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A ROLE FOR p75 RECEPTOR IN NEUROTROPHIN-3 FUNCTIONING DURING THE DEVELOPMENT OF LIMB PROPRIOCEPTION

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Abstract—Neurotrophin-3 is indispensable for the development of limb proprioceptive neurons and their end organs, muscle spindles. To determine whether the low-affinity p75 receptor potentiates the actions of neurotrophin-3, we examined the development of the proprioceptive system in p75 null mutant mice that had either normal or decreased tissue levels of neurotrophin-3. Postnatal mice lacking both copies of the p75 gene had fewer sensory neurons in dorsal root ganglia, but normal complements of muscle spindles in fast hindlimb muscles, although the slow soleus muscle showed a 50% loss of spindles. However, compound mutants lacking both copies of the p75 gene as well as one copy of the neurotrophin-3 gene displayed a dystonic/ataxic phenotype similar to that observed previously in neurotrophin-3 null mutants devoid of proprioception. The compound mutants also exhibited a commensurate loss of parvalbumin-expressing (proprioceptive) neurons in dorsal root ganglia. The degree of deficiency of spindles (and presumably proprioceptive neurons) in the compound mutants exceeded the sum of deficits in single mutants lacking either both copies of p75 genes or one copy of neurotrophin-3 gene, suggesting a synergistic interaction between the p75 receptor and neurotrophin-3. Neuronal deficits in the compound mutants were present prior to embryonic day 14, indicating an early role for the p75 receptor in sensory neuronogenesis.

Collectively, these data indicate that the p75 receptor is not essential for the survival and differentiation of most limb proprioceptive neurons when neurotrophin-3 is expressed at normal levels. However, the p75 receptor may act in synergy with neurotrophin-3 to enhance the survival of proprioceptive neurons when tissue levels of neurotrophin-3 are a limiting factor. © 1999 IBRO. Published by Elsevier Science Ltd.

Key words: neurotrophin-3, p75 receptor, muscle spindles, proprioception, sensory neurons.

The neurotrophin receptor p75 is a transmembrane glycoprotein that binds all members of the neurotrophin family with more or less equal affinity.[4,7,40] As an accessory molecule to the tyrosine kinase (trk) family of receptors or through a signaling pathway independent of trk receptors, the p75 receptor may mediate either cell survival or death, depending on the presence or absence of neurotrophins and the stage of neuronal development.[6,13,14]

In the presence of neurotrophins, p75 enhances neurotrophin binding and modulates trk signaling, allowing trk receptors to respond to lower concentrations of neurotrophins.[2,20,21,37] Thus, the affinity of trkA-expressing cells for nerve growth factor (NGF) is enhanced by p75 activation, and an optimal signal may be obtained only by co-stimulation of trkA and p75 receptors.[30] Blocking p75 receptors decreases binding of NGF to trkA receptors on sympathetic neurons.[26] In the absence of the p75 gene, cultured trkA-expressing neurons require a higher dose of NGF for survival.[12,29] Enhancement of NGF/trkA signaling by the p75 receptor may be important during development, when neurons are competing for limited amounts of target-derived neurotrophic factors.

Whether the p75 receptor also plays a role in neurotrophin-3 (NT-3)/trkC functioning is not known. Targeted deletion of either NT-3 or its cognate receptor trkC leads to complete absence of proprioceptive neurons in lumbar dorsal root ganglia (DRGs) and spindles in limb muscles.[17,18,22,36] The p75 receptor might potentiate the effect of NT-3 on neuronal survival, because most proprioceptive neurons co-express trkC and p75. Approximately one-half of trkC- expressing neurons also express p75 in DRGs of adult mice.[41] The incidence of trkC and p75 co-localization in neurons could be even higher during embryonic development, when expression of both trkC and p75 is widespread in DRGs.[33,43] If p75 facilitates the survival of proprioceptive precursors or

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Abbreviations: DiI, 1,1′-dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; DR, dorsal spinal root; DRG, dorsal root ganglion; E, embryonic day; MG, medial gastrocnemius; NGF, nerve growth factor; NT-3, neurotrophin-3; P, postnatal day; WT, wild-type.

neurons, it could do so by potentiating NT-3/trkC functioning prior to the stage of axonogenesis, when the neurons depend on intraganglionic NT-3.[16] Alternatively, p75 could play a role at a later stage, when axonal projections are already formed and proprioceptive neurons are supported retrogradely by target-derived NT-3.[19,31,42] Retrograde axonal transport of neurotrophins is reduced in p75-deficient mice.[11]

Analyses of mice lacking either one or both copies of the NT-3 gene showed a direct relationship between the level of NT-3 in tissue and the number of proprioceptive neurons in DRGs and spindles which ultimately form in muscles.[1,17] If p75 enhances NT-3 signaling, this would become more apparent when levels of NT-3 are decreased. Levels of NT-3 are decreased in mice lacking one copy of the NT-3 gene.[15] We therefore crossed p75 null mutants into a line carrying the NT-3 gene deletion to generate mice completely lacking p75 and exhibiting a decreased level of NT-3.

EXPERIMENTAL PROCEDURES

Animals

The p75 and NT-3 null mutant mice were generated on a 129-Balb/c background, as described previously.[17,28] Compound p75−/−NT-3+/− mutants were produced by mating p75+/− and NT-3+/− mice. Genotyping was carried out by polymerase chain reactions. Primers for p75 genotyping were PGK (5′-GGGAACTTCCTGACTAGGG G-3′), p75-1 (CCTCGCATTCGGCGTCAGCC) and p75-2 (CGATGCTCCTACGGCTACTA) (sequences kindly provided by Guy Guidry, NINDS, NIH). Primers for NT-3 genotyping were PGK, NT-3-3 (CCTGGCTTCTTTACAT CTCG) and NT-3-4 (TGGAGGATTATGTGGGCAAC). The polymerase chain reaction fragments from mutant alleles are larger than those from wild-type (WT) alleles. Mice were staged by setting the morning after mating as embryonic day (E) 0.5 and the day of birth postnatal day (P) 0. All animal experiments were carried out in accordance with the institutional Animal Review Board, and all efforts were made to minimize animal suffering and reduce the number of animals used.

Neuron counts

Animals were anesthetized with sodium pentobarbital (50 mg/kg). Adult mice were perfused with fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer. L4 DRGs and individual hindlimb muscles were excised, postfixed in OsO_4 and embedded in Eponate 12. Tissues were serially cross-sectioned at 1 μm and stained with Toluidine Blue for spindle and DRG neuronal counts. For embryonic tissues, E14.5 embryos from anesthetized pregnant mothers were cut in half transversely, fixed overnight in 4% paraformaldehyde buffered with 0.1 M sodium phosphate at 4°C, and processed for paraffin sectioning with an automatic tissue processor (Tissue-Tek VIP, Miles Scientific, Naperville, IL). Sagittal serial sections were cut at 5 μm and stained with Cresyl Violet for DRG neuronal counts.

For counts of DRG neurons from plastic sections, a modification of the empirical method of Coggeshall *et al.*[9] was used, as detailed elsewhere.[42] Sections were examined at ×250 at 60-μm intervals throughout the length of the L4 DRG. The number of nuclei visible in each section was counted and summed. The lengths of 50 randomly sampled nuclei were then measured in a series of serial sections. The total number of nuclei counted in the sample was multiplied by 60 (the sample interval) and divided by the mean nuclear length to give the total number of neurons in the entire DRG. The DRG counts obtained with the empirical method were comparable to test counts obtained from three specimens using an optical dissector technique.[8] To obtain DRG neuron counts in paraffin-embedded sections at E14.5, cells with Nissl-stained cytoplasm and clear nuclei with nucleoli were counted as neurons from every eighth section at 40-μm intervals. Counts of L4 DRG neurons obtained from plastic sections are higher than those reported for paraffin-embedded material,[17,19] but the percentage differences between the control and experimental values are comparable using the two counting techniques (see tables in Results). The numbers of myelinated nerve fibers in muscle nerves and dorsal spinal roots (DRs) were obtained at ×1000. Muscle nerves were examined 5 mm proximal to the point of entry of the nerve into the muscle, and DRs were examined 0.5 mm proximal to the DRG. Complements of muscle spindles were determined by counting the numbers of spindle equators in serial plastic sections. Statistical analyses of the data were performed with a Statview program using a *t*-test and the Wald and Wolfowitz runs test.

Immunocytochemistry was used to determine the number of neurons expressing parvalbumin in DRGs. The spinal cord and attached DRGs of newborn mice were fixed overnight in 4% paraformaldehyde buffered with 0.1 M sodium phosphate at 4°C. L4 DRGs were dissected, cryoprotected with 30% sucrose in phosphate-buffered saline and embedded in Tissue-Tek OCT compound (Miles Scientific) for cryostat sectioning. Serial 10-μm sections were cut with a Reichert–Jung cryostat, mounted on frosted slides (Fisher Scientific, Malvern, PA), warmed to room temperature, rinsed with phosphate-buffered saline and incubated with blocking solution for 1 h. Sections were exposed to primary antibody at room temperature overnight and subsequently incubated with a secondary antibody conjugated with fluorochrome for 1–2 h, coverslipped with glycerol–phosphate-buffered saline and observed with a Zeiss fluorescent microscope. The monoclonal anti-parvalbumin antibody was used (1:1000; Sigma, St Louis, MO).

Labeling of Ia afferents

1,1′-Dioctodecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) labeling was used to determine the density of group Ia afferent projections to spinal motoneurons. Axonal labeling with DiI (Molecular Probes, Eugene, OR) was carried out as described previously.[17] In brief, E14.5 cervical spinal cords with attached DRGs were dissected, pinned on 3% agarose gel and submerged in 4% paraformaldehyde. DiI dissolved in ethanol was microinjected into C5–C6 DRGs. The injected dye diffuses into both sensory and motor fibers associated with the DRG, and stains their afferent and efferent projections. After incubation at 42°C for seven to 10 days, spinal cords were rinsed several times in phosphate-buffered saline and embedded in 2% agarose gel. Cross-sections (50 or 100 μm) of the spinal cord were cut using a Vibratome and viewed with a rhodamine filter on a Zeiss fluorescent microscope.

RESULTS

p75−/−neurotrophin-3+/− compound mutants exhibit a dystoniclataxic phenotype suggestive of deficient proprioceptive functioning

In total, 21 p75−/− and 18 p75−/−NT-3+/− mice were studied. Most p75−/− mutant mice could not be distinguished phenotypically from WT

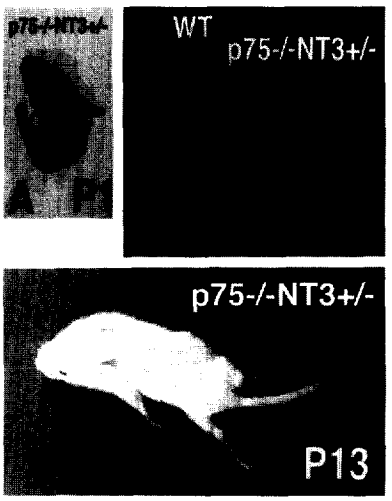

Fig. 1. Compound p75−/−NT-3+/− mutants exhibit dystonic/ataxic posturing of limbs, similar to that described previously for NT-3−/− mice.[17] The abnormalities are present at all stages of postnatal (P) development, including P1. The P13 mutant shows particularly severe limb abnormalities. Also note that p75−/−NT-3+/− mutants are smaller than WT mice (B).

littermates except for a lower (10–30%) body weight. An occasional p75−/− mutant showed slightly unstable gait and mild posturing of extremities, as reported previously.[29] These motor abnormalities were manifested during the first two postnatal weeks and disappeared by adulthood. NT-3+/− mutants had a normal behavioral phenotype.[17] In contrast, the motor patterns of p75−/−NT-3+/− mutant mice were grossly abnormal (Fig. 1). Newborn and adolescent p75−/−NT-3+/− animals displayed dystonic extensor posturing of limbs as well as abnormal ataxic gait with frequent falls, strikingly similar to the postural and gait deficits observed in NT-3 or trkC null mice deficient in proprioception.[17,18,22] These abnormalities were most prominent during the first two postnatal weeks and lessened with increasing survival time (see below). Compound homozygous mutants (p75−/−NT-3−/−) died at birth.

Only a few p75−/−NT-3+/− mutants survived longer than three weeks, and four of 60 mutants reached at least four to six months of age. The dystonia/ataxia lessened with increasing survival time, as if the CNS motor programs dependent on proprioceptive input had adjusted to a proprioceptive deficit over time. However, a new motor abnormality emerged at the age of three to four weeks, characterized by intermittent flexor spasms and uplifting of hindlimbs. Whether this behavioral symptom was related to the proprioceptive system or reflected dysfunction of another component of the sensory or motor system in these long-term survivors was not clear.

The ataxic/dystonic phenotype of p75−/−NT-3+/− mice was similar to the phenotype observed in NT-3−/− mice, which completely lack the limb proprioceptive system.[17] We therefore compared constituents of the proprioceptive system in p75−/−NT-3+/− mutants with those of p75−/−, NT-3+/− and WT (NT-3+/+, p75+/+) animals (Tables 1–3).

Table 1. Complements of spindles in hindlimb muscles of p75−/−, neurotrophin-3+/− and p75−/−neurotrophin-3+/− mice

Muscle	Mean number of spindles (*n*)	Percentage of WT
Soleus		
WT	11.4±0.9 (7)	
p75−/−	5.1±1.5 (19)	45*
NT-3+/−	5.4±1.0 (15)	48*
p75−/−NT-3+/−	0.9±0.8 (15)	8*
Medial gastrocnemius		
WT	10.5±1.1 (8)	
p75−/−	10.9±1.5 (18)	104
NT-3+/−	5.2±0.9 (6)	50*
p75−/−NT-3+/−	2.8±1.4 (13)	27*
Plantaris		
WT	9.7±0.7 (9)	
p75−/−	10.4±0.6 (13)	107
NT-3+/−	4.5±0.5 (2)	46*
p75−/−NT-3+/−	2.3±1.7 (6)	24*
Lumbrical		
WT	1.8±0.4 (9)	
p75−/−	1.8±0.4 (5)	100
NT-3+/−	1.3±0.4 (4)	70*
p75−/−NT-3+/−	0.6±0.7 (8)	35*

Comparison of numbers of spindles in the soleus, medial gastrocnemius, plantaris and lumbrical muscles among WT, p75−/− and NT-3+/− single mutants, and in p75−/−NT-3+/− compound mutants. NT-3−/− mice have no spindles regardless of limb muscle.[17] Data from P0–P1 and P18–P21 animals were pooled, and are shown as means±S.D. (*n*). *$P<0.001$ (*t*-test between WT and each mutant).

Deficits in complements of muscle spindles in p75−/−neurotrophin-3+/− compound mutants exceed those observed in p75−/− or neurotrophin-3+/− single mutants

Complements of muscle spindles reflect numbers of proprioceptive neurons, because there is a 1:1 relationship between spindles and group Ia proprioceptive neurons.[44] We compared the density of spindles in soleus, medial gastrocnemius (MG), plantaris and lumbrical muscles among p75−/−, NT-3+/−, p75−/−NT-3+/− and WT animals (Table 1). Spindles develop prenatally and their density in muscles does not change after birth.[23] No statistically significant difference between spindle counts at P0–P1 and P18–P21 was observed. Data from newborn (P0–P1) and young adult (P18–P21) mice were therefore pooled. As expected, the number

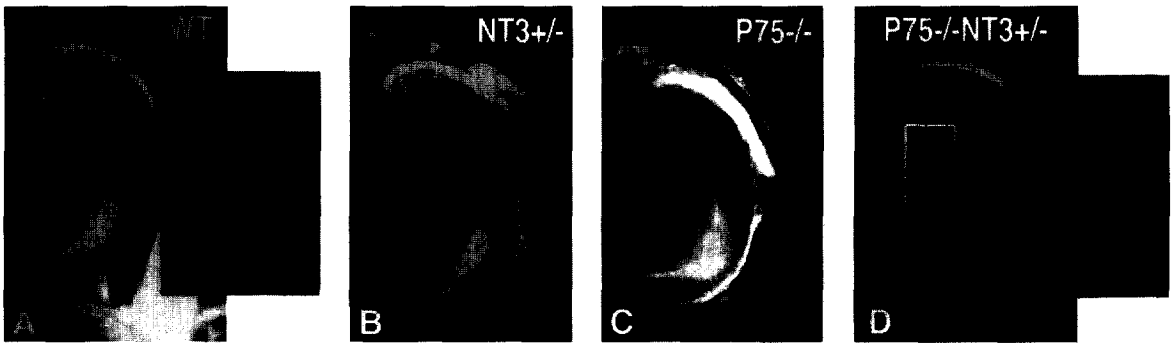

Fig. 2. Comparison of density of group Ia afferents projections to spinal motoneurons among mutant and WT mice. DiI labeling of central projections from cervical DRG to the spinal cord in WT (A), NT-3+/− (B), p75−/− (C) and p75−/−NT-3+/− (D) mice at E14.5. A and D include a higher magnification view of the target area of Ia afferent projections. Note that DiI-labeled Ia afferents are rare in p75−/−NT-3+/− mice, reflective of the paucity of proprioceptive neurons (D).

of spindles in NT-3+/− mutants was roughly one-half the normal spindle complement, regardless of the muscle examined.[17] Unexpectedly, spindle content varied among different muscles in p75−/− mice. MG, plantaris and lumbrical muscles exhibited no reduction in spindle density, even in one P75−/− mouse examined at six months of age. The only p75−/− muscle displaying a deficit of spindles was the soleus, which had a 50% loss. Thus, when the level of NT-3 is normal, the p75 deletion has no effect on complements of spindles in most hindlimb muscles.

Fewer spindles were present in hindlimbs of p75−/−NT-3+/− compound mutants relative to p75−/− or NT-3+/− single mutants. Similar to p75−/− mutants, the extent of spindle deficiency in p75−/−NT-3+/− mutants differed among the four muscles examined (Table 1). Significantly, MG, plantaris and lumbrical muscles in p75−/−NT-3+/− mutants exhibited a larger spindle deficit than the sum of spindle deficits in p75−/− (no deficit) and NT-3+/− (about 50% reduction) single mutants (Tables 1, 3). The soleus, a muscle deficient in spindles in p75−/− mutants, exhibited the greatest relative spindle deficit among the p75−/−NT-3+/− muscles examined, and 31% of double mutant soleus muscles were entirely devoid of spindles in both the P0–P1 and P18–P21 age groups. The complete absence of spindles in nearly one-third of p75−/−NT-3+/− soleus muscles underscores a role for p75 in proprioceptive development of this muscle.

Central and peripheral projections of proprioceptive neurons are deficient in p75−/−neurotrophin-3+/− mutants

Projections of proprioceptive afferents were studied as an index of density of proprioceptive neurons (Fig. 2). Central projections of group Ia neurons also synapse on motor neurons of the ventral spinal cord. The density of these projections reflects the number of Ia neurons in DRGs.[34] Using DiI tracing, we examined Ia projections in p75−/−, NT-3+/− and p75−/−NT-3+/− embryos at E14.5, a stage at which afferent–motor neuron contacts are already established in WT mice.[24] p75−/− and NT-3+/− embryos displayed an intraspinal pattern of Ia projections similar to that of WT littermates. In contrast, Ia projections to motor neurons appeared to be severely reduced in p75−/−NT-3+/− mutants, although not completely absent as characteristic of NT-3−/− animals.[17] Thus, numbers of group Ia proprioceptive neurons must already be severely reduced in DRGs of p75−/−NT-3+/− mutants at E14.5.

In the periphery, proprioceptive neurons project into muscles. A decreased number of proprioceptive neurons would therefore be expected to result in a decreased number of myelinated fibers in the muscle nerves. The soleus muscle nerve was examined because the soleus was deficient in spindles in all mutants studied. The density of myelinated fibers was decreased in all mutant soleus muscle nerves, with deficits relative to the number of myelinated fibers in WT mice (76 ± 2, mean ± S.E.M., $n=2$) ranging from mild in NT-3+/− (52 ± 4, $n=6$, $P<0.001$) to moderate in p75−/− (36 ± 2, $n=4$, $P<0.001$) and to severe in p75−/−NT-3+/− (26 ± 2, $n=8$, $P<0.001$) mice. The number of remaining myelinated fibers in p75−/−NT-3+/− soleus nerve was close to that observed in NT-3−/− mutants (22 ± 1, $n=4$), which totally lack proprioceptive afferents.[24,25] We assume that the deficit of myelinated fibers in the soleus muscle nerve reflected a deficit of proprioceptive as well as non-proprioceptive afferents, compounded by a concomitant deficit of fusimotor axons (data not shown).

Proprioceptive neurons are deficient in p75−/−neurotrophin-3+/− mutants

Given the 1:1 ratio between spindles and group Ia neurons,[44] the difference in spindle complements between single and compound mutants was assumed to reflect a difference in the number of proprioceptive

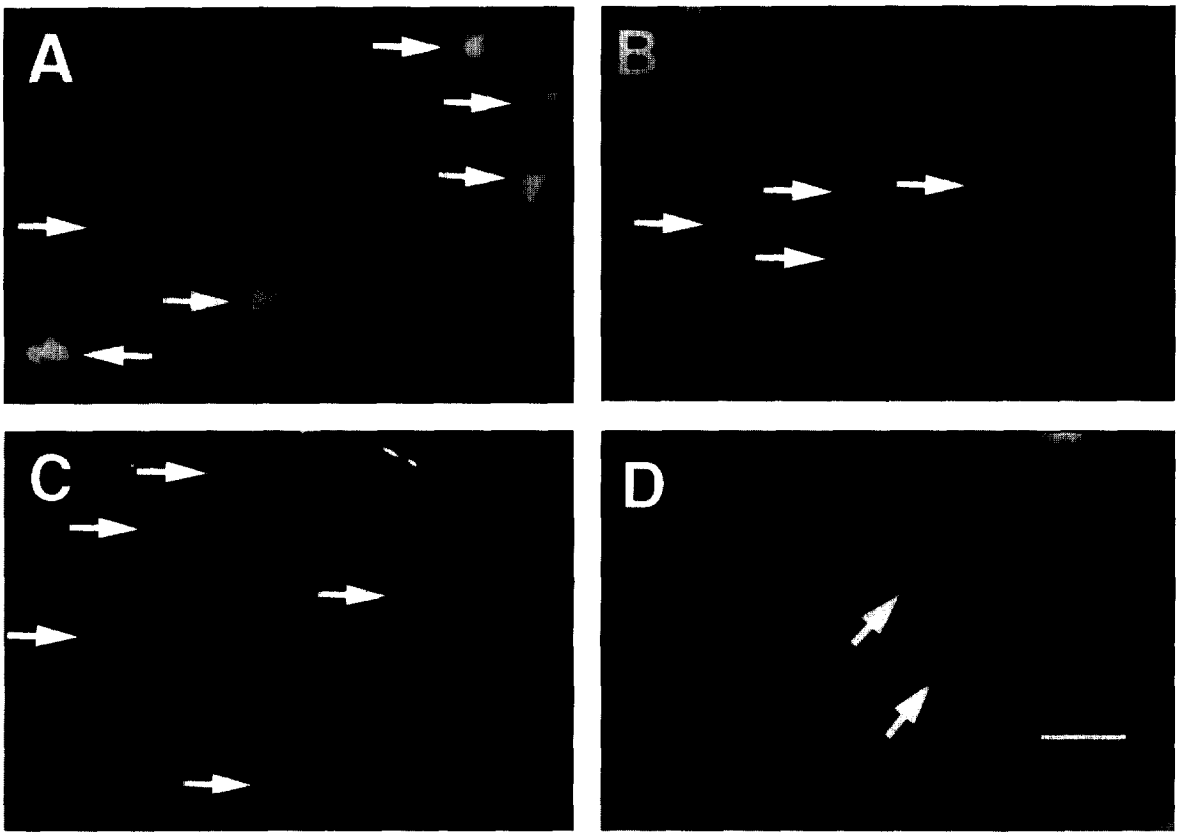

Fig. 3. Comparison of numbers of parvalbumin-positive sensory neurons among WT and mutant DRGs. Neuronal counts were obtained from alternate 10-μm serial sections of entire L4 DRGs. *Significant at $P<0.001$ relative to WT. Photographs show the representative parvalbumin staining in the L4 DRGs of WT (A), NT3+/− (B), p75−/− (C), and p75−/− NT3+/− compound mutant (D). Scale bar=50 μm.

neurons in these mice. To address this issue, we immunoreacted newborn DRGs with an antibody for parvalbumin, as shown in Fig. 3. Parvalbumin has been used as a marker of DRG proprioceptive neurons.[5,10,17,42] No parvalbumin-positive neurons are present in NT-3 null mutants that lack proprioceptive neurons.[1,17] We found that DRGs of p75−/−NT-3+/− compound mutants exhibited a more severe (about 90%) loss of parvalbumin-positive neurons than DRGs of NT-3+/− or p75−/− single mutants, consistent with a greater loss of spindles in compound than single mutants (or than would be predicted if the p75−/− and NT-3+/− mutations acted independently). Based on the observation of a mild spindle deficit in p75−/− mice, we would have predicted a smaller loss of proprioceptive neurons than the observed 50% deficiency of parvalbumin neurons in p75−/− L4 DRGs. The reason for this deviation from expectation is unclear. One possible explanation is that, in the absence of p75, the mechanical properties of spindle afferents are altered[35] and parvalbumin expression is down-regulated in corresponding proprioceptive neurons.[5] Moreover, parvalbumin expression may not be limited to proprioceptive neurons alone, because some non-proprioceptive neurons appear to express parvalbumin.[44] Non-proprioceptive neurons are also depleted in p75−/− mice (see below).

The overall deficit of sensory neurons in p75−/− neurotrophin-3+/− compound mutants exceeds that in p75−/− or neurotrophin-3−/− single mutants

To address whether more sensory neurons are missing in the compound than single mutants (or than would be predicted if the p75−/− and NT-3+/− mutations acted independently), we compared the number of neurons among mutant and WT L4 DRGs at P18–P21 (Table 2). Our data support the observation that DRGs of p75−/− mice are smaller than those of WT mice[3,28,29] and have a 50% overall loss of neurons.[35] Examination of DRGs in NT-3+/− mutants showed a 17% loss of neurons, slightly less than that reported previously.[1] Double mutant p75−/−NT-3+/− mice displayed a more severe deficit (78%) of sensory neurons than the sum of deficits in single mutants. The neuronal deficit of p75−/−NT-3+/− DRGs exceeded that observed in NT-3 null DRGs.[17]

Table 2. Comparison of numbers of dorsal root ganglion neurons and dorsal spinal root myelinated fibers among wild-type, p75−/− and neurotrophin-3+/− single mutants, and p75−/−neurotrophin-3+/− compound mutants

		Mean number (*n*)	Percentage of WT
DRG neurons			
WT	Embryonic	4667 ± 374 (3)	
	Adult	8606 ± 726 (5)	
p75−/−	Embryonic	2592 ± 135 (3)	55*
	Adult	3848 ± 689 (8)	45*
NT-3+/−	Embryonic	3570 ± 265 (4)	76*
	Adult	7120 ± 728 (6)	83*
p75−/−NT-3+/−	Embryonic	1152 ± 78 (5)	25*
	Adult	1872 ± 326 (9)	22*
NT-3−/−	Adult	2404 ± 674 (4)	28*
DR myelinated fibers			
WT		2686 ± 244 (7)	
p75−/−		1239 ± 162 (10)	46*
NT-3+/−		1762 ± 149 (6)	66*
p75−/−NT-3+/−		465 ± 54 (12)	17*
NT-3−/−		555 ± 123 (8)	21*

Adult mice were examined at P18–P21 (P16–P21 for NT-3−/− mice) in plastic-embedded sections and embryonic mice were examined in paraffin-embedded material at E14.5. Note that the number of DRG neurons is reduced in mutant mice at E14.5. Also note that the neuronal deficit in p75−/−NT-3+/− DRGs exceed that of NT-3−/− DRGs. Data are shown as means ± S.D. (*n*). *$P<0.001$ (*t*-test between WT and each mutant).

To corroborate that the missing neurons in DRGs included proprioceptive neurons, we counted the number of myelinated fibers in DRs (axons of proprioceptive neurons are myelinated). A deficit of myelinated DR fibers was present in all mutants examined (Table 2). Similar to spindle deficits, the deficit of myelinated DR fibers in p75−/−NT-3+/− mice exceeded that in p75−/− or NT-3+/− mice. Thus, large DRG neurons (such as proprioceptive), which give rise to myelinated nerve fibers, were deficient in DRGs of p75−/−NT-3+/− mice. However, large sensory neurons serving modalities other than proprioceptive must also be deficient in p75−/−NT-3+/− mice, because the extent of myelinated fiber loss (82%) exceeded the proportion of proprioceptive fibers (40%) among myelinated DR fibers.[22]

Although proprioceptive neuron numbers are reduced in p75−/−NT-3+/− mice, most of the missing DRG neurons in the compound mutants must belong to the non-proprioceptive category. About 75% of DRG neurons were lost in p75−/−NT-3+/− mutants (Table 2), yet proprioceptive neurons represent only 15–19% of the total DRG neuronal population.[22,41] Moreover, the approximately 50% loss of neurons in p75−/− DRGs was assumed to reflect an absence of non-proprioceptive rather than proprioceptive neurons, given the relative preservation of spindles in these mutants. Similarly, NT-3−/− mutants lack approximately 70% of DRG neurons, reflecting both proprioceptive and non-proprioceptive deficits.[1,17,19] Thus, large and possibly similar populations of non-proprioceptive neurons may be affected by the p75 and NT-3 gene deletions.

Cell death is increased in embryonic sensory ganglia of p75−/−neurotrophin-3+/− mutants

Complements of spindles and proprioceptive neurons were already reduced at birth in mutant mice; thus, the gene deletions must have impacted neuronal development during embryogenesis. To address this issue, we compared the number of E14.5 DRG neurons between mutant and WT embryos (Table 2). By E14.5, neuronogenesis is complete in lumbar DRGs and the number of neurons is close to that observed in postnatal DRGs.[19] Mutant L4 DRGs were smaller than WT DRGs at E14.5. In addition, the numbers of L4 DRG neurons in p75−/− as well as p75−/−NT-3+/− embryos were reduced relative to WT, similar to those observed at P18–P21. The presence of neuronal deficiencies in mutant DRGs at E14.5 indicates that p75 plays a role at the earliest stages of sensory neuron development, similar to NT-3.[16,19,24]

To address whether an increased rate of cell death accounted for the neuronal loss in p75−/− and p75−/−NT-3+/− mutants, we examined the number of pyknotic cells in L4 DRGs at E14.5 (Table 3). Relative to WT, the p75−/− and p75−/−NT-3+/− mutants had a 7.5- and 12.1-fold higher rate of cell death, respectively ($P<0.001$). The presence of more densely distributed pyknotic nuclei in p75−/− and p75−/−NT-3+/− mutants relative to WT indicated that excessive cell death was occurring in these mutants at E14.5. In contrast, NT-3+/− animals had a relatively low level of cell death at this stage, indicating that most NT-3-deprived neurons had already died by E14.5, as reported by Fariñas *et al.*[19] Thus, neuronal death rather than lack of

Table 3. Comparison of the rates of cell death among wild-type, neurotrophin-3+/−, p75−/− and p75−/− neurotrophin-3+/− L4 dorsal root ganglia at embryonic day 14.5

WT	NT-3+/−	p75−/−	p75−/−NT-3+/−
1.70±0.6	2.9±0.5	12.6±1.3*	20.3±2.1**

The cell death rates were obtained by dividing the number of pyknotic cells by the number of total neurons in the L4 DRGs (×100). Values are not statistically different between WT and NT-3+/− DRGs. *Values differ between WT and p75−/− or NT-3+/− DRGs at $P<0.001$. **p75−/−NT-3+/− DRGs differ from WT, NT-3+/− or p75−/− DRGs at $P<0.0001$ (ANOVA with post hoc *t*-test).

proliferation may account for the neuronal deficits in postnatal DRGs of p75−/− and p75−/−NT-3+/− mutants.

DISCUSSION

Mutant phenotypes

The density of spindles in muscles parallels the density of group Ia proprioceptive neurons in DRGs.[44,45] We observed only a limited spindle deficit in mice lacking the p75 receptor alone, indicating that p75 is not critical for the development of most proprioceptive neurons when NT-3 is expressed at WT levels. However, a severe spindle deficiency was observed in p75−/−NT-3+/− mutants, suggesting a role for p75 in the survival of proprioceptive neurons when levels of NT-3 are decreased.

The writhing limb movements and abnormal gait of p75−/−NT-3+/− mice were previously observed only in the complete absence of proprioception, such as in mutants devoid of NT-3 or trkC or overexpressing NT-3 in the spinal cord.[17,22,32] It seems unlikely that the transient movement disturbance in p75−/− mutants stems from the minor spindle deficiency, given that NT-3+/− mice with a greater spindle loss are functionally intact.[17] Rather, abnormalities of transducer function in sensory end organs[35] or a skeletomotor dysfunction[29] might underlie the gait problem of p75−/− mice.

Synergistic effects of p75 and neurotrophin-3 on the survival of proprioceptive neurons

Comparison of spindle numbers among muscles of p75−/−, NT-3+/− and p75−/−NT-3+/− mutants provides insight into the interaction of the p75 and NT-3 genes in differentiation and survival of proprioceptive neurons (Table 4). Deletion of p75 alone had a limited impact on the proprioceptive system, and significant deficits of proprioceptive neurons occurred only when the absence of p75 was compounded by decreased levels of NT-3. The MG, plantaris and lumbrical muscles, three predominantly fast limb muscles, had a 63–72% deficit of spindles in p75−/−NT-3+/− mutants, whereas no deficit was seen in p75−/− mice, and only a 50% or lesser deficit in NT-3+/− mice. Thus, deletion of the p75 gene significantly potentiated the deficit of spindles in p75−/−NT-3+/− compound relative to NT-3+/− single mutants. Based on these spindle numbers, we estimate that approximately 15–35% of proprioceptive neurons innervating the three muscles were lost in p75−/−NT-3+/− compound mutants (Table 4) as a consequence of the p75 deletion. Presumably, these neurons were lost owing to the absence of a synergistic effect of p75 and NT-3 normally operative in NT-3+/− mice that exhibit decreased tissue levels of NT-3.

Although the somata of afferents innervating all four muscles reside in the L4 DRG, the soleus was the only muscle examined that had a reduced complement of spindles in p75−/− mice. It was also the only predominantly slow muscle among the four limb muscles surveyed. It is unclear whether the spindle deficit in p75−/− mice is related to some neurogenic or myogenic factor specific to the slow limb muscles. However, the soleus muscle behaves differently from the other limb muscles in other experimental situations involving proprioception. Supernumerary spindles form in nerve-crushed MG but not soleus muscles in neonatal rats.[45] Unlike MG or plantaris muscles, the soleus muscle also fails to display supernumerary spindles in mice overexpressing NT-3.[42] Thus, some aspects of the regulation of developing proprioceptive neurons by p75 or NT-3 are different in the soleus relative to other limb muscles. Nevertheless, the deficit of spindles in p75−/−NT-3+/− soleus muscles exceeded the sum of spindle deficits in p75−/− and NT-3+/− soleus muscles, indicating that the synergistic effect of p75 and NT-3 is also operative during the proprioceptive development in slow muscles.

Lumbar proprioceptive neurons form at E10.5–E11.5.[19,27] They almost certainly express p75, because p75 expression is widespread in early DRGs, with a peak at E11.5–E12.5.[33,34] The neurons also express trkC[35] and require NT-3 prior to axonogenesis, either at the precursor stage or shortly thereafter.[16,19] One possibility is that p75 enhances NT-3/trkC functioning at these early stages when NT-3, either produced within DRGs[16] or diffusing into the ganglion,[19] is critical for survival. This enhancing effect of p75 would then be revealed by decreased survival of proprioceptive neurons in p75−/−NT-3+/− mice in which the p75 gene is deleted and NT-3 levels are decreased. That expression of p75 potentiates the ability of each of the trk receptors to mediate cell survival in the presence of limiting amounts of neurotrophins has been reported.[20,21]

Alternatively, p75 might act at a later stage of development, when continued survival of differentiated proprioceptive neurons depends on retrograde transport of NT-3, initially from mesenchymal tissues

Table 4. Summary of the synergism between the p75 receptor and neurotrophin-3

	Predicted proportion for p75−/−NT-3+/− mutants (%)	Actual proportion in p75−/−NT-3+/− mutants (%)	Change relative to predicted (%)
No. of spindles in soleus	22 (0.45 × 0.48)	8	−14
No. of spindles in MG	52 (1.04 × 0.50)	27	−25
No. of spindles in plantaris	49 (1.07 × 0.46)	24	−25
No. of spindles in lumbrical	70 (1.0 × 0.7)	35	−35
No. of DRG neurons in adults	37 (0.45 × 0.83)	22	−15
No. of DRG neurons in embryos	42 (0.55 × 0.76)	25	−17
No. of DR myelinated fibers in adult	30 (0.46 × 0.66)	17	−13

Values shown are the proportion of WT values. Predicted proportion is based on the product of individual proportions in single p75−/− or NT-3+/− mutants, as shown in Table 1. Actual proportion is the number of spindles in mutants/number of spindles in WT. Note that the deficit in the seven parameters examined in compound p75−/−NT-3+/− mutants exceeds the predicted deficits (i.e. products of deficits in single p75−/− and NT-3+/− mutants). A statistically significant trend of actual proportions being less than predicted proportions (i.e. actual deficits being greater than predicted deficits) was confirmed by Wald and Wolfowitz runs test for all seven parameters.

and subsequently from muscles.[19,31,42] Thus, p75 may either serve as a recruiting molecule to facilitate uptake of NT-3 into the nerve terminal or enhance NT-3 binding to trkC receptors. Indeed, retrograde axonal transport of neurotrophins is reduced in nerve fibers of adult p75−/− mice, although the transport of NT-3 is less affected than transport of NGF or brain-derived neurotrophic factor.[11] Transport of NT-3 from retina to brain in chick embryos is also reduced when p75 is inactive.[38,39] The facilitatory effect of p75 on retrograde transport of NT-3 may be of functional importance during normal development, when tissue levels of NT-3 are a limiting factor, such as in neurons with marginal access to NT-3, for reasons of axon topography. The observation that cells are dying at E14.5 in p75-deficient but not WT DRGs is consistent with the possibility that p75 deletion affects neuronal survival at a stage when neurons depend on target-derived NT-3. Neurons in tissue culture are not dependent on retrograde axonal signaling, which may explain why no alteration in sensitivity to NT-3 was observed in neurons isolated from p75-deficient mice.[12,28,29]

Contrasting effects of p75 on the development of proprioceptive and non-proprioceptive neurons

The deficit of proprioceptive neurons in p75−/− mice was greatly exceeded by that of non-proprioceptive neurons. Thus, our observations do not support the suggestion that all types of sensory neurons are equally depleted in p75-deficient DRGs.[3,35]

The differential impact of the p75 deletion on proprioceptive and non-proprioceptive neurons suggests that mechanisms underlying the neuronal loss may be different for the two classes of sensory neurons. The smaller non-proprioceptive neurons are also born later than the larger proprioceptive neurons. NT-3 supports the generation of non-proprioceptive DRG neurons at E12.5–E13.5 through an effect on the proliferation of neuronal precursors, and this potentiating effect may not be mediated by trkC.[19] One may therefore speculate that p75 facilitates the NT-3 effect on non-proprioceptive precursor cells at this later stage. It may not be coincidental that the degree of non-proprioceptive deficiency in p75−/− mice was similar to that reported for NT-3−/− mutants.[1,17,19] By E14.5, cell death in DRGs (although still ongoing to some degree) is largely complete in p75−/− mutants, consistent with the possibility that p75 acts at the stage (E12.5–E13.5) when non-proprioceptive neurons are generated.[19] Thus, both NT-3 and p75 may have a role in the expansion of the neuronal precursor pool from which non-proprioceptive neurons arise, although the exact mechanisms involved remain to be determined.

CONCLUSIONS

Our observations indicate that the p75 receptor is non-essential for the development of proprioceptive neurons to fast, but not slow, hindlimb muscles when NT-3 is expressed at normal levels. However, p75 may act in synergy with NT-3 to enhance the survival of all proprioceptive neurons when tissue levels of NT-3 are a limiting factor. In contrast, non-proprioceptive neurons are dependent on the presence of p75 regardless of tissue levels of NT-3.

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