). We observed no substantial change in PACSIN1 levels in contralateral, ipsilateral or the uninjured (naive) model of DRG neurons one day following sciatic nerve crush injury (Fig 3.5 A). Five days after sciatic nerve crush injury both protein and mRNA expression were reduced in the ipsilateral DRG. PACSIN1 mRNA expression was reduced by 60% (Fig 3.5 B) Large diameter cell bodies, containing the PACSIN1 intracellular protein, appeared to be the most affected: less PACSIN immunoreactivity was observed in the large diameter neurons. These findings suggest that PACSIN1 mRNA expression is not only reduced in the PN following injury, but cell bodies containing PACSIN1 are also being transported away from the DRG of the injured nerve. In contrast, the contralateral nerve PACSIN1 mRNA expression was not affected by the ipsilateral crush injury. The contralateral portion showed no significant change in PACSIN1 mRNA expression compared to the uninjured, naïve mouse model. (Interesting work but confusingly described; too much dependence on ipsilateral and contralateral and big and small bodies; please rephrase this paragraph)

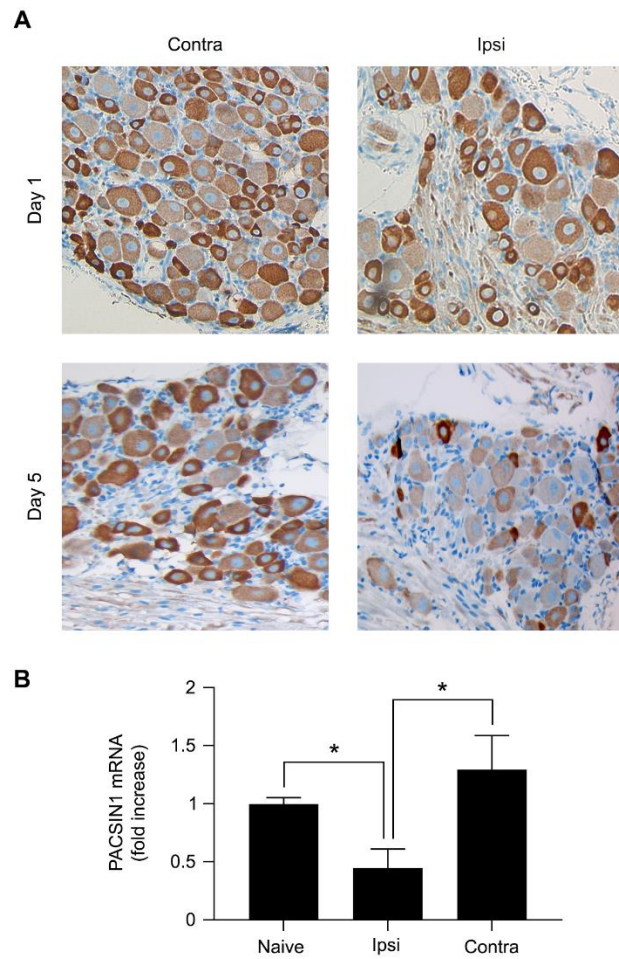**Figure 3.5: PACSIN1 mRNA expression is decreased in mouse DRG's following sciatic nerve crush injury** (A) Immunofluorescence microscopy was performed to show PACSIN1 expression in mouse DRG 1dpc and 5dpc. PACSIN1 immunoreactivity (red) in cell bodies. (B) Quantification of mRNA results. Paired t-test performed. Data are expressed as the mean ± STD. (n=6–8, *P<0.01 compared with the vehicle, naïve, control).

Chapter 4

Discussion

Over the course of this thesis project, for the first time, we characterized the neuronal-specific LRP1 ligand, PACSIN1, and its role in the PNS. We discovered that PACSIN1 is capable of binding and activating LRP1-dependent cell signaling. In addition, PACSIN signaling requires the LRP1 coreceptor / NMDAR for activation of c-Jun and ERK1/2. Finally, we show that indeed PACSIN1 is neuro-specific, being founs in both small and large diameter DRG neurons. After crush injury, large diameter neurons appear to decrease PACSIN1 expression.

SC survival immediately after peripheral nerve injury is essential for nerve regeneration (Meier et al., 1999; Jessen and Mirsky, 2005). The activation of the SC repair program is key in facilitating functional recovery from PNS injury and in preventing neuropathic pain (Flütsch et al., 2016). When activated by injury, SCs synthesize growth factors (Meyer et al., 1992; Zhang et al., 2000; Li et al., 2005) and extracellular matrix proteins (Akassoglou et al., 2000), phagocytose myelin, migrate, and provide scaffolds that are essential for peripheral axon regeneration (Jessen and Mirsky, 2008). Characterizing the molecular signals that assist in SC survival after nerve injury has implications for understanding disease mechanisms in peripheral neuropathies (Berger and Schaumburg, 1995; Suter and Scherer, 2003).

The SC's ability to detect peripheral nerve injury prior to transforming phenotypically to a repair phenotype, is vital to the injured peripheral nerve's success in recovery. An important receptor in the detection of peripheral nerve injury is LRP1 and it is vital in the activation of the SC repair program (Flütsch et al, 2016). Furthermore, LRP1 ligands have been implicated in neuronal survival (Hayashi et al., 2007; Fuentealba et al., 2009) and in neurite outgrowth (Mantuano et al., 2008a, b; Shi et al., 2009; Yoon et al., 2013). Thus (?), identifying LRP1 ligands that play a role in the SC repair program is vital to characterizing the SC and its disease mechanisms in peripheral neuropathies.

LRP1 is up-regulated rapidly in SCs after nerve injury (Campana et al., 2006) and capable of activating ERK1/2 and c-Jun in response to known LRP1 ligands (Flütsch et al, 2016). In the injured nerve, these ligands were not fully characterized or well known. Binding of ligands to LRP1 regulates diverse cell signaling pathways in Schwann cells (Mantuano et al., 2008a, b, 2010), including the nerve repair pathway. Identifying and characterizing novel ligands is vital in further understanding the mechanisms involved in the activation of the SC repair program. We were able to isolate extracellular proteins released after nerve injury that bind specifically to the LRP1-binding domains CCR2 and CCR4, using novel LRP1 capture technology and Mass Spectrometry (Fig 3.2). Doing so, we identified the intracellular neuronal ligand, PACSIN1, which appears to be regulated after sciatic nerve injury. We were able to characterize PACSIN as a novel LRP1 ligand that is endogenous and carried in large and small diameter neurons. The combination of upregulated LRP1 and PACSIN1 following a nerve injury may lead to the activation of the SC repair program *in vivo*, thereby contributing to nerve repair.

When the LRP1 gene is deleted in SCs in mice, numerous abnormalities are observed following sciatic nerve injury, including accelerated demyelination, increased infiltration of the nerve by inflammatory cells, and abnormal regeneration (Orita et al., 2013). Similarly, injecting RAP directly into injured sciatic nerves of rodents blocks LRP1-dependent cell signaling and promotes Schwann cell death (Campana et al., 2006; Mantuano et al., 2008a, b). This confirms the importance of the role of LRP1 in not only nerve repair but maintenance. Therefore, characterizing PACSIN1's role in LRP1-dependent cell signaling is vital. We hypothesize that in nerve injury, the newly discovered LRP1 ligand, PACSIN1, activates c-Jun and ERK1/2 in an LRP1-dependent manner and is necessary for nerve repair. To test this hypothesis, we established primary Schwann cell control cultures and genetically silenced LRP1 cultures. We then probed for ERK1/2 and c-Jun in response to the LRP1 ligand, PACSIN1. When PACSIN1 was added to the cultures, ERK1/2 and c-Jun were activated in control cells, but failed to activate in LRP1 silenced cells. This confirms that LRP1 gene silencing in rat Schwann cells blocks phosphorylation of ERK1/2 and c-Jun in response to the LRP1 ligand, PACSIN1. Furthermore, this confirms the importance of LRP1 as a necessary receptor for PACSIN1 to induce activation of the SC repair program.

Rat SC's have been shown to express NMDA-R which is an essential signaling receptor in response to proteins known to be present in the injured PNS (Mantuano et al, 2015). Based on NMDA-R interaction with LRP1, the NMDA-R functions as an essential signaling co-receptor for LRP1 ligands, including α2- macroglobulin (α2M), tissue-type plasminogen activator (tPA) and apolipoprotein E (Bacskai et al., 2000; Qiu et al., 2002; Samson et al., 2008; Martin et al., 2008; Sheng et al., 2008; Mantuano et al., 2013).We now show that NMDA-R is required for activation of ERK1/2 and c-Jun in response to LRP1 ligand, PACSIN1 in Schwann cells *in vitro*. These results suggest that NMDA-R functions as a co-receptor in PACSIN induced cell signaling. Whether PACSIN1 is capable of directly binding NMDA-R remains unknown. Binding of both LRP1 and NMDA-R has been shown for other LRP1 ligands such as tPA (Chevilley et al, 2015). Furthermore, our results support a model in which the NMDA-R can be an important regulator in PACSIN1 ability to activate Schwann cell repair in Schwann cell physiology. For the first time, we show that PACSIN1 is an activator of the SC repair program by inducing phosphorylation of c-Jun and ERK ½ in a LRP1/NMDA dependent manner.

Following nerve injury, axonal regeneration depends on the intrinsic growth capacity of neurons and the reaction of glial cells, which expresses proteins that inhibit or promote axonal regeneration. Injured neurons and cells will secrete multiple neurotrophic factors, adhesion molecules, and extracellular matrix molecules that will facilitate peripheral nerve regeneration (Glenn Yiu and Zhigang He, et al 2006). Growth factors play a key role in regeneration such as nerve growth factor (NGF), neurotrophin-3 (NT-3), vascular endothelial growth factor (VEGF), neuregulin-1 (NRG1) and Insulin-like growth factor 1 (IGF-1) (Thompson et al, 2016). However, PACSIN1 is a unique intracellular protein, in that it is neuronal specific and is only released from neurons following peripheral nerve injury. Further studies examining PACSIN1 signaling in vivo are warranted. In our studies, PACSIN1 was shown to be present in the cell bodies of large diameter and small cell bodies in the DRG. Following nerve injury, PACSIN1 levels are reduced, and particularly in large neurons. The mechanisms underlying this reduced expression remain unclear. Several possibilities exist for this reduced expression of PACSIN1 after injury. One possibility for the reduction in PACSIN1 expression may be that PACSIN1 is transported form the DRG

or transcription/translation is reduced. Furthermore, myelination may be a reason that larger diameter axons are affected the most following injury. (Fig 3.4), We examined mRNA expression in DRG's because it would give us better insight into where PACSIN1 gene was store prior to injury and if cell bodies containing PACSIN1 where released from neuron in a way that facilitates the activation of the SC repair program. The cell bodies containing PACSIN1in DRG's show no significant difference in mRNA expression between naïve WT uninjured mice and day 1 and day 5 contralateral injured mice. This suggest that contralateral to the injury site can function as a comparative control. We hypothesized that mRNA expression would decrease in ipsilateral DRG's compared to contralateral in the same mouse. Figure 3.4 shows that there is a significant decrease in ipsilateral DRG mRNA expression compared to contralateral. This confirms immunostaining of DRG's cell bodies containing PACSIN1 (fig. 3.4). This suggest that mouse DRG's house PACSIN1 mRNA in cell bodies and translate PACSIN1 mRNA into protein within sensory neurons. Following a nerve injury, these cell bodies will release PACSIN1 containing cell bodies gradually over time, decreasing the cell body count and size to the site of injury.

Conclusion

We have shown that the neuro-specific intracellular ligand PACSIN1 binds to LRP1/ NMDAR complex post nerve injury resulting in the activation of transcription factors c-Jun and ERK1/2, two transcription factors that are vital in the SC repair program following peripheral nerve damage. When LRP1 is blocked or NMDAR is antagonized, c-Jun or ERK 1/2 are not activated in response to the PACSIN1 ligand, suggesting that LRP1/NMDAR do not function independently in the presence of PACSIN1 binding. Furthermore, PACSIN1 mRNA is present in uninjured DRG of mice and is released gradually from DRG's following the peripheral nerve injury, with significant downregulation of PACSIN1 expression 5 days after injury. PACSIN1 is possibly transported to the site of injury from cell bodies containing the intracellular ligand in the DRG.

Future Directions

PACSIN1 is neuro-specific and has been shown to be expressed in the DRG and nerve of mouse and rats. However, how PACSIN1 is transported to the site of injury is not known. An animal model developed to study neuropathic pain is the partial sciatic nerve ligation (PSNL) model, which mimics some of the major features observed in clinical neuropathic pain (Seltzer Z et al., 1990). The PSNL model of neuropathic pain refers to a rodent neuropathic pain model that is produced by tightly ligating the dorsal third to half of the common sciatic nerve at the upper-thigh level (Seltzer et al.,1990).

The PSNL model could well serve as a way for understanding PACSIN1 transport along axons following peripheral nerve injury. Following injury, PACSIN1 is released gradually from DRG's, eventually to the site of injury. If we can track the location of PACSIN1 release and accumulation following injury, we can create a model for PACSIN1 transport. We can examine whole sciatic nerve and DRG, both ipsilateral and contralateral, following a PSNL. We hypothesize that PACSIN1 is released from DRG's in an anterograde fashion to regenerating nerve fibers.

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