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proNGF, sortilin, and p75^{NTR}: potential mediators of injury-induced apoptosis in the mouse dorsal root ganglion

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Abstract

The nerve growth factor precursor (proNGF) may function as a death-inducing ligand that mediates its apoptotic effects via p75^{NTR}. ProNGF-induced apoptosis is postulated to be dependent upon membrane expression of the sortilin receptor, which interacts with p75^{NTR} to promote a high affinity-binding site for proNGF. Here, we explore the expression of proNGF, sortilin and p75^{NTR} in the mouse lumbar dorsal root ganglion (DRG) to understand the potential for this trimeric signaling complex to function in injury-induced neuronal death of DRG neurons. Our results reveal the expression of all 3 components within the DRG and that a subpopulation of neurons coexpresses sortilin and p75^{NTR}. Following sciatic nerve transection, the expression of these proteins appears insensitive to injury; however, the majority of small p75^{NTR}-sortilin coexpressing neurons are lost 25 days after sciatic nerve transection. These results propose proNGF-induced, p75^{NTR}-sortilin mediated neuronal death as a critical aspect of nerve-injury induced death in the DRG.

Keywords

cell death; DRG; mouse; proNGF; sortilin; p75^{NTR}; peripheral nerve

Introduction

Traumatic nerve injury frequently leads to loss of function due to neuronal degeneration and death. The molecular mechanisms involved in injury-induced neuronal death are poorly understood, but are thought to involve apoptosis, or programmed cell death (Ekstrom, 1995). Recently, several molecules have been identified as key players in the induction of neuronal death. Sortilin, a type I membrane receptor expressed in neuronal tissues and thought to be involved in membrane trafficking, has been shown to interact with the p75 neurotrophin receptor (p75^{NTR}) in the induction of apoptosis upon binding unprocessed nerve growth factor (NGF), also known as proNGF (Nykjaer et al., 2004).

Neurotrophins are growth factors released by target tissues that can regulate cell survival and death signaling. Neurotrophins, such as NGF, are synthesized as precursors known as proneurotrophins. These pro-forms are susceptible to intracellular as well as extracellular cleavage by numerous proteolytic enzymes including furin, plasmin and MMP (Lee et al., 2001). Proneurotrophins have long been thought to be inactive precursors to their mature

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counterparts, but recent evidence has identified proNGF as the predominant form of NGF in many tissues, recognizing it as a potentially biologically important molecule (Lee et al., 2001; Yardley et al., 2000; Fahnestock et al., 2001; Pedraza et al., 2005; and Reinshagen et al., 2000). Neurotrophins are capable of mediating profound diverse biological effects depending on processing of the pro-forms and their interaction with two cell surface receptors, p75^{NTR} and the *trk* family of tyrosine kinase receptors. For example, mature NGF preferentially binds to *trk* A and promotes cell survival. However, recent reports have demonstrated that proneurotrophins, such as proNGF, selectively bind to p75^{NTR} and activate its pro-apoptotic signaling cascade (Nykjaer et al., 2004). The formation of a signaling complex in which the sortilin receptor interacts with p75^{NTR} is required for proNGF-mediated neuronal death to occur (Nykjaer et al., 2004). ProNGF simultaneously binds sortilin (via the pro-domain) and p75^{NTR} (via the mature domain) and thereby acts as a crosslinker, creating a tertiary complex that results in the activation of neuronal apoptosis.

Evidence identifying proNGF as a physiological ligand for p75^{NTR} under pathological conditions continues to build. Recent reports demonstrate that proNGF expression is induced after brain injury and causes death of adult corticospinal neurons upon binding p75^{NTR} *in vivo* (Harrington et al., 2004). Likewise, proNGF mediates cell death of oligodendrocytes following spinal cord injury *in vivo* (Beattie et al., 2002) and has been suggested to be responsible for the neurodegeneration found in the brains of Alzheimer's disease patients (Pedraza et al., 2005).

In this report, we investigate whether members of the proNGF-p75^{NTR}-sortilin signaling complex could potentially be responsible for the injury-induced apoptosis of primary sensory neurons in the lumbar L4/5 dorsal root ganglia (DRG). The results presented here demonstrate that members of this signaling complex are present in the L4/5 DRG and their expression is not significantly altered soon after injury. Moreover, there is a population of L4/5DRG neurons that coexpress both sortilin and p75^{NTR} and that these neurons may be susceptible to injury-induced neuronal death 25 days following sciatic nerve transection. These data propose this signaling complex as a potential mechanism driving injury-induced apoptosis in certain L4/5 DRG neurons.

Results

Sortilin is present in the mouse L4/5 DRG and its expression is not responsive to injury

Immunocytochemistry was performed to identify L4/5 DRG neurons that express sortilin. Cell counts of sortilin-immunoreactive neurons revealed that 73.8% \pm 4.82 of L4/5 DRG neurons express the sortilin receptor (Fig. 1). Neurons that displayed sortilin immunoreactivity displayed a diffuse cytoplasmic distribution of sortilin, as well as superficial membrane staining. (Fig. 1). The cytoplasmic distribution of sortilin observed here is consistent with previous studies that localize over 90% of sortilin expression to intracellular vesicles including the golgi apparatus and endo- and lysosomal compartments (Petersen et al., 1997 and Mazella et al., 1998). Next, we evaluated the responsiveness of sortilin expression to injury. L4/5 DRG were examined for sortilin expression 3 days following sciatic nerve transection. At this time point, injury results in little neuronal death but effects the expression of many neuropeptides (Shi et al., 2001 and McKay Hart et al., 2002). Cell counts of sortilin-immunoreactive neurons after injury revealed a similar pattern on staining and 72.8% \pm 3.50 of neurons were sortilin-positive (Fig. 1). An unpaired t-test determined that there was no significant difference in the number sortilin-expressing neurons between injured and uninjured groups ($p = 0.3573$). To substantiate this result, we used Western blot analysis to quantify sortilin peptide expression. Using the same antisera, we were able to detect a band of 95 kDa, which is consistent with the molecular weight of sortilin (Petersen et al., 1997) (Fig. 2A). Peptide expression was quantified by analyzing band intensity and no significant difference in the number of neurons expressing

sortilin was observed between injured and uninjured groups ($p = 0.8643$). Collectively, these findings support the view that sortilin protein expression is not altered in response to injury of peripheral axons of L4/5 DRG neurons.

p75^{NTR} is present in the L4/5 DRG and its expression is not responsive to injury

DRG neurons expressing p75^{NTR} were identified using a primary polyclonal antibody and neuronal counts revealed that 66.1% \pm 2.83 of L4/5 DRG neurons from uninjured mice express p75^{NTR} (Fig. 1). According to the literature, p75^{NTR} expression is upregulated in response to injury, throughout various disease states, and during development (King et al., 2000; Syroid et al., 2000; and Crockett et al., 2000). However, these studies were carried out in the central nervous system. Similarly, we assessed if p75^{NTR} expression was altered by sciatic nerve transection. Analysis of L4/L5 DRGs 3 days following sciatic nerve transection revealed a similar pattern of p75^{NTR} and counts of 67.2% \pm 4.43 p75^{NTR}-positive neurons (Fig. 1). An unpaired t-test confirmed no significant difference in the average number of neurons expressing p75^{NTR} between groups ($p = 0.8248$). To confirm these results, we employed Western blot analysis to re-examine the effects of injury upon p75^{NTR} expression. Application of the primary polyclonal antibody resulted in the presence of a ~75 kDa band along with bands of approximately ~55, ~135, and ~155 kDa in size (Fig. 2B). These multiple bands were present in samples taken from both injured and uninjured mice. Band intensity was analyzed to detect any alteration in peptide expression. An unpaired t-test did not detect any significant difference in p75^{NTR} expression between groups ($p = 0.7836$), confirming our immunocytochemical results.

Sortilin is expressed in several neuronal subpopulations within the lumbar DRG

Next, we sought to determine if sortilin expression is confined to a specific neuronal subpopulation within the DRG. These neurons can be divided into 3 broad subcategories based on expression of neuronal markers. The first class consists of neurons that do not express the classic neuropeptides generally associated with pain transmission; however, they will bind the isolectin IB4, which is specific for small, non-peptidergic sensory neurons (Kashiba et al., 2001). The second subpopulation consists of neurons that produce calcitonin gene-related peptide (CGRP), which exclusively identifies the small- and medium-sized peptidergic sensory neurons (Silverman et al., 1988). Finally, there is a class of neurons that can be identified using the neuronal marker N52, which is specific for large, myelinated neurons. Uninjured L4/5 DRG neurons that express both the sortilin receptor and a specific neuronal marker were counted. Immunocytochemistry revealed 13.6% \pm 1.16 of sortilin-positive neurons express IB4. Alternatively, 31.7% \pm 3.69 of IB4-positive neurons expresses the sortilin receptor (Table 1, Fig. 3). Approximately 20.6% \pm 1.47 of sortilin-expressing neurons coexpress CGRP, while 44.1% \pm 3.10 of CGRP-positive neurons express sortilin (Table 1, Fig. 3). Finally, approximately 18.2% \pm 2.57 of neurons that express sortilin also coexpresses N52. On the contrary, 52.8% \pm 4.95 of N52-expressing neurons display sortilin coexpression (Table 1, Fig. 3). In conclusion, sortilin expression is found within the small non-peptidergic neurons, the small-medium sized peptidergic neurons, and finally the large, myelinated neurons. These data indicate that sortilin expression is not confined strictly to one primary sensory neuronal population, but rather it is found within all classes of L4/5 DRG neurons.

proNGF is the predominant form of NGF present in the mouse L4/5 DRG

Next, we sought to determine if the pro-form of nerve growth factor is present in the mouse DRG and if its expression is responsive to injury. To date, this was the first assessment of proNGF expression in the mouse L4/5 DRG. Western blot analysis using a polyclonal antibody designed to detect the pro-form of proNGF detects bands of approximately ~35, ~40, ~44, and ~87 kDa in size (Fig. 4A). Each band is present in all of the treatment groups (no injury and

sciatic nerve crush or transection). The ~35kDa band closely resembles the molecular weight of the unglycosylated NGF precursor, as detected by Yardley and colleagues (Yardley et al., 2000). The ~40, ~44, and ~80 kDa bands most likely represent post-translationally modified forms of proNGF (Yiangou et al., 2002 and Lobos et al., 2005). To address the effects of injury upon proNGF expression, band intensity was analyzed between treatment groups. ANOVA did not reveal a significant effect of group on band intensity, indicating that proNGF expression is not altered in mouse L4/5 DRG neurons in response to peripheral sciatic nerve injury ($p = 0.9990$). Next, we inquired if the bands detected by our proNGF antibody truly represented peptides of proNGF nature. We used a polyclonal antibody aimed at identifying the mature domain of NGF. First, using a commercial source of recombinant NGF peptide, we ensured that this particular antibody would indeed detect mature NGF (Fig. 4B). Similarly, the same bands of ~35, ~40, ~44, and ~87 kDa were again detected in each treatment group. On the contrary, a band of ~91 kDa was also present which was not detectable with the use of proNGF antisera, which suggested the band represented at ~91 kDa to not be of true NGF origin (Fig. 4C). Interestingly, we were not able to distinguish a band for mature NGF (~13 kDa), signifying that mature NGF is not present in detectable quantities under injured or uninjured conditions in the mouse L4/5 DRG. Again, band intensity was analyzed and no significant difference in peptide expression was detected between groups using one way ANOVA ($p = 0.9868$).

Certain DRG neurons co-express sortilin and p75^{NTR}

Next, we examined if there exists within the L4/5 DRG a unique population of neurons that coexpress the sortilin and p75^{NTR} receptors, making them particularly vulnerable to proNGF-induced neuronal death. To examine this matter, we used immunocytochemistry to identify which neurons were positive for both sortilin and p75^{NTR}. First, the total number of neurons expressing either the sortilin or the p75^{NTR} receptor was determined. Next, we assessed the percentage of sortilin-positive neurons coexpressing p75^{NTR} and vice versa, the number of p75^{NTR}-positive neurons coexpressing sortilin in uninjured animals (Table 2). Analysis revealed 52.5% \pm 3.77 of sortilin-positive neurons express p75^{NTR}. Alternatively, 50.7% \pm 3.84 of p75^{NTR}-positive neurons coexpress the sortilin receptor. Three days following sciatic nerve transection, 54.5% \pm 2.82 of sortilin-positive neurons continue to coexpress p75^{NTR} and 49.9% \pm 0.83 of p75^{NTR}-positive neurons maintain sortilin expression. These data suggest that there is no significant change in the number of neurons coexpressing sortilin and p75^{NTR} 3 days after injury.

To determine if this neuronal subpopulation was vulnerable to death following injury, we extended the time frame at which neuronal death was assessed and examined the number of sortilin-p75^{NTR} coexpressing neurons 25 days following sciatic nerve transection. We observed a 59.4% loss of sortilin-p75^{NTR} coexpressing neurons compared to uninjured animals 25 days following sciatic nerve axotomy.

In addition, we sought to define which particular class of neurons among the sortilin-p75^{NTR} coexpressing neuronal population is particularly susceptible to injury-induced neuronal death. To identify this population, we measured the size of sortilin-p75^{NTR} coexpressing neurons present in uninjured mice but absent 25 days following sciatic nerve axotomy (Fig. 5). These data indicate that small neurons (0–200 μm^2) comprise 58.03% of the total number of neurons found in uninjured mice. However, neurons of an equivalent area were completely absent in the injured group. Likewise, medium-sized neurons (200–400 μm^2) compose 22.79% of the uninjured population while only 1.84% of neurons measured were found of the same area within the injured group. Finally, 19.18% of the total neuronal population can be classified as large (<400 μm^2) in uninjured mice while this size class accounts for the majority of the total neuronal population (98.16%) in injured mice. These results are represented in a histogram-based model by a large shift to the right, indicating that the percentage of small and medium-

sized neurons is drastically reduced in injured mice while the large-sized neuronal population is maintained (Fig. 8). These data suggest that among sortilin-p75^{NTR} coexpressing DRG neurons, the small and medium-sized neuronal populations are included in those vulnerable to injury-induced neuronal death.

Discussion

Peripheral nerve injuries are very common, with several hundred thousand cases occurring each year within the United States alone (Wiberg et al., 2003). Neurodegeneration occurs as a result of traumatic nerve injury. To date, the mechanisms underlying this pronounced neuron loss remain to be revealed. It is clear that a better understanding of the molecular mechanisms involved in injury-induced neuronal death is critical to the creation of new therapeutic strategies aimed at preventing the sensory deficits that result from peripheral nerve injuries.

The purpose of this study was to identify a possible mechanism for the neuronal death that occurs amongst the primary sensory DRG neurons following sciatic nerve injury. Recently, several key players have been implicated in the induction of neuronal death; including the sortilin and p75^{NTR} receptors and the death-inducing ligand, proNGF. Sortilin and p75^{NTR} cooperate to promote a high-affinity binding site for proNGF (Nykjaer et al., 2004). These 3 individual components thereby interact to create a trimeric signaling complex that is capable of inducing apoptosis. Our first goal was to confirm the existence of these pro-apoptotic components in the L4/5 DRG in order to determine if these participants might indeed be responsible for the injury-induced neurodegeneration observed here.

proNGF

The proneurotrophins, such as proNGF, have long been thought to be inactive precursors to their mature counterparts. However, recent publications show that proneurotrophins are detectable in the extracellular environment, indicating that they may have biologically important roles (Lee et al., 2001). In fact, proNGF is the dominant form of NGF present in many tissues (Chen et al., 1997; Harrington et al., 2004; Yardley et al., 2000; Fahnstock et al., 2001; Pedraza et al., 2005; Fahnstock et al., 2004; Seidah et al., 1996 and Reinshagen et al., 2000). Recently, it has been reported that proNGF induces apoptosis in cells coexpressing sortilin and p75^{NTR} *in vivo*, identifying proNGF as a pathological ligand (Pedraza et al., 2005; Harrington et al., 2004; Beattie et al., 2002; and Volosin et al., 2006). The many isoforms represent the multiple cleavage and glycosylation sites available, which render proNGF susceptible to post-translational modification. Here, all of the isoforms were present in each of the treatment groups; no injury, and sciatic nerve crush or transection. Furthermore, none of the isoforms displayed any modification in response to injury.

Interestingly, we were not able to detect the presence of mature NGF (~13 kDa) in the C57Bl/6 mouse L4/5 DRG. This finding is not altogether surprising since several other studies have reported the presence of proNGF but not mature in tissues such as rat DRG, brain, spermatid, prostate and skin (Chen et al., 1997; Delsite and Djakiew, 1999; Reinshagen et al., 2000; Yardley et al., 2000; Fahnstock et al., 2001). Taken together, these data suggest that proNGF is present as the predominant form of NGF in the mouse L4/5 DRG and furthermore, that proNGF expression is not modified following sciatic nerve injury, in contrast to other neurotrophins such as brain derived neurotrophic factor (BDNF) (Karchewski et al., 2002).

Sortilin

Sortilin, a member of the Vps10p family of receptors, cooperates with p75^{NTR} to promote high-affinity binding of proNGF. We first sought to determine if the sortilin receptor was expressed in the mouse DRG and our results indicate that the majority of L4/5 DRG neurons express

sortilin (~73%). Furthermore, a similar percentage of neurons continue to express sortilin 3 days after injury. Since there is no significant neuronal loss in L5 mouse DRG three days following sciatic nerve axotomy (Shi et al., 2001), these results suggest that sortilin peptide expression is not altered in response to injury.

Our studies also revealed that sortilin is expressed by the majority of L4/5 DRG neurons and is generally expressed by all 3 neuronal subpopulations examined, including IB4, CGRP, and myelinated N52+ large neurons. Although sortilin expression was found in all subpopulations of DRG neurons, it is unclear why sortilin expression is not found within all neurons of the DRG. One explanation is that sortilin might define a target specific pattern of expression. For example, neurons which project to muscle and skin might express sortilin, but not neurons navigating to viscera. These questions can be addressed in the future using retrograde labeling studies to help determine if sortilin expression is target dependent.

p75^{NTR}

p75^{NTR} is a member of the tumor necrosis factor (TNF) receptor superfamily (Roux and Barker, 2002). To date, there has been no enzymatic activity associated with p75^{NTR}, but its associations with several cytoplasmic interactors, such as NRIF, TRAF6, and NUAGE, allow it to induce apoptosis via a cytoplasmic signaling cascade (Kenchappa et al., 2006). The exact molecular events involved in activation of the p75^{NTR} apoptotic pathway are not completely understood but are thought to involve the activation of c-Jun N-terminal kinase (JNK). Further downstream events include phosphorylation of c-jun, and activation of p53 as well as the “BH3-domain only” family members Bad and Bid, which results in mitochondrial release of cytochrome c and the consequent activation of caspases (Nykjaer et al., 2005). p75^{NTR} is a requirement for proNGF-induced neuronal death to occur. This study demonstrates the presence of p75^{NTR} in a majority of DRG neurons (66%), localizing the third and final component of the death-inducing complex to the DRG. Furthermore, the data presented here establish that p75^{NTR} expression is found in 67% of neurons following injury, indicating the p75^{NTR} expression is not altered following peripheral sciatic nerve injury in the DRG. This result was surprising as previous studies have described dramatic alterations in p75^{NTR} expression after injury (Byers et al., 1992, Arendt et al., 1995, Zhou et al., 1996; Syroid et al., 2000, King et al., 2000, Xie et al., 2003). One possible explanation for these inconsistencies is the timeframe at which p75^{NTR} expression was assessed after injury. Although the expression of many peptides is modified 3 days after injury, the discrepancies could potentially be attributed to the designated stage of examination in this study. Importantly, however, studies by Zhou et al., 1996 revealed that increases p75 after injury is due to selective upregulation within glial cells in the DRG, and that neuronal expression of p75 concomitantly decreases. Overall, these responses culminate in an overall lack of change in p75 expression in the DRG (Zhou et al., 1996), thus supporting our findings that overall p75 protein levels do not change after injury.

We found 4 distinct forms of the p75^{NTR} receptor by western blot analysis. The band of ~ 75 kDa represents the full-length receptor, while the band of ~ 55 kDa most likely represents the intracellular domain (ICD) which is cleaved by γ -secretase in response to binding proapoptotic ligands in neurons (Kenchappa et al., 2006 and Humpert et al., 2007). Higher molecular weight bands of ~155 and ~135 kDa potentially represent a p75^{NTR} dimer and accompanying post-translational modifications, respectively.

Previous studies have shown that p75^{NTR} expression is confined to specific subclasses of neurons within the DRG. p75^{NTR} is coexpressed in almost all of the neurons that express the *trk A* or *trk B* receptor (CGRP-positive subpopulation) while only approximately 50% of the *trk C*-expressing neurons (N52-positive neuronal subpopulation) are positive for p75^{NTR} expression. Interestingly, p75^{NTR} expression is not found in the small, non-peptidergic sensory

neurons (IB4-positive neuronal subpopulation), theoretically rendering this class of neurons resistant to proNGF-induced neuronal death (Wright and Snider 1995). Results presented here indicate that small sortilin-p75^{NTR} coexpressing neurons (< 200µm²) are lost 25 days after injury. Reportedly, small non-peptidergic neurons do not express p75^{NTR}. Therefore, our results suggest that the small peptidergic DRG neurons are included in those that are lost after injury.

Sortilin-p75^{NTR} coexpressing neurons

Here we define a unique DRG neuronal subpopulation coexpressing sortilin and p75^{NTR}, making this distinct neuronal class particularly vulnerable to proNGF-induced neuronal death. Previous studies have established a 54% loss of DRG neurons amongst mice 28 days following sciatic nerve transection (Shi et al., 2001). We report in this study that neither p75^{NTR} nor sortilin peptide expression is altered three days following sciatic nerve transection. Therefore, we are able to attribute a reduction in p75^{NTR} and sortilin immunoreactivity to a loss of neurons due to injury-induced apoptosis. In our study, injury to the peripheral sciatic nerve induced a 60% loss of sortilin-p75^{NTR} coexpressing neurons, suggesting that these neurons are vulnerable to injury-induced death. That injury did not lead to death of all of sortilin-p75^{NTR} coexpressing neurons may be explained by the fact that these surviving neurons are supported by mechanisms rendering them insensitive to proNGF-induced cell death. However, our results do suggest proNGF is a contributing factor to injury-induced neuronal cell death in adult mouse DRG. Related to this point, a recent paper studying the ability of proNGF to induce cell death in retinal ganglion neurons reported that proNGF induced cell death during development, but does not promote injury-induced neuronal death in retinal ganglion neurons, suggesting that this apoptotic mechanism is not active in all settings (Nakamura et al. 2007). Finally, data from Nykjaer et al. 2004 assessed the need for both receptors by culturing neurons only expressing sortilin or p75 to determining the apoptotic effects of pro NGF upon these specific signaling molecules. In addition, these authors transfected neurons with either sortilin, p75, or both receptors and found that proNGF actions require both receptors, suggesting that both receptors are required for proNGF-induced apoptosis. Future studies in the mouse DRG will be necessary to confirm the importance of both receptors in injury-induced cell death of these neuronal subpopulations. Collectively, these results indicate that it will be very important to define proNGF's actions in each model and stage of development.

Conclusion

In conclusion, we have shown that proNGF, sortilin, and p75^{NTR} are present in mouse DRG neurons, identifying them as potential mediators of neuronal death. Furthermore, we have demonstrated that the number of sortilin-p75^{NTR} coexpressing neurons is reduced dramatically in response to injury. These data suggest a pathological role for proNGF *in vivo* in mediating apoptosis of L4/5 DRG neurons following sciatic nerve injury. By identifying a likely mechanism for cell death in a particularly vulnerable population, we can now focus on potential therapeutic strategies aimed at preventing the death of these neurons thereby preventing loss of sensory function.

Experimental Procedure

Materials

Phenylmethylsulfonyl fluoride, leupeptin, pepstatin A, aprotinin, antipain, sodium fluoride, sodium orthovanadate, ethylenediaminetetraacetic acid disodium dihydrate and all other reagent grade chemicals were purchased from Sigma (St. Louis, MO). Normal donkey/goat sera, as well as porcine gelatin, were obtained from Jackson Immunoresearch (West Grove, PA). 12.5% Tris-HCl gels and 0.4 µm nitrocellulose membranes were purchased from Bio Rad (Hercules, CA).

The anti-sortilin rabbit polyclonal antibody, specific for the extracellular (luminal) domain of sortilin, was generously donated by Dr. Claus Munck Petersen, University of Aarhus, Denmark. The polyclonal antibody specific to the extracellular domain of the p75^{NTR} was a gift from Dr. Louis Reichardt, Howard Hughes Medical Center. The anti-nerve growth factor beta and the anti-pro-NGF polyclonal antibodies were purchased from Chemicon International (Temecula, CA) and Sigma, respectively. Primary antibodies for the neuronal markers CGRP and N52 were purchased from Chemicon and Sigma, respectively. The secondary antibodies, Alexa Fluor 488/555 and horseradish peroxidase conjugated affinity purified secondary antibody, were purchased from Molecular Probes and Chemicon, respectively. IB4 was purchased from Molecular Probes (Eugene, OR).

Animal Model

The experiments were carried out on six-week old, male C57BL/6 mice (Charles River, Wilmington, MA) and were conducted according to the guidelines from the National Institutes of Health and the University of Kansas Medical Center Animal Care and Use Protocol. The animals were housed in 12/12-h light/dark cycle and allowed free access to food and water. Anesthesia was induced by an intraperitoneal injection of a 1.25% avertin solution (2.5 g of 2,2,2-tribromoethyl alcohol and 5 ml tert-amyl alcohol [Sigma] dissolved in 200 ml deionized water, 0.2 ml/10 g body weight). Sciatic nerves were bilaterally exposed at the mid-thigh level (between 18 and 22 mm distal to the DRG) and transected or not injured. Following transection, a 5 mm portion of the sciatic nerve was resected to prevent regeneration. The sciatic nerves of mice in the uninjured group were viewed anatomically without further intrusion. Sciatic nerves of the crush and transected groups received either mild pressure applied with forceps for approximately five seconds or were completely severed, respectively. The surgical incision was closed by applying Nexaband[®] liquid tissue adhesive (Webster Veterinary Supply, Raleigh, NC). Mice were allowed to survive for three days following surgical treatment of the sciatic nerves for western blot or immunocytochemical analysis. For neuronal survival assessment, L4/5 DRG were bilaterally removed 25 days following sciatic nerve transection.

Western blot Analysis

L4/5 dorsal root ganglia (DRG) (n = four mice per treatment group) were pooled together for optimal protein concentration. Tissue was placed in liquid nitrogen upon dissection and homogenized using a PRO250 homogenizer (PRO Scientific, Monroe, CT) in 500 μ L ice cold RIPA buffer (1% triton, 1% deoxycholic acid, 0.1% SDS, 15 M NaCl, 50 mM Tris pH 7.5, 1% NP-40, and 1 mM PMSF) for 30 seconds. Samples were then centrifuged for 30 minutes at 15,000 rpm at +4 $^{\circ}$ C. Total protein concentration was determined using the Bradford Assay method (Bradford, 1976). Samples were denatured for 10 minutes at 100 $^{\circ}$ C in bromophenol blue. Protein samples (50 μ g/lane) were separated by SDS-page on 12.5% gels for the detection of mature NGF, proNGF, sortilin, and p75^{NTR}. Recombinant mouse NGF (R&D Systems, 20ng/lane) was loaded as a positive control to test the specificity of the anti-mature NGF antibody. All protein loading was normalized to cyclophilin A (1:1,000). Primary antibodies were diluted as follows: anti-sortilin (1:4,000), anti-p75^{NTR} (1:2,000), anti-mature NGF (1:2,000), and anti-proNGF (1:2,000). Membranes were incubated in the secondary antibody (HRP-conjugated affinity-purified secondary antibody; 1:20,000) for one hour at +4 $^{\circ}$ C. The proteins were detected using an enhanced chemiluminescence reagent kit (Pierce, Rockford, IL) and imaged using a ChemiDoc[™] XRS Imager (Bio Rad). Relative band density was analyzed with Quantity One software (Bio Rad) by applying the following equation:

$$\text{Band Intensity} = \frac{\text{density (the total intensity of pixels enclosed in the volume box)}}{\text{intensity (the area of the volume box [mm}^2\text{])}}$$

Immunocytochemistry

Sortilin and p75^{NTR} expression was assessed in L4/5 DRG obtained from mice with bilaterally uninjured or transected sciatic nerves (n = 10/treatment group). Mice were deeply anesthetized with avertin (0.2 ml/10 g body weight) and transcardially perfused with ice cold saline followed by 4% paraformaldehyde solution in 0.1 M phosphate buffer (PBS) (pH 7.4). L4/5 DRG were dissected and allowed to post-fix in the same solution for one hour. Following post-fixation, the tissue was cryoprotected using 30% sucrose. 12 μ m sections were cut using a cryostat maintained at -20°C and mounted onto Superfrost Plus microscope slides (Fisher, Chicago, IL). Immunocytochemistry was performed using previously published protocols (Christianson et al., 2003). Briefly, sections were blocked for 1 hour in 1.5% normal goat/donkey serum, 0.5% porcine gelatin, and 0.2% Triton X-100 in Superblock buffer (pH 7.4, Pierce, Rockford, IL). Sections were incubated overnight with primary antisera to sortilin (1:5,000) or p75^{NTR} (1:2,000) at $+4^{\circ}\text{C}$ under humidified conditions. The primary antibody was removed by two washes in PBS-triton (0.5%) and sections were then incubated with secondary antibodies (Alexa Fluoro 488 and 555; 1:2,000) for one hour at $+4^{\circ}\text{C}$. Fluorescently labeled sections were rinsed and coverslipped with PBS and viewed using a Nikon E800 microscope attached to a Magnafire digital camera.

Sortilin expression within DRG neuronal subpopulations

Sortilin expression in DRG neuronal subpopulations was defined by removing L4/5 DRG bilaterally from uninjured C57Bl/6 mice (n = 5). A polyclonal primary sortilin antibody (1:5,000) was applied to 14 μ m sections in conjunction with the individual neuronal markers anti-CRGP (1:500), IB4 (1:20), and N52 (1:400). Immunocytochemistry was performed as described above.

Sortilin-p75^{NTR}-coexpressing neurons

To identify DRG neurons that coexpress sortilin and p75^{NTR}, L4/5 DRG from C57BL/6 mice (n = 5) were removed and perfused three days following no injury or sciatic nerve transection. Primary polyclonal sortilin and p75^{NTR} antibodies were used together on 14 μ m DRG sections. The number of neurons coexpressing both receptors was counted in random, non-sequential sections of DRG. Approximately ten sections per DRG per animal were counted to arrive at an average number of neurons coexpressing both receptors.

Analysis of Neuronal Survival

The number of neurons in L4/5 DRG coexpressing the sortilin and p75^{NTR} receptors was assessed in C57BL/6 mice (n = 7/treatment group) that were perfused 25 days following no injury or bilateral sciatic nerve transection. To identify a subpopulation of neurons vulnerable to sortilin-p75^{NTR}-mediated proNGF-induced neuronal death, immunocytochemistry was performed on DRG sections using primary antisera specific for the sortilin and p75^{NTR} receptors. The number of neurons coexpressing sortilin and p75^{NTR} were counted in both injured and uninjured groups of mice. The area of neurons was measured, in square microns (μm^2), using NIH Image J software. Neurons were categorized into different size-based groups based on their area as follows: small neurons (0–200 μm^2), medium neurons (200–400 μm^2), and large neurons (<400 μm^2). The number of neurons in each group was counted and regarded as a percentage of the total number of neurons within the overall treatment group (no injury or 25 days post-sciatic nerve transection).

Statistical Analysis

Statistical analysis was performed using either ANOVA or an unpaired t-test to determine a significant difference between treatment groups. Statistical significance was set at $P < 0.05$. The variations in number of neurons are given as mean \pm S.E.M.

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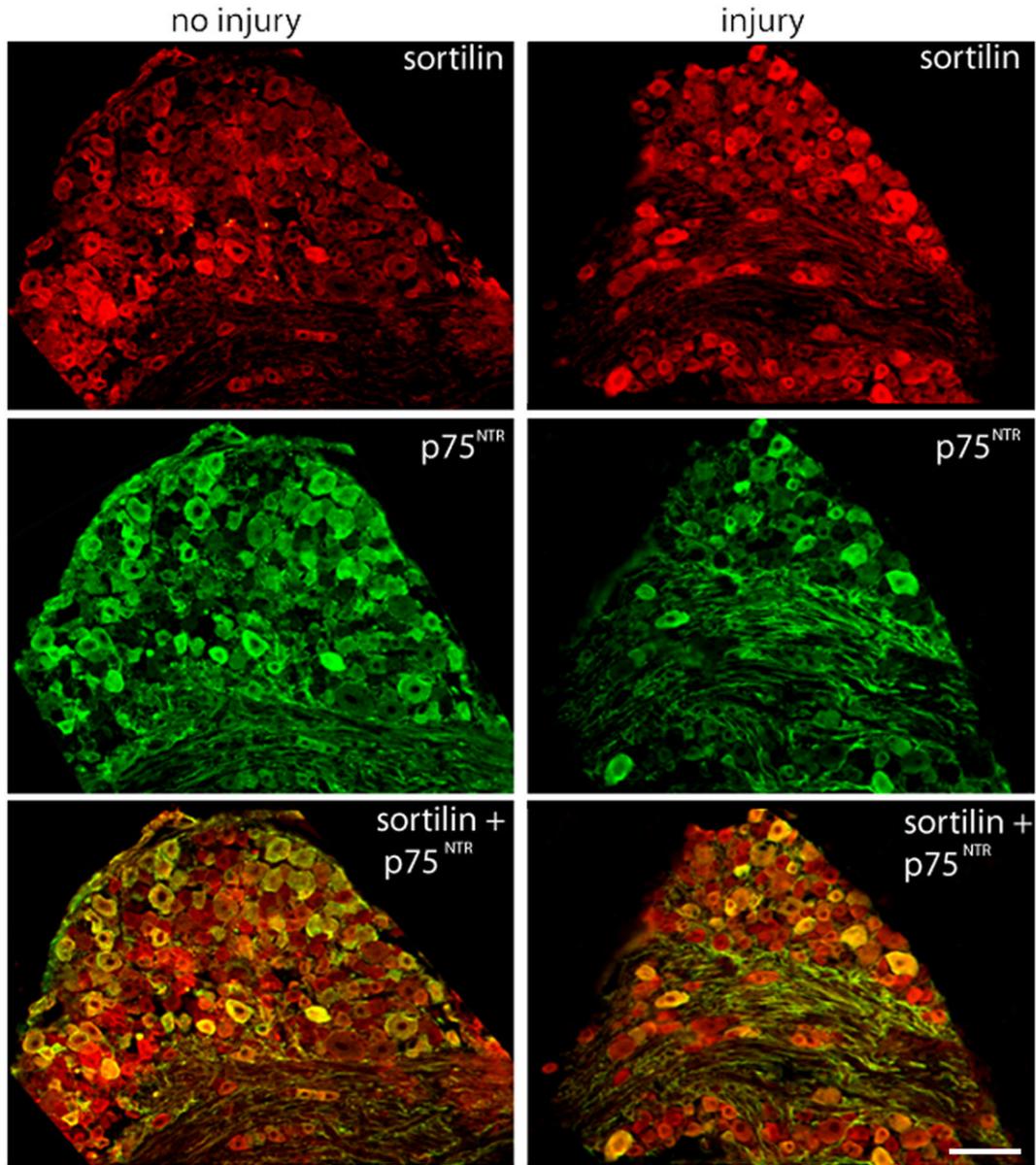


Figure 1.

Immunocytochemistry was performed on L4/5 DRG of uninjured and injured C57BL/6 mice to identify neurons that express sortilin (red, top panels) or p75^{NTR} (green, middle panels). Top left) On average, 73% of neurons express sortilin in uninjured mice. Top right) Similarly, 72.8% of neurons express sortilin 3 days following sciatic nerve transection. Middle left) 66.1% of neurons express p75^{NTR} in uninjured mice. Middle right) Similarly, 67.2% of neurons express p75^{NTR} three days after sciatic nerve transection. Bottom left) Merged image illustrating the overlap of sortilin and p75 with no injury (left) and following injury (right) Scale bars equal 100µm for each panel.

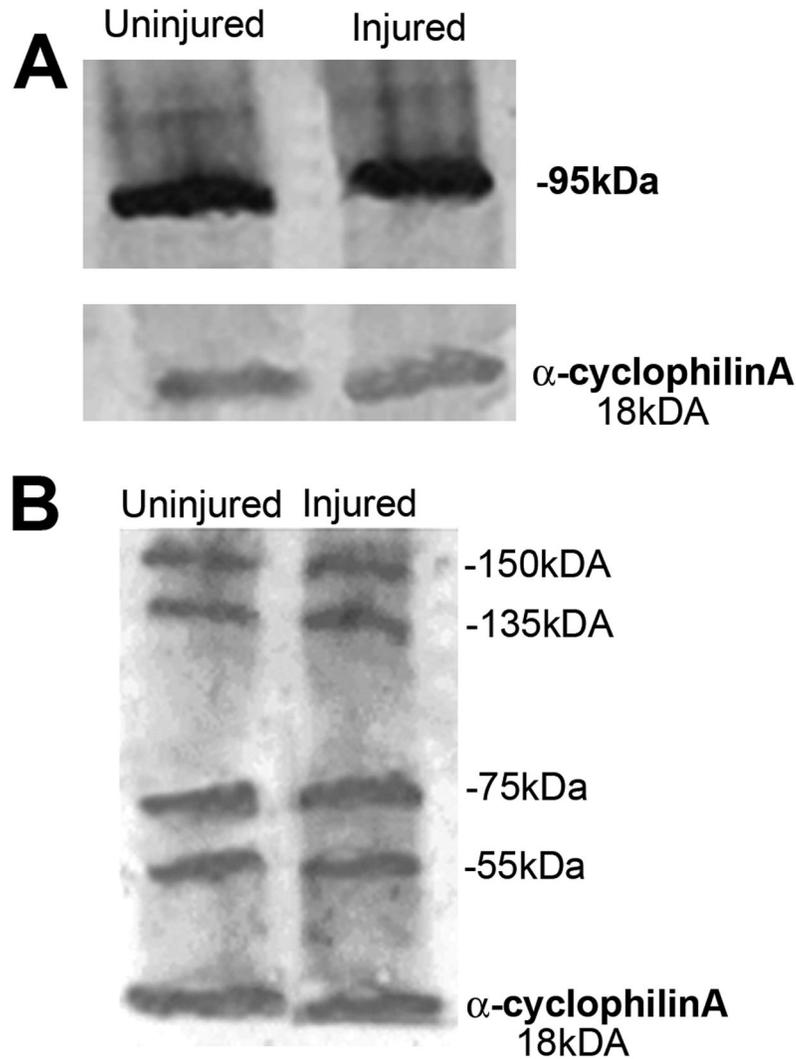


Figure 2.

Immunoblotting analysis was performed using L4/5 DRG removed 3 days following no injury (lane 1) or sciatic nerve transection (lane 2). A) A polyclonal antibody against sortilin recognized a band of 95 kDa that was determined to be the sortilin receptor. All protein loading was normalized to cyclophilin A (18 kDa). An unpaired t-test performed on band intensity revealed no significant difference in sortilin expression between injured and uninjured groups ($p = 0.8643$), suggesting that sortilin expression is not altered in DRG neurons following sciatic nerve injury.

B) Immunoblotting analysis was performed using L4/5 DRG removed 3 days following no injury (lane 1) or sciatic nerve transection (lane 2). A polyclonal antibody designed to detect p75^{NTR} recognized a band of 75 kDa that was identified as the p75 receptor. Bands of ~55, ~135, and ~155 kDa represent possible cleaved or post-translationally modified forms of p75^{NTR}, respectively. All protein loading was normalized to cyclophilin A (18 kDa). An unpaired t-test performed on band intensity revealed no significant difference in p75^{NTR} expression between injured and uninjured groups ($p = 0.7836$), suggesting that p75^{NTR} expression is not altered in DRG neurons following sciatic nerve injury.

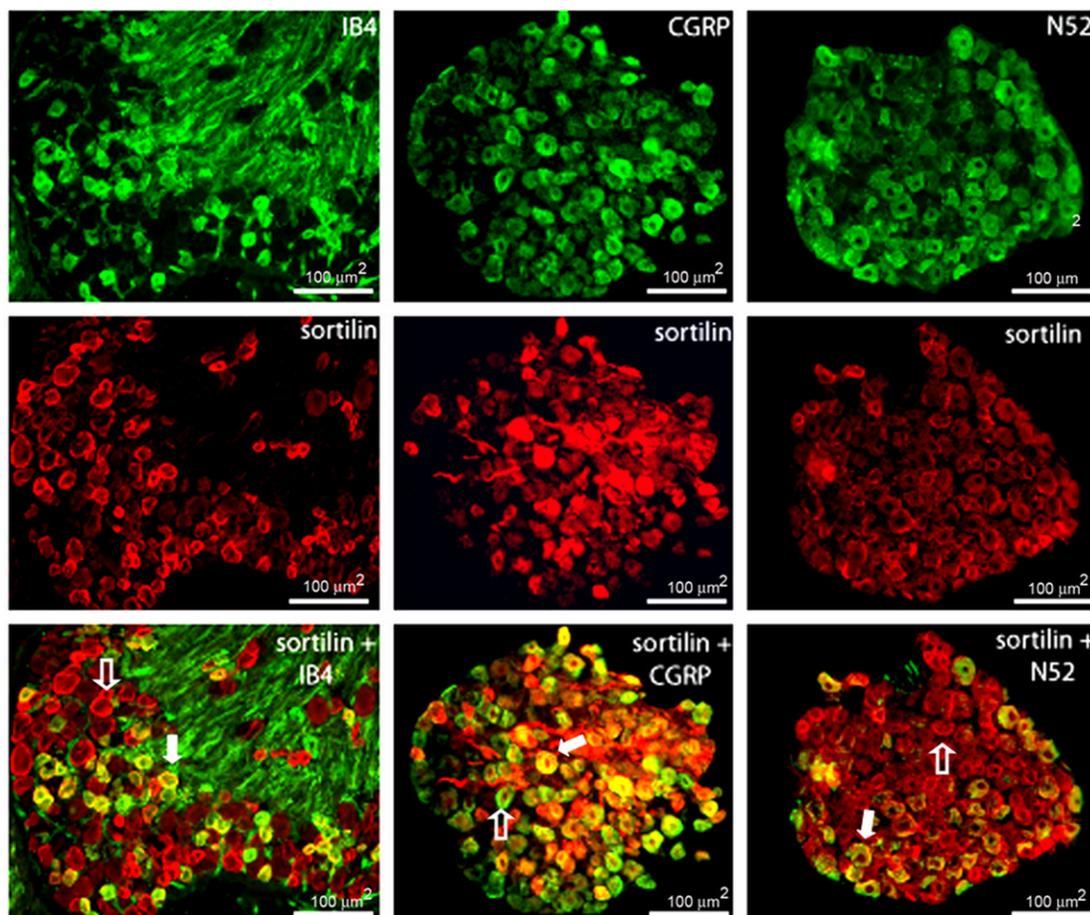


Figure 3.

Immunocytochemistry was performed on L4/5 DRG from uninjured C57BL/6 mice using a primary polyclonal sortilin (red) antibody in conjunction with the neuronal markers (green) IB4, CGRP, and N52. 13.6% of sortilin-expressing neurons coexpress IB4. Alternatively, 31.7% of IB4-expressing neurons coexpress the sortilin receptor. 20.6% of sortilin-expressing neurons coexpress CGRP while 44.1% of CGRP-expressing neurons coexpress sortilin. 18.2% of sortilin-expressing neurons coexpress N52; 52.8% of N52-expressing neurons coexpress sortilin. Filled arrows represent neurons that coexpress sortilin and a specific neuronal marker; open arrows identify neurons that express sortilin in the absence of neuronal markers.

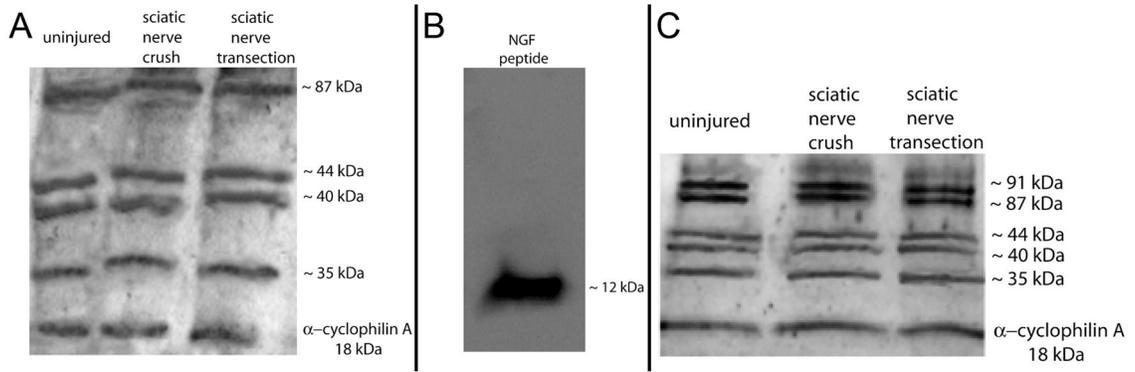


Figure 4.

A) Immunoblotting analysis was performed using L4/5 DRG from the following groups of mice: no injury (lane 1), three day post-sciatic nerve crush (lane 2), or transection (lane 3). A polyclonal antibody specific for the pro-domain of proNGF detected multiple bands of ~35, ~40, ~44, and ~80 kDa. The ~35 kDa band was determined to identify the unglycosylated NGF precursor. All higher molecular weight bands likely represent post-translationally modified forms of proNGF. Statistical analysis using ANOVA revealed no significant difference in band intensity amongst groups ($p = 0.999$), suggesting that proNGF expression is not altered in response to injury in the DRG. A polyclonal antibody specific for mature NGF detected bands of similar size, including a band of ~91 kDa (see panel C). Because this band was not detectable when using the pro-NGF antibody, this band was determined to not be of true NGF origin. B) Commercially prepared NGF (20ng/lane) was used to test the specificity of the mature antibody. C) Mature NGF (12 kDa) was not present in detectable concentrations. No significant change in peptide expression was detected by ANOVA between treatment groups ($p = 0.9868$). All protein loading was normalized to cyclophilin A (18kDa).

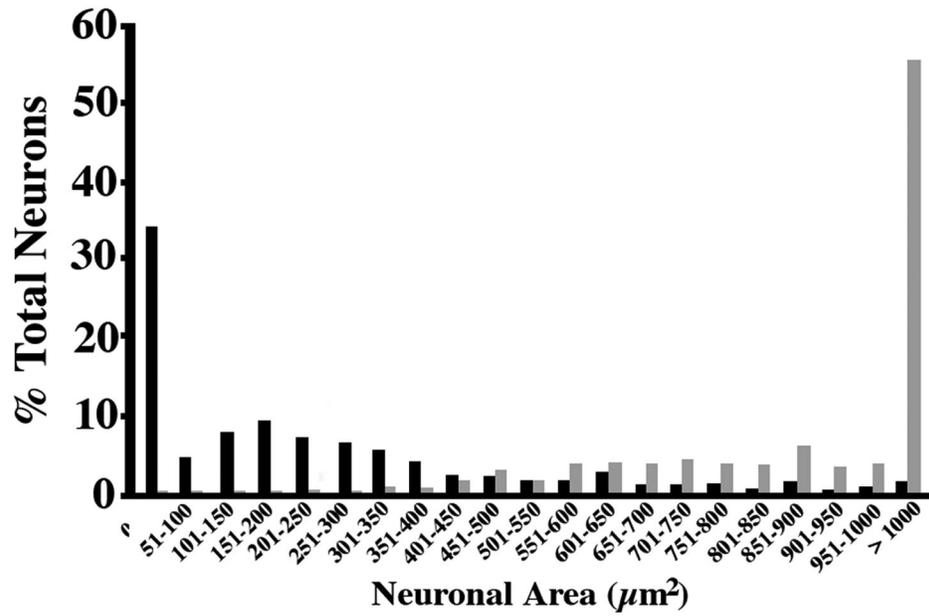


Figure 5. Size-frequency histogram illustrating the distribution of neuronal area (μm^2) in uninjured (black bars) and sciatic nerve transected (grey bars) L4/5 DRG 25-days following sciatic nerve axotomy. Small neurons ($0\text{--}200\mu\text{m}^2$) comprise over half (58.03%) of the L4/5 DRG neuronal population in uninjured mice (black bars). This population of neurons is completely lost 25-days following sciatic nerve transection (grey bars).

Table 1

	Total No. of Immunopositive Neurons	No. of Sortilin Expressing Neurons that Coexpress CGRP, IB4, or N52
Sortilin	778	
CGRP	334	106 (13.6 %)
IB4	365	161 (20.6 %)
N52	269	142 (18.2%)

Values represent the percentage of immunopositive neurons in L4/5 DRGs from 6-week-old mice (n = 3)..

Table 2

	Sortilin Expressing Neurons	p75 ^{NTR} Expressing neurons	Sortilin+/p75 ^{NTR} Expressing neurons	% Sortilin that Express p75 ^{NTR}	% p75 ^{NTR} that Express Sortilin
No Injury	780	806	409	52.5 %	507 %
Injury	759	829	414	54.5 %	49.9 %

Values represent the percentage of positively labeled neurons in L4/5 DRGs from 6-week-old mice (n = 3). No significant differences were observed between non-injured and injured groups.