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NEURONAL DEATH IN THE DORSAL ROOT GANGLION AFTER SCIATIC NERVE INJURY DOES NOT DEPEND ON SORTILIN

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Abstract—Injury to the sciatic nerve induces loss of sensory neurons in the affected dorsal root ganglia (DRGs). Previous studies have suggested the involvement of the neurotrophin receptors p75 neurotrophin receptor ($p75^{NTR}$) and sortilin, proposing that sensory neuron subpopulations undergo proneurotrophin-induced apoptosis in a similar manner to what can be observed in the CNS following injury. To further investigate this hypothesis we induced sciatic nerve injury in sortilin-deficient mice, thereby preventing apoptotic signaling of proneurotrophins via the sortilin-$p75^{NTR}$ receptor complex. Using an unbiased stereological approach we found that loss of sortilin did not prevent the injury-induced loss of DRG neurons. This result demonstrates that previous findings linking $p75^{NTR}$ and proneurotrophins to loss of sensory neurons need to involve sortilin-independent pathways and suggests that proneurotrophins may elicit different functions in the CNS and PNS.

Key words: sortilin, $p75^{NTR}$, dorsal root ganglion, injury, cell death.

INTRODUCTION

During embryonic development, peripheral sensory neurons depend on stimulation by neurotrophic factors for survival, differentiation and targeting of axonal connections. Neurotrophins act via two structurally unrelated receptors, the p75 neurotrophin receptor ($p75^{NTR}$) and Trk tyrosine kinase receptors (TrkA, -B, and -C), which can mediate survival as well as apoptotic signaling (Chao, 2003; Nikoletopoulou et al., 2010). Neurotrophins are synthesized as precursor proteins, pro-neurotrophins, which are subsequently processed into their mature counterparts in the trans-Golgi network prior to secretion. Later it was described that the pro-neurotrophins can also be released and cleaved extracellularly. However, consensus remained that only the mature neurotrophins were biologically active signaling molecules, inducing either pro-survival signaling and differentiation via the Trk receptors or neuronal apoptosis via the $p75^{NTR}$. It is now recognized that also pro-neurotrophins (proNGF, proBDNF) can induce neuronal apoptosis by engaging in a complex of $p75^{NTR}$ and the sorting receptor sortilin (Nykjaer et al., 2004; Jansen et al., 2007). Several studies have documented upregulation of pro-neurotrophins as well as increased co-expression of $p75^{NTR}$ and sortilin under conditions of CNS injury and neuronal apoptosis (reviewed in (Nykjaer and Willnow, 2012)), arguing that both receptors are important in the apoptotic signaling by pro-neurotrophins following neuronal injury. However, the relevance of pro-neurotrophin-induced apoptosis via the sortilin-$p75^{NTR}$ complex for pathologies of the peripheral nervous system remains largely to be investigated.

In the current paper we focus on the role of sortilin following peripheral nerve damage. A previous study has investigated the effect of $p75^{NTR}$ in relation to the loss of sensory neurons in the fifth lumbar (L5) dorsal root ganglia (DRG) 42 days after sciatic nerve injury (Sørensen et al., 2003). It was demonstrated that mice deficient for $p75^{NTR}$ did not experience any neuronal loss, whereas 23% of the L5 neurons were lost in normal mice. This observation argues that death signaling via the $p75^{NTR}$ receptor is required for the observed apoptosis of sensory neuron following sciatic injury. Subsequent studies have found sortilin and $p75^{NTR}$ to be co-expressed in DRG neuron subpopulations (Arnett et al., 2007; Fan et al., 2008; Vaegter et al., 2011). Arnett et al. described that the majority of small $p75^{NTR}$–sortilin co-expressing DRG neurons are lost following sciatic nerve injury (Arnett et al., 2007). Furthermore, Fan et al. demonstrated that proBDNF antiserum increases the survival of DRG sensory neurons after sciatic transection. Together, these observations suggest that pro-neurotrophin-$p75^{NTR}$–sortilin-mediated neuronal death may explain critical aspect of injury-induced neuronal death in the DRG. On the other hand, no increase in pro-NGF, sortilin or $p75^{NTR}$ was observed by Arnett following sciatic injury. Although $p75^{NTR}$ thus appears to be critically involved, this receptor has other functions in relation to neurotrophin signaling (Skeldal et al., 2011) and it is possible that the molecular mechanisms involved in death of DRG neurons

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Abbreviations: DRG, dorsal root ganglia; IHC, immunohistochemistry; $p75^{NTR}$, p75 neurotrophin receptor; *Sort1−/−*, sortilin; Trk, tyrosine kinase receptors; wt, wild type.

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is independent of pro-neurotrophin signaling and that the role of pro-neurotrophin in this system is more indirect. As apoptotic signaling by pro-neurotrophins is critically dependent on the $p75^{NTR}$–sortilin complex (Nykjaer et al., 2004; Jansen et al., 2007), we aimed to address the relevance of this signaling pathway in the DRG neurons following sciatic nerve injury by investigating the loss of DRG neurons in sortilin-deficient mice.

EXPERIMENTAL PROCEDURES

Surgery: All surgical procedures were approved by the Danish Animals Experiments Inspectorate. Adult (8–10 weeks old) transgenic mice lacking sortilin (*Sort1*−/−) and backcrossed > 10 generations on c57bl/6jBomTac background have been described previously (Jansen et al., 2007; Vaegter et al., 2011). C57bl/6jBomTac (Taconic) were used as wild-type (wt) controls. 8 *Sort1*−/− and 6 controls were subjected to sciatic nerve ligation on the right side. Briefly, mice were anesthetized with i.p. injection of ketamine (100 mg/kg) and Xylazine (15 mg/kg), the skin opened and the sciatic nerve exposed using blunt dissection of the muscle layer (Richner et al., 2011). The nerve was ligated at mid-thigh level using 6–0 suture and dissected distally. The skin was closed with Vetbond tissue adhesive (3 M). The animals received pain relief (NSAID) the following week. After 42 days the mice were deeply anesthetized and sacrificed. The L4–L5 ganglia were dissected on both sides and fixed in 4% paraformaldehyde for 24 h.

Immunohistochemistry: 8-wk-old mice were decapitated, the L3–L5 DRGs carefully dissected and fixed in 4% PFA for 4 h. Following incubation in 30% sucrose for 24 h at 4 °C they were embedded in TissueTek (Sakura Finetek) and cryosectioned (10 μm) using the CryoJane® Tape-Transfer System (Leica Biosystems). The sectioned tissue was mounted on precoated adhesive slides (Surgipath®, Leica) and kept at −80 °C until use. Following permeabilization and blocking (5% Donkey serum, 1% BSA and 0.3% Triton X-100 in TBS for 30 min), the tissue was incubated with primary antibodies diluted in 50 mM Tris-base buffer (TB-buffer) (pH 7.4), overnight in a humidified chamber at 4 °C. Antibodies used were directed against sortilin (AF2934, R&D Systems, diluted 1:100), $p75^{NTR}$ (MC9651, a gift from professor Moses Chao, diluted 1:1500), peripherin (ab4666, Abcam, diluted 1:8000) or NF200 (ab8135, Abcam, diluted 1:1000). Following 3x wash in TBS the sections were incubated with AlexaDye-conjugated secondary antibodies (1:300, Invitrogen) for 4 h and mounted with Fluorescent Mounting Medium (Dako, S3023). Hoechst nuclear stain (cat. 861405, Sigma–Aldrich, diluted 1:30,000) was added to the first wash. Immunohistochemistry on sections from wt and knockout controls (*Sort1*−/− *or* $NGFR^{-/-}$) was performed in parallel to control antibody specificity. Images were acquired with a Zeiss LSM780 confocal microscope with a 20× objective. Estimates of colocalization were done as previously described (Vaegter et al., 2011). Briefly, non-adjacent DRG sections were stained against sortilin and $p75^{NTR}$, and images acquired on the Zeiss LSM780 system (threshold settings based on parallel slides from knockout mice). The percentage positive neurons for each marker and the percentage double-positive cells were counted. $N = 3$ mice with six independent sections from each were counted.

Stereology: The number and volume of DRG neurons were estimated using an unbiased stereological approach, as described in detail in (Dorph-Petersen et al., 2001, 2009, n.d.). Briefly, the tissue was incubated in 30% sucrose for 24 h, embedded and cut on a cryostat and stained with cresyl violet acetate to visualize Nissl substance. The data were collected with an Olympus BX 50 light microscope equipped with a Prior motorized stage, a Heidenhain microcator, Olympus UPlanSApo 60× oil lens (NA = 1.35), and an Olympus DP70 digital camera controlled by newCAST (Visiopharm) software. Every second section was sampled systematically and the number of neurons estimated by the optical fractionator using nucleoli as counting units, with Q-weighted section thickness. The individual neuronal volume was estimated by the isotropic rotator (based on isotropic uniformly random (IUR) sections), utilizing the Cavalieri estimator and two-dimensional nucleator, with five test rays in the newCAST software.

Statistics: Within the same group of animals, the paired Student's t-test was used to evaluate the effect of nerve ligation using the non-ligated side as control. Comparisons of ganglia or cell type between genotypes were evaluated by an ANOVA after testing for normal distribution. All values are given as mean ± S.E.M. (Standard Error of the Mean).

RESULTS

Sortilin and $p75^{NTR}$ co-expression in DRG neurons

To validate the co-expression of sortilin and $p75^{NTR}$ in DRG neurons we performed immunohistochemistry (IHC) analysis of cryosectioned DRGs from uninjured wt mice. A similar analysis was performed previously by Arnett et al. (2007) and Fan et al. (2008), however unfortunately the anti-sortilin antibodies utilized in both studies are not sufficiently specific for sortilin in the IHC analysis of DRGs in our hands, resulting in unacceptably high staining in the DRGs from *Sort1*−/− mice (data not shown). Different antibodies against sortilin were therefore systematically tested in wt mice with the sortilin-deficient mice as negative control, and we find the AF2934 from R&D Biosystems to be the most specific for IHC. Similarly, we utilized DRG tissue from $p75^{NTR}$-deficient mice as negative controls for the $p75^{NTR}$ staining, assuring high specificity of both the sortilin and $p75^{NTR}$ visualizations.

DRG neurons can be divided into two main subgroups based on appearance and function. The larger A-cells give rise to myelinated Aα, Aβ and Aδ axons involved in proprioception, low-threshold mechanical sensation (touch) or fast nociceptive response, respectively. The smaller B-cells on the other hand give rise to unmyelinated axons involved in the slower nociceptive response from noxious thermal, mechanical or chemical stimulation (Patapoutian et al., 2003). Co-expression with NF200 and peripherin, markers for A and B cells