In vivo analysis of Trk receptor signalling in the mouse nervous system
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Chapter 6

Trk receptor signalling requirements for neuronal survival and maintenance of target innervation in TrkB- and TrkC-dependent sensory neurons.


Part of this chapter is contained in:

Distinct requirements for TrkB and TrkC signaling in target innervation by sensory neurons
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SUMMARY
The mechanisms by which Trk receptors signal to mediate neuronal survival, differentiation and function are incompletely understood. In TrkA the intracellular tyrosine (Y490) that binds the Shc adaptor is critical in mediating survival and differentiation. We have generated a point mutation in this tyrosine in TrkB and TrkC in the mouse. We find that in the inner ear, TrkB- and TrkC mediated survival is largely Shc site independent. In contrast, TrkB-mediated target innervation is Shc site dependent, whereas TrkC-mediated target innervation is Shc site independent. Here, we report that in trkB<sup>shc/shc</sup> mice TrkB-dependent baroreceptor innervation of the aortic arch is lost. In trkC<sup>shc/shc</sup> mice, TrkC-dependent DRG proprioceptive neurons show a minor reduction in number and their function appears unaffected. In addition, NT3-dependent skin mechanoreceptors are unaffected by the mutation. These results show that the observed difference in Shc site dependency for target innervation is independent of cell type. They argue that a difference in signaling mechanism exists between TrkB and TrkC.

INTRODUCTION
Neurotrophins play a variety of roles during peripheral and central nervous system development (Henderson, 1996). Members of the neurotrophin family are secreted ligands for the Trk receptor tyrosine kinase family (Barbacid, 1994). In particular, NGF binds TrkA, BDNF and NT4 bind TrkB and NT3 show high-affinity for TrkC. In addition, NT3 can bind TrkA and TrkB with low-affinity in a way that is physiologically relevant (Farinas et al., 1998) (Farinas et al., 2001) (Davies et al., 1995).

During development more neurons are generated than are required during adult life (Jacobson et al., 1997). The neurotrophin hypothesis states that neurons compete for access to the neurotrophin survival factor that is secreted by the target tissue (Hamburger, 1992) (Davies, 1996), a mechanism that is thought to support correct target innervation. This phenomenon has been well studied in the sensory neurons of the dorsal root ganglia (DRG). The DRG contain a wide variety of bipolar sensory neurons that connect to a peripheral target with one axon and to a spinal cord neuron with the other. Null mutations have confirmed that neurotrophins-Trk interactions play a crucial role in mediating specific sensory neuron survival (Snider, 1994). For instance, targeted deletion of NGF and trkA eliminates small-diameter nociceptive neurons and their cutaneous afferents (Crowley et al., 1994) (Smeyne et al., 1994). In contrast, in mice lacking the catalytic form of trkC the 20% of large-diameter proprioceptive neurons are lost, resulting in loss of innervation of Golgi-tendon organs and muscle spindles (Klein et al., 1994). In NT3 knockouts some trkB-positive neurons die in addition to the proprioceptors (Farinas et al., 1998), explaining why these mice loose 60% of their DRG neurons (Ernfors et al., 1994) (Farinas et al., 1994).

Mechanoreceptive sensory neurons in the DRG are difficult to classify due to a lack of molecular markers. Traditionally, the wide variety of mechanoreceptors has been described on the basis of the conduction velocities of their axons (unmyelinated C-fibers and myelinated A<sub>α</sub>, A<sub>β</sub> and A<sub>δ</sub> fibers). Further functional classes can be delineated on the basis of thresholds and adaptation responses of fibers to discrete stimuli. For instance, cutaneous mechanoreceptors showing low threshold responses to tactile and vibration stimuli have A<sub>β</sub> and A<sub>δ</sub> conduction velocities. One type of these fibers, D-hair afferents, sequentially depend on NT4 and NT3 for their survival (Airaksinen et al., 1996) (Stucky and Lewin, 1998) (Stucky et al., 1998). Slowly adapting mechanoreceptors (SAM) on the other hand require NT3 for survival, while depending on BDNF for their mechanosensitivity (Airaksinen et al., 1996) (Carroll et al., 1998).

Another peripheral ganglion well studied for its neurotrophin dependence is the nodosepetrosal ganglion (NPG). The NPG contains neurons that are responsible for visceral sensory
TrkB- and TrkC-dependent neurons

innervation. All neurons express TrkB (Huang et al., 1999) and nearly all are lost in BDNF-NT4 double mutants and TrkB mutants (Conover et al., 1995) (Silos-Santiago et al., 1997). The ligands are expressed exclusively in the different target organs of the NPG and thereby support separate neuron subpopulations. BDNF supports the survival of 50% of the NPG neurons (Ernfors et al., 1994) (Jones et al., 1994) (Conover et al., 1995) (Liu et al., 1995) (Erickson et al., 1996). These include arterial chemoafferents that innervate the carotid body and baroreceptor innervation of the aortic arch (Erickson et al., 1996) (Brady et al., 1999).

Figure 6-1. Cell size determination of lumbar L4 DRG neurons in P4 wild-type and trkC<sup>shc/shc</sup> mice. (A) Cell size histogram of lumbar DRG neurons. The number of cells falling into each size category is expressed as a percentage of the total number of cells measured, and averaged per three wild-type or mutant animals, respectively. (B) From the size measurements the cell area above which in wild-type animals the 19% largest, TrkC-positive cell bodies can be found was determined. This was normalised to one hundred percent and the percentage of cell sizes larger than this critical size in trkC<sup>shc/shc</sup> mice was calculated.

We have previously shown that a mutation in a juxtamembrane tyrosine residue of the TrkB receptor, called TrkB-Shc, result in a mild increase in cell death in BDNF-dependent vestibular and nodose neurons (Minichiello et al., 1998). In addition, vestibular neurons suffer a postnatal, progressive degeneration of their afferents (Chapter 5). In mice carrying the equivalent mutation in TrkC (TrkC-Shc), the effects on cochlear neuron survival are reminiscent of the TrkB-Shc results. However, in these cochlear neurons target innervation remains unaffected (Chapter 4). Together, these results could indicate that the two receptors have different requirements for eliciting their biological effects. Alternatively, these differences could be due to a difference between vestibular and cochlear neurons. Here, we address these possibilities by studying survival and target innervation in other TrkB and TrkC dependent neurons. We show that these neurons have phenotypes similar to those found in the inner ear ganglia.

RESULTS

In the TrkC null mutants the large-diameter proprioceptive of the Dorsal Root Ganglia (DRG) are lost (about 19% of the DRG) (Klein et al., 1994). We have asked whether the Shc site on the TrkC receptor is required to maintain the survival of this subgroup of sensory neurons. Due to their low abundance in the DRG, total cell count would not detect small reductions of proprioceptive neurons.
We used cell size profiling to determine whether any large-diameter DRG neurons were lost and whether any cell shrinkage of neurons had occurred. This method was previously used to detect the loss of small-diameter nociceptors in the NGF knockout mouse (Crowley et al., 1994). Serial paraffin sections (8 µm) of L4 DRG’s were obtained from P4 mice and stained with cresyl violet. Images were collected every sixth section throughout the ganglion and cell profiles were recorded using NIH Image (wildtype: 3 animals, n=932 cells; trkCshc/shc: 3 animals, n=1413 cells). Every surface area measurement was categorised in 10 µm² intervals and expressed as a percentage of the total number of profiles in each individual animal. No major shift towards smaller area sizes was observed (average area size wild type 26.7 ± 1.4 µm² and trkCshc/shc mutants 25.1 ± 1.7 µm², p=0.5), suggesting that no major cell shrinkage has occurred. (Figure 6-1A). In addition, the wild-type and mutant data did not differ significantly in any of the 10 µm² intervals. Even at surface area intervals smaller than 10 µm² large-diameter proprioceptors could not be readily distinguished from medium-diameter neurons with this method (data not shown). In order to renormalise the large-diameter cells in this analysis to 100%, we determined in our wildtype samples the average surface area above which we find the 19% of largest cells. In our wildtype animals this area size is 39 µm². We then determined the percentage of profiles above this threshold in trkCshc/shc mutants and found a small reduction in mutant animals that was not statistically significant (Figure 6-1B; trkCshc/shc 69 ± 25% compared to wildtype 100 ± 4%, p=0.21).

Figure 6-2. Analysis of proprioceptive neuron cell bodies in the L4 DRG of P0 mice. A quantitative analysis by in situ hybridisation of TrkC mRNA expression (A) reveals that there are significantly fewer proprioceptive neurons in trkCshc/shc mutants compared to wild-type animals. (B-C) Photomicrograph of DIG-labelled TrkC-positive neurons in wild-type and trkCshc/shc DRG sections (arrows in B and C, respectively) reveals no obvious difference in number or distribution of stained cells in the DRG.

In order to quantify the large-diameter proprioceptive neurons in a different manner, we highlighted them by in situ hybridisation using the extracellular domain of TrkC as a probe (kind gift of L. Tessarollo). Serial paraffin sections (8 µm) of P0 DRG’s were stained and counted every sixth section. In trkCshc/shc mutants only a minor loss of TrkC positive profiles was observed (68 ± 3%, p<0.002; n=4 ganglia) compared to heterozygote littermates (100 ± 5%; n=6 ganglia) (Figure 6-2A). The staining pattern was specific and cellular morphology appeared similar in wild-type and mutant tissue (Figure 6-2B,C). In all, these results confirm that a minority of DRG proprioceptive neurons die in the trkCshc/shc mice and indicate that the remaining neurons are not atrophic. The fact that no
proprioceptive phenotype is observed in the trkC^shc/shc mutants also indicates proper functioning of the remaining neurons.

**Figure 6-3. Physiological recordings of saphenous nerve fibers.** Recordings made from large diameter sensory fibers in the saphenous nerve indicate no loss of slowly-adapting mechanoreceptors (SA) in trkC^shc/shc mutant mice (A). In contrast a large depletion of SA fibers has been observed in NT-3 heterozygote mice (data replotted from (Airaksinen et al., 1996) for comparison). (B) No loss of D-hair mechanoreceptors was noted in trkC^shc/shc mutant mice. In contrast, nearly all D-hair receptors were lost in the trkB^shc/shc mutant (data replotted from (Minichiello et al., 1998)). (C-F) Representative plastic sections of the purely cutaneous saphenous nerve taken from wild type (C), trkC^shc/+ (D) and trkC^shc/shc (E) and NT-3 +/- (F) mice. Scale bars, 50µm (C-F).

We extended our study to other TrkC-dependent sensory neurons, the DRG mechanoreceptors. NT-3 is required in the post-natal period to maintain the survival of both slowly adapting mechanoreceptors (SAM) innervating Merkel cells and D-hair mechanoreceptors (Airaksinen et al., 1996). We have asked whether the Shc site on the TrkC receptor is also necessary for NT-3 to maintain the survival of these sub-groups of sensory neurons. We used an in vitro skin nerve preparation to record from single cutaneous sensory neurons in the saphenous nerve (Koltzenburg et al., 1997). For each genotype 3-7 mice were used and between 60-92 single Aβ-fibers and Aδ-fibers were recorded. We found no selective loss of Aβ-fibers (conduction velocity >10 m/sec) characterised as SAMs in trkC^shc/+ or trkC^shc/shc compared to wild type (Figure 6-3A). Thus, as in the wild type mice approximately 60% of Aβ-fibers in both trkC^shc/+ or trkC^shc/shc mice were found to be SAM and the remaining receptors could be characterised as rapidly adapting mechanoreceptors (RAM). This was in contrast to mice heterozygote for the NT-3 gene mutation where the proportion of SAM neurons amongst Aβ-fibers falls to around only 15% (Figure 6-3A, data replotted from Airaksinen et al., 1996). In NT-3 deficient mice a loss of D-hair receptors that have Aδ-fiber conduction velocities between 1-10 m/sec is also observed. However, in mice with a trkC shc mutation no loss of D-hair receptors was observed; the proportion of D-hair receptors recorded in wild type, trkC^shc/+ trkC^shc/shc mice was 42% (n=19), 41% (n=22) and 44% (n=41), respectively (Figure 6-3B). The remaining receptors recorded with Aδ-fiber conduction velocities for each genotype could be characterised as nociceptors (Koltzenburg et al., 1997). To confirm these physiological findings we also recorded
images of sagitally section saphenous nerve from wild-type and mutant mice (Figure 6-3C-F). We also counted the number of myelinated axons remaining in the saphenous nerve in trkC<sup>shc</sup> mutants. Here we found that the number of myelinated axons present in trkC<sup>shc/+</sup>, trkC<sup>shc/shc</sup> was not different (470 ± 11 and 459 ± 8 respectively, P=0.09 t-test, n=5 nerves per genotype). This represents a small reduction (12%) compared to counts of axons taken from wild type mice (518 ± 6 n=2 nerves). However, the loss of axons in mice that are only heterozygote for the NT3 mutation leads to a much larger reduction in axon number of around 30-35% (data not shown).

**Figure 6-4. Aortic arch innervation.** Representative photomicrographs of sagittal sections through the aortic arch of a control trkB<sup>shc/+</sup> mouse (A) and trkB<sup>shc/shc</sup> mutant mouse (B). Staining with anti-PGP 9.5 reveals nerve fibers (similar results were obtained in total of n=3 animals for all genotypes). Rostral is to the right and caudal to the left. Representative pictures are shown from lateral (A,C) and medial (B,D) sections. Arrows indicate baroreceptor fibers within the wall of the arch. Arrowheads point to the aortic depressor nerve. Note the small size of the aortic depressor nerve and the sparseness of vessel innervation in trkB<sup>shc/shc</sup> mutant mouse compared to control mouse. Scale bars, 20µm (A-D).

We next asked, if equivalent defects could be found other sensory neurons that depend on signalling through TrkB receptors. We analysed visceral target innervation by nodose-petrosal ganglion cells. Nodose-petrosal neurons are TrkB-dependent, yet heterogeneous with respect to their response to BDNF and NT4, and previous studies have shown that approximately 50% of them die in either BDNF or NT4 knockout mice (Erickson et al., 1996), and other references within). Mutation of the Shc site in TrkB causes a partial loss of nodose-petrosal neurons, that primarily involves the NT4-dependent subset (Minichiello et al., 1998), (Fan et al., 2000). To determine whether target innervation of the surviving BDNF-dependent nodose neurons was affected in the trkB<sup>shc/shc</sup> mutants, we examined baroreceptor innervation of the aortic arch, which contributes to the neuronal circuits controlling blood pressure (Yardley et al., 1983) (Brady et al., 1999). Baroreceptor innervation in new-born trkB<sup>shc/+</sup> and trkB<sup>shc/shc</sup> mice was analysed in sagittal sections cut through the region of the aortic arch and stained with antibodies against anti-protein gene product (PGP) 9.5 to reveal nerve fibers. Representative pictures are shown from lateral (Figure 6-4A,C) and medial (Figure 6-4B,D) sections. Heterozygotes displayed a normal pattern of baroreceptor innervation, consisting of a dense plexus of nerve fibers distributed circumferentially in the outer wall of the arch (arrows in Figure 6-4A,B and data not shown). In contrast, sparse fibers were observed in the aortic arch of trkB<sup>shc/shc</sup> mice, that only weakly ramified in the dorsal wall of the arch at the level of entry of the aortic depressor nerve (arrows in Figure 6-4C,D). Moreover, the depressor nerve, which is the source of baroreceptor innervation to the arch, appeared much reduced in size in trkB<sup>shc/shc</sup> mice compared to
trkBshc/+ animals (compare arrowheads in Figure 6-4A and D). These data indicate that BDNF signalling through the TrkB Shc site is required for the maintenance of peripheral baroreceptor fibers in the aortic arch, but, based on our previous studies (Minichiello et al., 1998) (Fan et al., 2000), is not required for the survival of their cell bodies in the nodose ganglion. This is suggesting that our observations in the vestibular organ may be applicable to other sensory systems as well.

**DISCUSSION**

We showed that vestibular neurons loose their afferents in the TrkB-Shc mice, but that cochlear neurons in the TrkC-Shc mice do not loose their target innervating fibers (Chapters 4 and 5, this thesis). Unlike most sensory neurons, inner ear ganglion neurons express two Trk receptors (Farinas et al., 2001). Our genetic results could mean the point mutations in TrkB and TrkC have highlighted different signalling mechanisms between the two homologous Trk receptors. Alternatively, they may reflect a particular property of inner ear neurons, or even a difference between vestibular and cochlear neurons. Here, we sought to establish whether the phenotype we observed in the inner ear is shared by other TrkB and TrkC-dependent sensory neurons.

We determined the TrkC-dependent subpopulation of the DRG in two different ways. In situ hybridisation experiments using an extracellular domain probe showed that approximately 30% of the proprioceptors died in the lumbar L4 DRG of P4 trkCshc/shc mice (Figure 2A). Although this probe could also have highlighted truncated TrkC isoforms, it has been reported that one prominent truncated TrkC isoform is downregulated towards birth (Menn et al., 1998). Using a cell size profiling approach we obtained a result similar to the in situ data, be it with much greater variation (figure 1B). Importantly, when we looked at all individual intervals (at either 5 or 10 µm² intervals) we saw that in none of these the wildtype and trkCshc/shc numbers showed a statistically significant difference (figure 1A). This is also reflected in the average cell surface area (wild type 26.7 ± 1.4 µm² and trkCshc/shc mutants 25.1 ± 1.7 µm², p=0.5). Lastly, the animals do not show any proprioceptive phenotype, indicating that a functional proprioceptive system is maintained. Together, these results show that in the trkCshc/shc mice a minority of proprioceptive DRG neurons dies, while the remaining neurons are not functionally impaired.

We next turned to two NT3-dependent subclasses of mechanoreceptors, the slowly adapting mechanoreceptors (SAM) innervating Merkel cells and the D-hair mechanoreceptors (Airaksinen et al., 1996). These mechanoreceptors make up a very small proportion of the DRG and there are no known markers to identify their cell bodies. We therefore determined the effects of the TrkC-Shc mutation on several classes of mechanoreceptors by recording nerve fibers in a purely sensory nerve, the saphenous nerve. The SA mechanoreceptors depend on NT3 and TrkC (Airaksinen et al., 1996); Fundin 1997), however our analysis shows that the numbers of slowly- and rapidly adapting mechanoreceptors are unaltered in trkCshc/shc mutant mice. D-hair receptors depend on NT4 and NT3 for survival at different periods during development (Stucky and Lewin, 1998). In NT4+/− animals all D-hair receptors die (Stucky et al., 1998), whereas in NT3+/− animals 50% of D-hair receptors die. Previously, we observed a near complete loss of D-hair receptors in trkBshc/shc (Minichiello et al., 1998). We find in our trkCshc/shc mutants no significant difference in the proportion of D-hair receptor and A-fiber (AM) Aδ fibers. These data show that the mutation has no significant effect on TrkC-dependent survival of these mechanoreceptors and that their fibers do not degenerate postnatally.

Nearly all nodose-petrosal ganglion (NPG) neurons depend on BDNF and NT4 for survival (Conover et al., 1995). Both TrkB and BDNF knockout mice die of a breathing deficit (Klein et al, 1993) (Erickson et al., 1996), most likely due to a loss of dopaminergic neurons innervating the carotid body and blood pressure sensors (Erickson et al., 1996) (Brady et al., 1999). In trkBshc/shc mice no breathing problems and no reduction of tyrosine hydroxylase immunoreactivity, a marker for the
dopaminergic subpopulation, were observed (Minichiello et al., 1998). Moreover, approximately half the neurons in the ganglion died, similar to the amount lost in either BDNF or NT4 knockout mice. In addition, in cultured nodose neurons from mutant mice the NT4-dependent survival was severely reduced, whereas the BDNF-dependent survival was largely unaltered compared to wildtype. Together, this shows that predominantly NT4-dependent neurons are lost in the trkB<sup>shc/shc</sup> mutants (Minichiello et al., 1998). Here, we studied whether the remaining BDNF-dependent neurons maintained their target innervation. The baroreceptor innervation to the aorta was severely reduced in trkB<sup>shc/shc</sup> mutants compared to wildtype animals. But, as shown previously, the number of dopaminergic cell bodies in the NPG was unchanged. This shows that these TrkB-dependent neurons share the loss of target innervation phenotype observed in the vestibular organs of trkB<sup>shc/shc</sup> mutants. In conclusion, since the cell populations that depend on TrkB versus TrkC signalling in the inner ear are different (vestibular versus cochlear neurons, Chapters 4 and 5), one might argue that different cellular contexts determine the different biological responses. However, two different TrkB-dependent neurons, vestibular and nodose neurons, show similar reductions in target innervation (Chapter 5 and 6), and three populations of TrkC-dependent neurons, cochlear, DRG proprioceptive, and D-hair mechanoreceptive neurons, all maintain target innervation and functionality (Chapters 4 and 6). This rather suggests that the observed differences between TrkB and TrkC reflect different signalling properties of the two related receptors.

**EXPERIMENTAL PROCEDURES**

**DRG cell profiling.** In order to determine cell loss and changes in cell morphology in the trkC<sup>shc/shc</sup> mutants we examined the distribution of cross-sectional areas of neuronal profiles in Cresyl Violet labelled DRG sections. Neonatal mice (P4) were perfused with PBS followed by 4% paraformaldehyde. After postfixation the spinal segments containing the L4 DRG’s were dissected, dehydrated and embedded in paraffin. Serial sections (8 µm) were stained in Cresyl Violet. Non-overlapping images covering the entire section were recorded every fifth section throughout the ganglion. Subsequently, in each image the surface area was measured only of cells that displayed a clear nucleus and nucleolus using the NIH Image programme. A cell size histogram was generated by classification of cells in 3, 5 or 10 µm<sup>2</sup> intervals. The amount of large-diameter proprioceptors was determined by measuring above which surface area the 19% largest cells are found in each of three wild-type animals. This point was then imposed on three mutant cell surface distributions resulting in the percentage of proprioceptors in the trkC<sup>shc/shc</sup> animals. To correct for DRG-wide cell body shrinkage we tested whether individual categories in the cell size histogram differed significantly from each other.

**In situ hybridisation.** In order to quantify the large-diameter proprioceptive neurons that are lost in the TrkC knockout (Klein et al., 1994) we highlighted them by in situ hybridisation using the extracellular domain of TrkC as a probe. Serial paraffin sections (8 µm) of P0 DRG’s were stained and counted every sixth section. DIG in situ hybridisations were performed according to standard protocol. Antisense riboprobes were transcribed in vitro and labeled by incorporation of digoxigenin-linked UTP (Roche) from linearized template containing the extracellular domain of TrkC (Tessarollo et al., 1993).

**Nerve histology and electrophysiology.** The saphenous nerve histology was carried out essentially as previously described (Airaksinen et al., 1996). Briefly, a length of saphenous nerve from mutant mice used for recordings was fixed in situ with a 2.5% glutaraldehyde solution in PBS. The nerve was left in fixative overnight and then osmicated for one hour to stain myelin and then embedded in plastic (technovit). Semi thin sections (2µm) were cut and the number of myelinated axons per nerve
counted. For the electrophysiological analysis an in vitro skin/nerve preparation was used to record from functionally single primary afferents in micro-dissected teased filaments of the saphenous nerve as described previously (Koltzenburg et al., 1997). Once the receptive field of single units had been identified with a mechanical search stimulus, its receptive properties were systematically examined with a range of quantitative stimuli. Using a probe fixed to a micrometer a standard ascending series of displacement stimuli were applied to the receptive field at 30 sec intervals. This stimulus series as well as the use of Von Frey hairs to determine mechanical threshold of single fibers allowed us to classify units as slowly and rapidly adapting mechanoreceptors.

**Baroreceptor innervation.** For the analysis of Baroreceptor innervations, tissue preparation and section immunostaining with PGP 9.5 antibody (Accurate, Westbury, NY) were done as described (Erickson et al., 1996).