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Distribution of neurotrophin receptors in the mouse neuromuscular system

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ABSTRACT The neurotrophins are a family of secreted proteins with critical roles in regulation of many aspects of neural development, survival and maintenance. Their actions on neural tissue are thought to be mediated by interaction with high affinity (trk family members) or low affinity ($p75^{NTR}$) cell surface receptors. In general, neurotrophins are considered to be supplied in limiting quantity by cells of a target tissue or synaptic partner. To date, alpha motoneurons have been shown surprisingly indifferent to loss of neurotrophic factors. Direct evidence for supply of a critical motoneuron factor(s) by skeletal muscle and a specific uptake mechanism *in vivo* remains elusive. We wished to directly establish whether targets in the periphery might be potential sources of neurotrophic support for motoneurons by examining whether neurotrophin receptors are present on motoneuron nerve terminals. We have used immunofluorescence techniques with a panel of antibodies against known neurotrophin receptors (trk A, trk B, trk C, $p75^{NTR}$) to map the locations of these receptors in the developing neuromuscular system of mice from our neurotrophin-3 (NT-3) knockout colony. To our surprise, we failed to locate immunoreactivity for any of these receptors in association with motor nerve endplates or terminal intramuscular axon branches, although they were found in association with a population of unidentified cells. We believe this result indicates that the neurotrophic relationship between alpha motoneurons and their target cells is not a simple one of neurotrophin supply by skeletal muscle cells and its uptake at the neuromuscular junction.

KEY WORDS: *neurotrophin, trk receptor, p75 receptor, neuromuscular junction*

Introduction

The neurotrophins are a family of related proteins with roles in neural development and maintenance (Davies, 1994). The effects of neurotrophins on receptive neural populations are thought to be mediated by interactions between the neurotrophin and two types of cell surface receptors, the high affinity *trk* receptors and the low affinity $p75^{NTR}$ receptor (Kaplan and Miller, 2000). For neurotrophins to mediate their effects neurons must have the means to receive the signal and for the signal to be transduced to the nucleus. In many cases (eg. the neuromuscular system) the site of putative signal receipt is distant from the cell body. The signal must therefore be received in the periphery and translocated to the nucleus. What are the mechanisms for neurotrophin signal receipt and translocation?

A current theory, for which there is significant supporting evidence (Reynolds *et al.*, 2000) holds that neurotrophins bind to surface receptors and induce their dimerization. These neurotrophin-receptor complexes are then internalised into coated vesicles that are sorted and targeted for microtubule-dependent retrograde transport to the cell body. On arrival at the cell body the neurotrophin-containing vesicles must signal to the nucleus before being destroyed. We are particularly interested to know whether the details of this model for neurotrophin recognition and uptake apply to spinal alpha motoneurons.

The cell bodies of alpha motoneurons reside in the lumbar lateral motor columns of the spinal cord. They are of particular interest in the study of neurotrophin function because they are the most widely used neurons for study of control of cell death, and because identification of the definitive motoneuron survival factor(s) has been elusive (Henderson *et al.*, 1993; Oppenheim *et al.*, 1993).

Abbreviations used in this paper: AChE, Acetylcholinesterase; BDNF, Brain Derived Neurotrophic Factor; CNS, Central Nervous System; DIC, Differential Interference Contrast; FGF-5, Fibroblast Growth Factor-5; NGF, Nerve Growth Factor; NT-3, Neurotrophin-3; NT-4/5, Neurotrophin 4/5; P0-14, Postnatal day 0-14; PBS, Phosphate Buffered Saline; PCR, Polymerase Chain Reaction; trkA-C, tropomyosin receptor kinase A-C; +/+, wild type; +/-, heterozygote; -/-, null mutant.

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Gene knockouts in mice for Nerve Growth Factor (NGF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5), and Brain-Derived Neurotrophic Factor (BDNF) have all been described (as have some multiple knockouts eg. Liu and Jaenisch, 2000) and the impact of loss of these trophic factors on spinal motoneuron survival has been relatively minor. In addition, other potential motoneuron survival factors have been sought (eg. FGF-5, McGeachie *et al.*, 2001) and found to have interesting roles, though not including promotion of motoneuron survival *in vivo*. While the survival of motoneurons through the normal cell death period does not seem to be critically dependent on the adequate supply of any single known neurotrophic factor, we have recently reported that alpha motoneurons are not indifferent to neurotrophin presence (Woolley *et al.*, 1999). Although we reported normal alpha motoneuron numbers in NT-3 null mice, motoneuron cell bodies were significantly smaller than those of their NT-3 +/+ or +/- littermates. This effect could be mediated via either a lack of NT-3, or it may be independent of NT-3 and be instead due to a lack of proprioceptive input to motoneurons, a secondary consequence of the null mutation. To help differentiate between these alternatives, we asked the question whether alpha motoneurons are receptive to NT-3 derived from target muscle?

In asking this question, the first and most obvious issues to address were whether the target might be a potential source of NT-3 for these cells, and whether the motoneurons have a means for its uptake. Several studies have assayed for either NT-3 mRNA or protein (Ernfors *et al.*, 1990; Maisonpierre *et al.*, 1990; Copray and Brouwer, 1994; Kaisho *et al.*, 1994; Zhou and Rush, 1994; Griesbeck *et al.*, 1995; Katoh-Semba *et al.*, 1996; Bartlett *et al.*, 2001) and found both to be present in skeletal muscle and motoneurons. Motoneuron cell bodies have neurotrophin receptors (Merlio *et al.*, 1992; Buck *et al.*, 2000; Copray and Kernell, 2000) and can retrogradely transport neurotrophins (DiStefano *et al.*, 1992; Yan *et al.*, 1993; Curtis *et al.*, 1998). However, the location and method of neurotrophin uptake *in vivo* remain unclear.

In looking at these issues, it occurred to us that our understanding of the roles of the neurotrophins in neuromuscular development/maintenance were clouded somewhat by lack of published material examining the precise cellular locations of the neurotrophins and their receptors at specific developmental stages. For instance, the *in situ* work of Griesbeck *et al.* (1995) is often cited as evidence for the presence of NT-3 in skeletal muscle, and immunoassay invariably shows the presence of NT-3 in muscle (Kaisho *et al.*, 1994; Katoh-Semba *et al.*, 1996). Skeletal muscle is a complex and dynamic tissue comprising several cell types. The *in situ* hybridisation and immunoassay techniques do not usually distinguish which cells are positive for NT-3 or its receptors, and so an examination of the specific cellular locations of neurotrophins and their receptors in the neuromuscular system is warranted.

To begin to address these issues, we have used a panel of antibodies against all known neurotrophin receptors to screen for their presence in mouse motor nerve terminals and near terminal axon branches, and in non-neural cells within the neuromuscular system. We used the same technique to identify the locations of NT-3 immunopositive cells. In contrast with our expectations we find no evidence for the presence of neurotrophin receptors (as recognised by our panel of antibodies) on alpha motoneuron intramuscular axon branches or terminals, although we are able to describe their presence and some temporal changes in their distribution in non-neural cells. We are able to confirm the presence of NT-3 in the target region, and can show the locations of NT-3 immunopositive cells. We believe these results argue against a simple model for neurotrophic support of motoneurons by skeletal muscle fibres.

Results

Rationale for Animals Used

We did our investigation on animals at three ages (postnatal day zero (P0), P14, 3.5 months) and on all three genotypes (null, heterozygous, wild type) from our NT-3 knockout mouse colony. This was to allow us to describe possible changes in distribution of neurotrophin receptors in the neuromuscular system as a function of age and/or genotype since previous work has suggested neurotrophin-mediated regulation of receptor expression (Wyatt *et al.*, 1999). Null mutants die within a few days of birth, so these animals were not represented in the P14 or 3.5 month age groups.

Visualisation of Motor Nerve Endplates

As expected, the Karnovsky histochemical method revealed the presence of motor nerve endplates in virtually every section taken from the middle third of the muscle belly (Fig. 1A). To verify that these cholinesterase-positive areas were indeed innervated, we immunostained for synaptophysin (to show the fine motor nerve branches ramifying over the endplate) and neurofilament (to show intramuscular axons approaching the nerve terminal). In every case we found that cholinesterase positive locations were overlain by neural profiles (Fig. 1B). Although we did not repeat this protocol

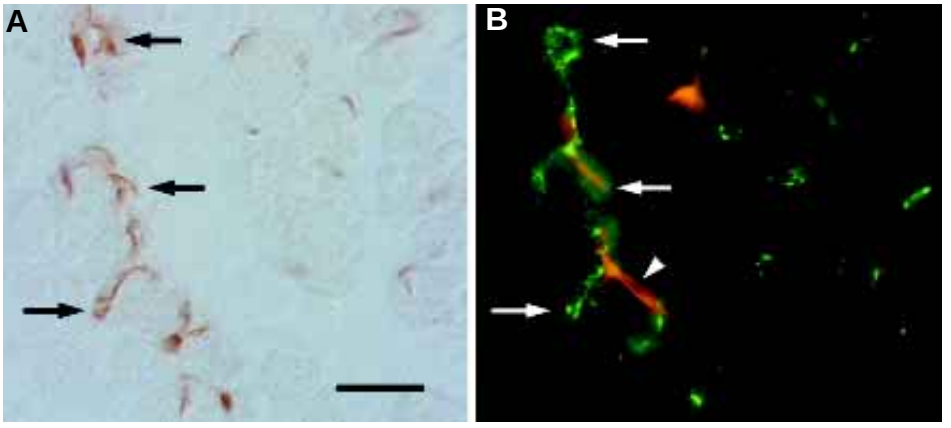
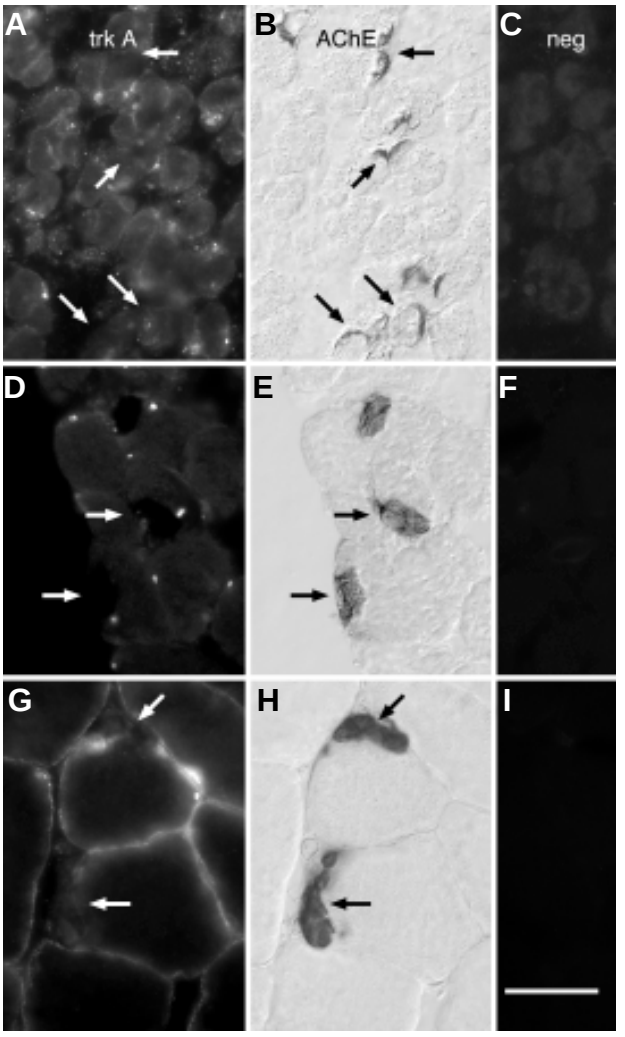


Fig. 1. A transverse section of P0 NT-3 +/- skeletal muscle. (A) *Histochemically stained for AChE, with positive sites (arrows) visible typically as red-brown arcs around the fibre perimeter.* **(B)** *The same section as (A), immunostained with antibodies against synaptophysin (green) and neurofilament (red) to label nerve profiles and terminal ramifications at endplate sites. Obliquely sectioned nerve profiles branch through the muscle (arrowhead), and without exception AChE positive profiles in (A) are overlain by synaptophysin immunopositive nerve terminals (arrows). Scale bar, 50 μm.*



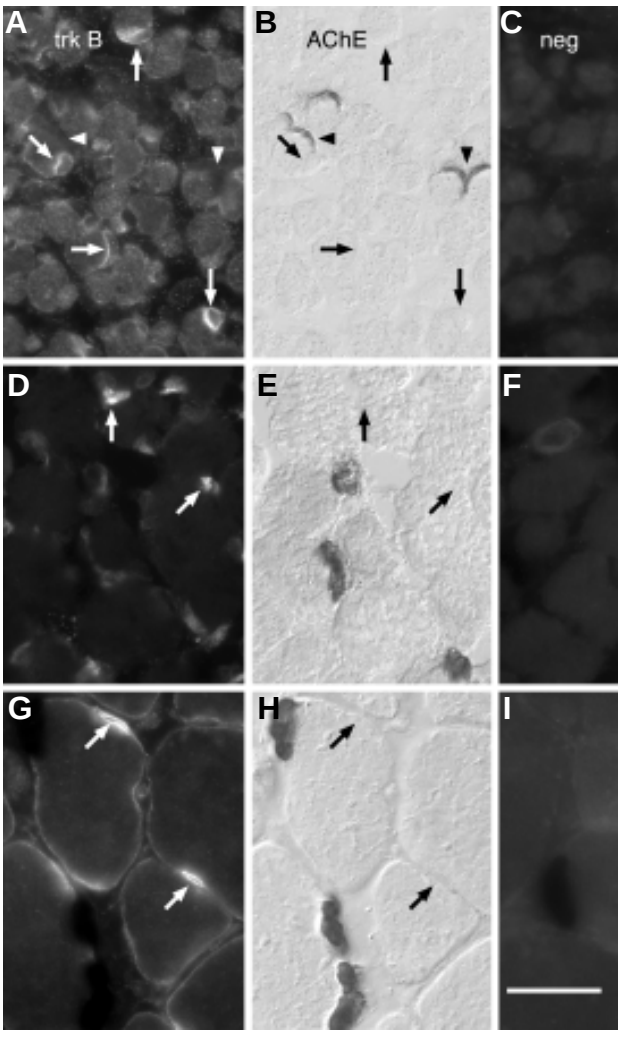


Fig. 2. (Left) Transverse sections of mouse skeletal muscle, immunostained for trk A (A,D,G), histochemically reacted for AChE (B,E,H), or incubated with secondary antibody alone (negative controls, C,F,I). (A) *P0 NT-3 -/- muscle showing widespread punctate immunostaining for trk A, but with no apparent association of immunopositive locations with the motor nerve endplates (arrows) evident in* **(B)**, *which depicts the same panel stained for AChE.* **(C)** *is a negative control of an adjacent section, photographed under identical conditions of illumination, and with an identical exposure time as panel A.* **(D-F)** *The same outcomes on sections of P14 NT-3 +/- muscle, while* **(G-I)** *show the staining pattern on 3.5 month NT-3 +/+ muscle. Scale bar, 50 μm.*

Fig. 3. (Right) Transverse sections of mouse skeletal muscle, immunostained for trk B (A,D,G), histochemically reacted for AChE (B,E,H), or incubated with secondary antibody alone (negative controls, C,F,I). (A) *P0 NT-3 -/- muscle showing widespread punctate immunostaining for trk B with highest levels concentrated around young myotubes (arrows). There is no apparent association of immunopositive locations with the motor nerve endplates (arrowheads) evident in* **(B)**, *which depicts the same panel stained for AChE.* **(C)** *A negative control of an adjacent section, photographed under identical conditions of illumination, and with an identical exposure time as panel A.* **(D-F)** *The outcomes on sections of P14 NT-3 +/- muscle; the most immunopositive cells (arrows) have a morphology suggesting they are neither nerve nor muscle.* **(G-I)** *The staining pattern on 3.5 month NT-3 +/+ muscle, with strongly immunopositive cells (arrows) at the muscle fibre periphery being dominant. Scale bar, 50 μm.*

in combination with the receptor immunohistochemistry, we have every confidence that the AChE-revealed motor nerve endplates were innervated, that fine nerve branches were present in each tissue section, and that immunopositive structures in motor nerve terminals were visible after AChE immunohistochemistry.

Neurotrophin Receptor and NT-3 Immunolocalisation

Positive controls. Neural tissues included in the sample as positive controls for the staining protocol and the reliability of the antibodies always gave the expected positive outcomes (not shown). In addition, the presence of immunopositive sites within muscle tissue served as an internal indicator of the success of the procedure.

trk A. We failed to locate any trk A immunoreactivity in close association with an identified motor nerve endplate at any age, or in any genotype (Fig. 2). However, discrete intramuscular structures were strongly immunopositive at all ages and in all genotypes (Fig. 2). These punctate trk A immunopositive sites were located throughout the muscle of the newborn, gradually becoming confined by 3.5 months to specific cells at the fibre periphery (Fig. 2 G,H). None of the immunopositive profiles were of a shape consis-

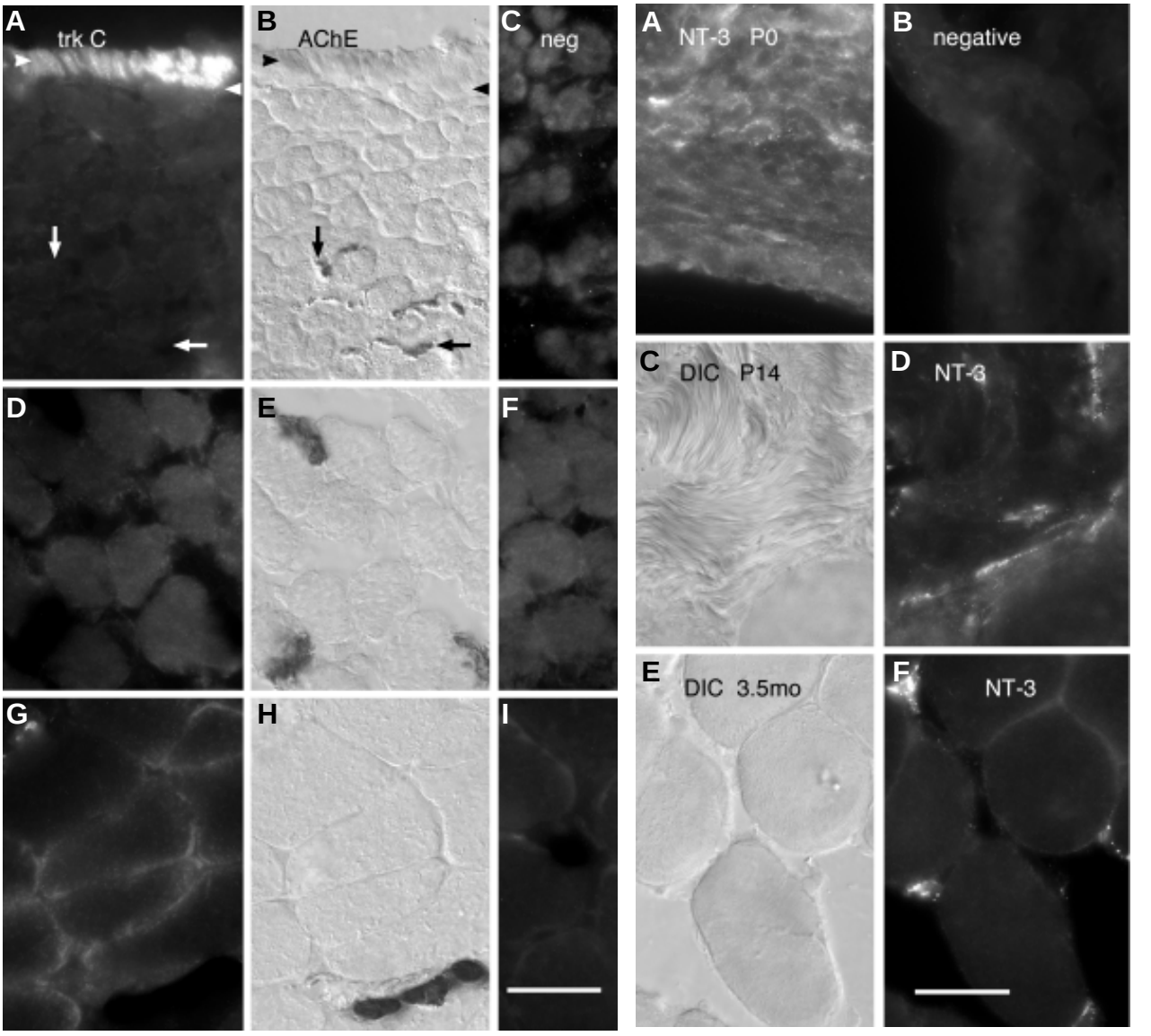


Fig. 4. (Left) Transverse sections of mouse skeletal muscle, immunostained for trk C (A,D,G), histochemically reacted for AChE (B,E,H), or incubated with secondary antibody alone (negative controls, C,F,I). (A) *P0 NT-3 -/- muscle showing no immunoreactivity in the muscle belly or in association with endplates (arrows, same section shown in* **(B)**, *but with strong immunoreactivity associated with intramuscular connective tissue (arrowheads).* **(C)** *is a negative control of an adjacent section, photographed under identical conditions of illumination, and with an identical exposure time as panel A.* **(D-F)** *The same outcomes on sections of P14 NT-3 +/- muscle.* **(G-I)** *The staining pattern on 3.5 month NT-3 +/+ muscle. Scale bar, 50 μm.*

Fig. 5. (Right) Transverse sections of NT-3 +/+ mouse skeletal muscle immunostained for NT-3. (A) *P0 muscle showing widespread NT-3 immunoreactivity, with a negative control section photographed under identical conditions of illumination and exposure shown in* **(B)**. **(C)** *A differential interference contrast (DIC) image of a region of muscle and connective tissue from a P14 animal, with the same section immunostained for NT-3 shown in* **(D)**. *Strongest immunoreactivity is associated with the connective tissue.* **(E,F)** *A DIC/fluorescence pair of images of the same section of 3.5 month muscle in which it is apparent that muscle fibres have very little NT-3 immunofluorescence while unidentified cells distributed through the muscle belly remain strongly positive. Scale bar, 50 μm.*

tent with axonal profiles, which are invariably obliquely sectioned and distinctive in appearance (Fig. 1B).

trk B. As for trk A, we failed to locate any trk B immunoreactivity in apparent association with a motor nerve endplate at any age, or in any genotype (Fig. 3). Muscle sections were again not completely immunonegative, with strong reactivity within the muscle in a pattern quite different to that seen with trk A. In muscles from newborn animals Trk B appeared to be strongly associated with the interface between young muscle fibres of dissimilar sizes (possible primary-secondary myotube pairs, Fig. 3A). By P14 the staining appeared restricted to cells which, judging by morphological criteria were neither nerve nor muscle (Fig. 3D). By 3.5 months the immunopositive profiles were spindle-shaped and generally located at the fibre periphery (Fig. 3G). Note the similarity in position and shape of trk A and B immunopositive cells at 3.5 months (Figs. 2G, 3G). At present the identity of these cells remains unknown.

trk C. Once again, we failed to locate any trk C immunoreactivity in close association with a motor nerve endplate or axon at any age, or in any genotype (Fig. 4). Trk C immunoreactivity was not usually present through the muscle belly, but was located on intra- and peri-muscular connective tissue (Fig. 4A). The strength of the putative connective tissue immunostaining did not appear to correlate with genotype, but did decline in strength with age.

p75. As for the high affinity neurotrophin receptors, we located no p75 immunoreactivity in close association with a motor nerve endplate or intramuscular axon at any age or in any genotype (data not shown). In contrast to the pattern seen for the trk receptors, we saw no discrete high-level immunostaining of any locations within the muscle. Instead, the staining pattern appeared pale and diffuse, only slightly above background.

Neurotrophin-3. Since most other studies have used low spatial resolution methods to establish the presence of NT-3 in muscle, we wished to determine where this protein was located at higher resolution. NT-3 was present at moderately high levels throughout the muscle belly in the +/- and +/+ P0 animals (Fig. 5). The overall amount of immunostaining gradually declined with age, but specific unidentified intramuscular cells remained strongly positive (Fig. 5) as did the occasional intrafusal fibre present in the section.

Discussion

Theories of neural development and maintenance are now intimately intertwined with our understanding of neurotrophic support. The neurotrophins are considered to play crucial roles in modulating not only nerve cell survival during development, but also neuronal excitability, synaptic efficacy, and morphology (Gonzalez and Collins, 1997; Munson *et al.*, 1997; Mendell *et al.*, 1999; Schuman, 1999; Poo, 2001). While some neuronal populations appear to be critically dependent on the presence of a single neurotrophin (Ernfors *et al.*, 1994), this appears not to be the case for motoneurons (Oorschot and McLennan, 1998). Nonetheless, it is still widely believed that motoneuron survival and maintenance is dependent on neurotrophic support (Gouin *et al.*, 1993; Henderson *et al.*, 1993; Yan *et al.*, 1993; Griesbeck *et al.*, 1995; Oppenheim *et al.*, 1995; Sendtner *et al.*, 1996), though that view is not ubiquitous (Greensmith and Vrbova, 1996).

Neurotrophic support hypotheses have at their heart the concept of target-derived neurotrophin(s) being produced in limiting amounts with neuronal competition for access to the substances. Invariably, these models propose receptor-mediated uptake of the neurotrophin, its internalisation into vesicles, and subsequent retrograde axonal transport to the cell body where the signalling cascade is initiated (Reynolds *et al.*, 2000). With regard to motoneurons, it is clear that their neurotrophin requirements are complex and that some, but not all known neurotrophin receptors are present on their cell bodies (Yan *et al.*, 1993; Copray and Kernell, 2000). While several studies have examined receptor distribution on motoneuronal cell bodies, none have yet looked to see which of these receptors are translocated to the periphery where they might act to mediate specific neurotrophin uptake.

Here, we contribute to this issue by providing data on the distribution of neurotrophin receptors at motor nerve endplates. In spite of our attempts to locate neurotrophin receptors using a high resolution mapping technique, we failed to find these proteins on motor nerve terminals or intramuscular axon branches. This failure would not appear to be due to a generalised technical failure of reagent or procedure, since the technique routinely detected specific immunopositive sites at non-neural locations within the muscle, and on the cell bodies of neurons on sections of CNS material. Germane to this discussion are the observations that the trk receptors may exist in non-catalytic or truncated forms (Klein *et al.*, 1990; Biffo *et al.*, 1995). It has been shown, at least for the relationship between BDNF and trkB that the noncatalytic form of the receptor is able to specifically recognise, bind, and internalise its ligand. Given that in the neuromuscular system the probable site of neurotrophin uptake is a long way from the signalling location, binding and uptake by a noncatalytic form of the receptor may be advantageous. This may allow specific recognition and sequestering from the region of supply without need for a primed signalling system to be retained intact for an extended period during its long journey to the nucleus. Subsequent to its uptake, the neurotrophin-receptor complex could be either activated in transit or, on reaching the cell body the neurotrophin might be released from the cell to act on functioning trk receptors at the cell body in an autocrine or paracrine fashion. Whether the antibodies we used in this study recognise only catalytic forms of the receptors (and therefore fail to detect the postulated truncated non-catalytic forms) is unknown. In light of current evidence, this would appear as a model worthy of further investigation and is a potentially viable explanation for the apparent disparity between our current observations and the published evidence for receptor-mediated neurotrophin uptake.

If one accepts, as seems reasonable, that motoneuron physiology is modulated by neurotrophic support from the target region, the absence of known receptors at or close to the neuromuscular junction is problematic. Could neurotrophin uptake be mediated by a non-receptor mediated mechanism? There is abundant evidence for the existence of a mechanism for non-receptor mediated uptake of substances present in the synaptic cleft (this has been exploited countless times in neural tracing studies utilising retrograde transport of Horseradish Peroxidase eg. Chu-Wang and Oppenheim, 1980). While the details of that method of vesicular uptake and transport share a number of striking similarities with the method proposed for receptor-mediated uptake (Chu-Wang and Oppenheim, 1980; Reynolds *et al.*, 2000), the competitive binding studies of Yan and colleagues (1993) strongly suggests that non-receptor mediated uptake is unlikely. Therefore, a receptor mediated mechanism seems probable (although not necessarily utilising a functional receptor), but what is the site of uptake and which cells are the sources of neurotrophin?

Neurotrophic support theories suggest that the cell providing the support has a synaptic relationship with the cell deriving the support, although there is little direct *in vivo* evidence for this in the neuromuscular system. Several studies have shown neurotrophin mRNA or protein in muscle using techniques that do not identify the cellular locations of the message or the protein (Henderson *et al.*, 1993; Kaisho *et al.*, 1994; Griesbeck *et al.*, 1995; Katoh-Semba *et al.*, 1996). One study used a high resolution but relatively low sensitivity technique to localise NT-3 mRNA in muscle spindles (Copray and Brouwer, 1994). Our work shows that NT-3 is present broadly through the muscle around the time of birth, but that the amount of NT-3 protein associated with muscle fibres falls dramatically in the early postnatal period while other (as yet unidentified

cells) remain strongly NT-3 positive into adulthood (Fig. 5). NT-3 protein is not present in association with motor nerve terminals at a level higher than in non-endplate muscle regions (our unpublished observations). Is it possible that neurotrophin receptors are localised on axons away from the neuromuscular junction, and that the site of uptake is the muscle environment in general, rather than the neuromuscular junction in particular? Evidence for neurotrophin receptors on immature proximal axons (Mckay *et al.*, 1996) and lack of functional receptors at neuromuscular junctions is consistent with a location removed from the neuromuscular junction. Indeed, the presence of neurotrophins in the muscle in general, rather than at the neuromuscular junction in particular obviates the need for a specific site for uptake and casts doubt on a role for neurotrophins in synaptic competition at the neuromuscular junction.

We have not yet identified the non-muscle cells that stain positively for NT-3 in mature animals. However, several studies have established that neurotrophins are expressed in many tissues with protein levels much higher than in nerve or muscle (Kaisho *et al.*, 1994; Katoh-Semba *et al.*, 1996). The inescapable consequence of this finding is that neurotrophins are likely to have functions other than those postulated for interactions between neural cells and their targets. It is clear that alpha motoneurons are not dependent on any single neurotrophin for their survival, and perhaps one of these other, as yet unspecified neurotrophic actions is the main role being played in this instance.

While immunoassay for neurotrophin levels in muscle generally gives low readings (Kaisho *et al.*, 1994; Katoh-Semba *et al.*, 1996) it is important to note that such techniques involve homogenisation of the tissue. The resulting neurotrophin level is then an average for the entire tissue and does not indicate cellular variation in protein levels. In the present study, we showed widespread NT-3 in the muscle of newborns but as the animals age the expression pattern changes into one in which muscle fibres appear NT-3 negative while nonmuscle cells (judging by their morphology) remain strongly positive. Previous studies have described age-related declines in neurotrophins and their receptors (Kaisho *et al.*, 1994; Johnson *et al.*, 1999).

In addition to describing the lack of immunoreactivity for known neurotrophin receptors at the neuromuscular junction, we have described age-related changes in the specific intramuscular distribution of neurotrophin receptors. As yet, we have not identified which cells are immunopositive for each receptor. High levels of trkA and trkB immunoreactivity were associated (particularly in the younger animals) with a cellular location peripheral to myotubes. The high, transient, and specific neurotrophin receptor distribution indicates a possible role for neurotrophin signaling in myogenesis. Retention of immunoreactivity for these receptors on a subset of cells with a peripheral fibre location (possible satellite cells?) suggests a possible role for neurotrophin signaling in activation of satellite cells to initiate muscle repair. Trk C immunoreactivity was low in the muscle belly, but in young animals it was strong on connective tissue. The presence of high levels of trkC immunoreactivity on connective tissue raises the possibility that NT-3/trkC signalling is especially important in directing the development of connective tissue by fibroblasts. Consistent with this interesting prospect is our recent unpublished observation that collagen fibrils in the region of the neuromuscular junction are overtly disorganised in NT-3 null mutants.

In conclusion, we have presented evidence that the neurotrophic interaction between alpha motoneurons and skeletal muscle fibres is far from simple or predictable. Our evidence indicates that the neuromuscular junction may not be a specific site for neurotrophin uptake by motoneurons, but that axonal uptake from the muscle region in general remains a possibility. If true, this proposal has important implications for our concept of the mechanism and purpose of neurotrophic support between motoneurons and muscle fibres. An alternate explanation for our findings is that the panel of antibodies we have used does not recognise non-functioning truncated neurotrophin receptors, and that these are present at the neuromuscular junction to mediate neurotrophin uptake in the way frequently proposed. We are currently working to differentiate between these alternatives.

Materials and Methods

Animals from our colony of NT-3 knockout mice were used for all experiments. The colony founders were originally purchased from Jackson Labs (Bar Harbor, Maine, USA, strain Ntf3^{tm1Jae}; stock number 002275). Animals were taken at postnatal day 0 (PN 0), PN14, and 3.5 months, and were genotyped at birth from tail tips using PCR protocols as described by Jackson Labs. Animals of each genotype were used (NT-3 null, -/-; heterozygous, +/-; wild type, +/+) at PN0, but due to the early death of the null mutants only heterozygous and wild type animals were available at the older ages.

Animals were killed by cervical dislocation or decapitation. Skeletal muscles (tibialis anterior, gastrocnemius, soleus) from the lower hindlimb (older animals) or entire hindlimbs (newborns) were embedded in OCT compound, snap frozen in isopentane cooled by liquid nitrogen, and stored at –80˚C until needed for sectioning. Brain, lumbar spinal cord, and dorsal root ganglia were similarly removed and frozen to provide neural tissue to be used as positive controls for the immunostaining protocol.

Serial transverse tissue sections were cut (10 μm, -20˚C) in a Leica CM1850 cryostat, and picked up on coated slides. Slides were air dried, washed in Phosphate Buffered Saline (PBS), and reacted with a standard immunohistochemical protocol. Primary antibodies raised against the neurotrophin receptors trkA, trkB, trkC, p75^{NTR} were purchased from Santa Cruz Biotechnology (Santa Cruz, California, USA, anti p75NGFR, rabbit polyclonal, cat # sc-5632; anti trkA, rabbit polyclonal, cat# sc-118; anti trkB, rabbit polyclonal, cat # sc-12; anti trkC, rabbit polyclonal, cat# sc-117) and applied to the sections at dilutions of 1:800 (anti trkB)-1:1000 (anti trkA, trkC, p75) in a humidified chamber overnight at 4˚C. After washing, incubation with the secondary antibody (goat anti rabbit IgG F(ab)$_2$ conjugated to Alexa Fluor® 488 from Molecular Probes, Eugene, Oregon, USA) took place in the dark humidified chamber at room temperature for 2 hours. Final washing was followed by histochemical reaction for AcetylCholinesterase (AChE) using the Karnovsky method (Karnovsky and Roots, 1964). Finally, specimens were mounted in GlycerGel mountant (Sigma cat #GG-1) into which the anti-fade agent 1,4-Diazabicyclo[2.2.2]octane (Sigma cat #D-2522) had been added at 25mg/10ml. To establish that the endplates under examination were indeed innervated, and that the AChE protocol did not interfere with or obscure immunoreactivity, several specimens were double immunolabelled with a combination of polyclonal rabbit anti-synaptophysin (diluted 1:800, Dako, Carpinteria, California, USA) and monoclonal mouse anti-neurofilament (diluted 1:1000, Sigma) antibodies. In this case the second antibodies were a combination of the anti rabbit Alexa Fluor® 488 and an anti mouse Alexa Fluor® 568, again from Molecular Probes. Negative control sections were covered with immunodiluent alone during the primary incubation step then subsequently processed as described. To check for the presence of NT-3 in muscle tissue, some specimens were immunolabelled with anti NT-3 (rabbit polyclonal, cat # AB-1532SP, Chemicon, Temecula, California, USA).

Specimens were viewed with fluorescence and Differential Interference Contrast optics on an Olympus BX-50 microscope. Digital photographs were taken with a SPOT-RT Slider digital camera (Diagnostic Instruments, Sterling Heights, Michigan, USA). To ensure that the relative brightness of immunolabelled and negative control specimens was retained exposure

readings for each immunolabelled photograph were noted, and negative control sections were photographed using identical illumination and exposure parameters to those of the experimental specimens.

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