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Neurotrophins and the Specification of Neuronal Phenotype

Gary R. Lewin

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Neurotrophins and the specification of neuronal phenotype

GARY R. LEWIN

Department of Neurobiochemstry, Max Planck Institute for Psychiatry, D-082152 Martinsried, Germany

SUMMARY

Nerve growth factor, brain derived neurotrophic factor and neurotrophin-3 all influence sensory neurons derived from the dorsal root ganglia. Traditionally these neurotrophins have been thought of as survival factors for sensory neurons during their development. Recent evidence from experiments where the *in vivo* levels of these proteins has been manipulated indicates that they may influence the development of specific sensory neuron phenotypes. In this review these experiments are discussed in relation to the mechanisms by which neurotrophins could influence the phenotypic fate of sensory neurons. The first mechanism requires that when a neuron becomes dependent for survival on a neurotrophin the availability of the factor simply influences the number of neurons surviving with a certain modality. This model requires that neurotrophin repsonsiveness is a determinant of the possible modalities that the neuron may acquire. The second mechanism requires that the availability of a given neurotrophin influences how many neurons can differentiate into different sensory neuron phenotype independent of survival. The available experimental data is discussed in relation to these two models.

1. INTRODUCTION

The mechanisms by which neuronal fate is determined is of central interest to neurobiologists. The nervous system, unlike other organ systems, contains a huge variety of different cell types which are uniquely specified to carry out a large number of different tasks. Neuronal phenotypes can be differentiated by a number of different criteria which have included anatomy, connectivity, and neurotransmitter content. For the most part, a given neuron will be adapted to its functional role by having a unique combination of the above attributes. Because any one of these attributes, for example neurotransmitter content, may be shared by a range of different neuronal types the process of fate determination must be a complex one. Factors in the peripheral targets of sympathetic neurons have been implicated in neurotransmitter choice, although it is still unclear what the identity of the these factor(s) is *in vivo* (Landis 1994). In this review, however, I will focus on sensory neurons of the dorsal root ganglion (DRG). Despite having their cell bodies in the same ganglion these neurons are highly specialized to carry out a wide range of different tasks. They are also very amenable to study during development and in maturity. Members of the neurotrophin family include nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (NT-6) all interact with sensory neurons (Lewin & Barde 1996). Experiments where the levels of the neurotrophins have been manipulated *in vivo* have resulted in selective changes in the numbers of sensory neurons with specific phenotypes. The data suggest that interactions between sensory neurons and the neurotrophins may be important in the determination of neuronal fate during development. Several examples of such changes in neuronal fate will be reviewed and the possible mechanisms by which neurotrophins could act in this context evaluated.

2. HETEROGENEITY OF SENSORY NEURONS

In the adult animal, sensory neurons subserve various functions ranging from signalling noxious stimulation of the skin to the rate of change of muscle length. The sensory stimulus that best excites the neuron was termed by Sherrington the adequate stimulus (Sherrington 1906). In the first physiological studies on sensory neurons, carried out in the 1960s and 1970s, much effort was expended classifying neurons according to their adequate stimuli. These studies mostly involved recording from functionally single axons in small filaments teased from peripheral nerves or occasionally dorsal roots (Perl & Burgess 1973). The number of physiologically determined types using these techniques amounts to 43 according to a summary of the literature made by Perl (1992). The very large number is, however, somewhat misleading as it includes sensory neurons innervating all somatic targets which includes skin, muscle, joint, viscera, kidney, and uterus. In this review I will concentrate on the biology of skin and muscle sensory neurons as these have probably been the best studied in terms of physiology and other aspects. Sensory neurons of the DRG can be conveniently divided up into those with myelinated axons and large light cell bodies and those

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Table 1. *Simplified classification of myelinated cutaneous afferents*

type	adequate stimulus	conduction velocity	psychophysical percept
rapidly adapting (RA[a])	skin or hair movement	fast A-β	flutter/vibration
slowly adapting type I (SAI)	indentation of touch dome	fast A-β	skin indentation
slowly adapting type II (SAII[b])	stretch of skin	fast A-β	none
D-hair receptor (D-hair)	small movement of hairs	slow A-δ	?touch
A-mechanonociceptor (AM[a])	noxious mechanical stimulus (pinch)	slow A-δ	pain

[a] The fibres types indicated can be further subclassified but for the purpose of the present discussion this has not been done.
[b] SAII in the cat project differently in the spinal cord (see Burgess & Horch 1973) and certainly subserve a different role fom SAI afferents in man. However, they are very rare in rodents where most of the work with neurotrophins has been carried out and therefore will not be considered in great detail (Lewin & McMahon 1991).

Table 2. *Simplified classification of myelinated muscle afferents*

type	adequate stimulus	end-organ	conduction velocity
Group Ia	rate of change of muscle length	muscle spindle intrafusal fibres equatorial region	fast
Group Ib	active tension	golgi tendon organ	fast
Group II	muscle length	juxta-equatorial region of spindle intrafusal fibre	fast
Group III	noxious damage	free nerve endings	slow

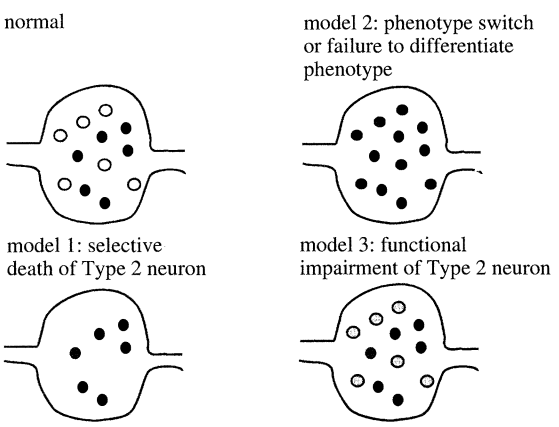

Figure 1. Schematic illustration of the way that the availability of neurotrophins might influence the number of sensory neurons developing with different phenotypes. Dark circles represent Type 1 neurons; light circles represent Type 2 neurons; grey circles represent functionally impaired Type 2 neurons.

with unmyelinated axons and small dark cell bodies. The former make up approximately 20–30% of the DRG (Lawson 1992), and are physiologically quite heterogeneous (see table 1 and 2). Sensory neurons with unmyelinated axons are somewhat more homogenous in their physiological properties as the vast majority are nociceptors (see Lewin & Mendell 1993).

When one classifies myelinated skin and muscle afferents according to the simplified criteria one comes up with the physiological types listed in tables 1 and 2. These sensory neurons have very distinctive connections centrally and therefore different functional roles (Brown 1981; Koerber & Mendell 1992). The subject of this review is how neurotrophins may operate during development to specify these functional roles.

Three models or mechanisms by which neurotrophins might specify sensory neuron phenotype will be examined. These are outlined schematically in figure 1 where an idealized situation with only two phenotypes is considered. In model 1 the relative number of one type of neuron is reduced by cell death. In model 2 only one phenotype of neuron develops either by a switch in the phenotype or by a failure of one type to differentiate. In this latter case it is not clear whether the total number of neurons would be altered or not. Finally, model 3 represents a situation where one aspect of the neuronal phenotype is altered leading to a functional impairment.

3. NERVE GROWTH FACTOR

(*a*) *Loss of nociceptors*

It has been known for sometime that NGF is a survival factor for subsets of sensory neurons during development. Experiments, carried out in the late 1970s and early 1980s, using blocking anti-bodies to NGF showed that small sensory neurons containing neuropeptides such as substance P and calcitonin gene related peptide require NGF for survival during embryonic development (for a review, see Lewin & Mendell 1993). The vast majority of these neurons are known to be nociceptors of one type or another (Kress *et al.* 1991). Indeed new born animals deprived of NGF during embryonic development are largely insensitive to noxious stimuli such as heat and pinprick (Aloe *et al.* 1980; Crowley *et al.* 1994). Later experiments confirmed that the neurons lost after anti-NGF treatments

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were nociceptors as the innervation of the nocireceptive spinal cord superficial laminae was missing in these animals (Ruit *et al.* 1990). Thus the availability of NGF during development is clearly necessary for the development of the nociceptive phenotype. The mechanism is very closely related to the classical neurotrophic theory that during embryonic development only those neurons expressing NGF receptors die and this conforms to model 1 outlined in figure 1. One implication of these findings is that the NGF-dependent population during embryonic development are already specified to become nociceptive or perhaps they already display a nociceptive phenotype during target dependent cell death (see Fitzgerald 1987).

(*b*) *Switching neuronal phenoptype*

In adults NGF ceases to become absolutely necessary for neuronal survival (but see Johnson & Yip 1984). In the early post-natal period anti-NGF treatment fails to kill sensory neurons after the second day of life (Lewin *et al.* 1992). Using electrophysiological techniques the number of functionally defined sensory neurons with myelinated axons (see table 1) was examined after various post-natal anti-NGF treatments (Ritter *et al.* 1991; Lewin *et al.* 1992). These experiments revealed highly specific losses of sensory neurons concerned with nociceptive stimuli (termed high threshold mechanoreceptors or A-fibre mechanonociceptors AM). The loss of these afferents could only be elicited during a critical period between post-natal day 4 and 11 (PND 4–11). More remarkably the loss of AM afferents was not associated with any significant cell death and this together with other considerations led us to conclude that the AM afferents had been converted to low threshold D-hair afferents (Lewin *et al.* 1992; Lewin & Mendell 1993). The increased numbers of D-hair afferents which had developed in the absence of NGF appeared to be indistinguishable from normal receptors in several respects. Their action potential characteristics were typical of D-hairs (narrow fast TTX sensitive spike) and not of AM fibres (broad TTX insensitive) (Ritter & Mendell 1992). Furthermore, the central neurons that these *de novo* D-hair afferents connected to were not those concerned with nociceptive stimuli (Lewin & Mendell 1996). Thus in this case the availability of NGF appeared to regulate the number of cells being committed to a given phenotype without changing neuronal number. Thus this phenomenon fits the scheme outlined in model 2 in figure 1. In our view the role of NGF in this case would be to stabilize the phenotype of AM afferents as the lack of a factor leads to an increased number of another afferent type. This interpretation raises the possibility that other factor(s) present are responsible for inducing the change in phenotype observed.

4. NEUROTROPHIN-3

(*a*) *Nociceptive neurons*

Very good evidence has accumulated to indicate that NT-3 may not interact with nociceptive neurons (see below). Many, if not all nociceptive neurons, appear to the tyrosine kinase receptor Trk A which is thought to be the primary NGF receptor (see review by W. B. Snider, this volume). However, there is some evidence that NT-3 may be able to bind to and activate Trk A receptors in cells lines and also in primary cultured embryonic neurons (Clary & Reichardt 1994; Davies *et al.* 1995). Is there any interaction between NT-3 and Trk A expressing small diameter sensory neurons *in vivo*? The results of some recent experiments in the developing chick embryo suggest that NT-3 could profoundly influence the phenotypic fate of such neurons. In these experiments a fibroblastic cell line secreting NT-3 was grown on the chorioallantoic membrane of developing chick embryos. In late stage embryos (E17-E21) the physiological properties of the sensory neurons were examined using a novel *in vitro* nerve skin preparation (Koltzenburg *et al.* 1994). Normally, most of the neurons recorded are sensory neurons with unmyelinated axons and nociceptive receptive field properties. By analogy to mammals these neurons probably respond to NGF and express Trk A receptors (see also Lewin *et al.* 1994). After prolonged NT-3 treatment virtually all these neurons with unmyelinated axons acquired receptive field properties more characteristic of low threshold mechanoreceptors (Lewin *et al.* 1994). This treatment was also accompanied by a marked reduction in the number of sensory terminals in nocireceptive spinal cord laminae (primarily lamina II) (Eide *et al.* 1994). These results suggested to us that excess NT-3 may influence the phenotype of presumptive nociceptive neurons to become more like that of low threshold receptors. One can speculate that the number of neurons that become committed to a nociceptive or low threshold phenotype is influenced by a balance between the availability of NT-3 and NGF. This hypothesis remains to be more rigorously tested however. The change in afferent phenotype induced by excess NT-3 was clearly not in every case complete as the conduction velocities (and therefore myelination) of the changed afferents were identical to controls. Thus this experimental manipulation appears to cause a change in the properties of sensory neurons which may not be complete but best conforms to model 2 outlined in figure 1.

(*b*) *Proprioceptive neurons*

Soon after the discovery of NT-3 it became apparent that this factor appeared to be a good candidate for a muscle afferent survival factor. Thus it was shown early on that NT-3 could support the survival in culture of almost all sensory neurons of the trigeminal mesencephalic nucleus most of which are known to be proprioceptive (Hohn *et al.* 1990). Experiments where chick sensory neurons of the DRG were retrogradely labelled from muscle nerves and then put in culture showed that the survival of these neurons was also selectively supported by NT-3 (Hory Lee *et al.* 1991). Further, experiments in the developing chick embryo demonstrated that function blocking antibodies to NT-3 could abolish the projection of presumptive muscle spindle afferents to the ventral motor pools (Eide *et al.*

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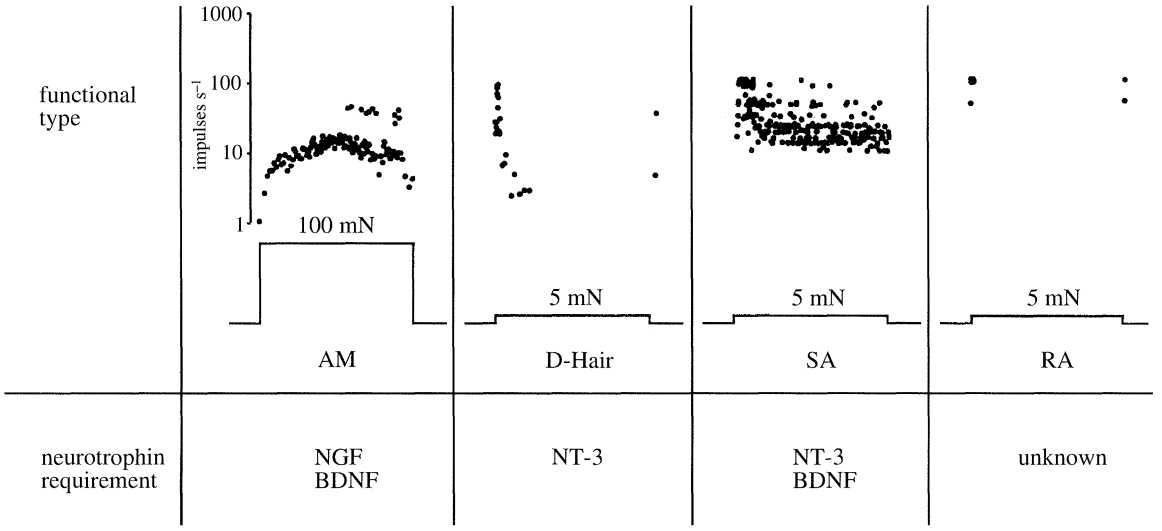

Figure 2. Combinatorial effects of neurotrophins. Four functionally defined types of myelinated cutaneous afferents are shown together with the neurotrophins known to influence them. For each functional type a typical response to the adequate stimulus is shown. Each neurons firing rate is shown in response to a standard force stimulus delivered to its receptive The AM fibre shown only responds to high stimulus strengths with a slowly adapting low frequency discharge. The D-hair responds with a high frequency discharge nly to the onst and offset of the lowest stimulus used (5 mN). SA and RA fibres shown differ only in that the former responds throughout the stimulus whereas the latter only responds to the onset or offset of the stimulus. These four functionally defined neurons can be uniquely defined by the neurorophins that they require for their survival or proper function *in vivo*.

1994; Oakley *et al.* 1995). At least during the period of naturally occurring cell death in the chick it appears that peripheral muscle derived NT-3 is required for the survival of muscle afferents (Oakley *et al.* 1995). Unfortunately, this data does not address the question of whether functionally defined subtypes of muscle afferents (see table 2) are selectively dependent on NT-3. At the time these NT-3 deprivation experiments are carried out there are no morphologically distinguishable end-organs in the muscle (Golgi tendon organs and muscle spindles). The fact that some muscle afferents innervating the dorsal horn of the spinal cord remain after anti-NT-3 treatments in chicks suggests that perhaps not all muscle afferents are equally susceptible to NT-3 deprivation (Eide *et al.* 1994).

Experiments on mice with null mutations of the NT-3 gene or the Trk C locus which encodes a functional receptor for NT-3 confirmed that proprioceptive afferents critically depended on NT-3 signalling (Snider 1994). However, it was clear that all subtypes of muscle afferents, except perhaps muscle nociceptors appeared to be missing in NT-3 knockout mice (Ernfors *et al.* 1994*a*; Farinas *et al.* 1994).Thus muscle spindle afferents (Group Ia and Group II) and Golgi tendon organ afferents (Group Ib) were not present (see table 2) (Ernfors *et al.* 1994*a*). Further investigation of the timing of the loss of muscle afferents in these animals has recently revealed that from the outset virtually no muscle afferents appear to innervate the muscle in NT-3 knockout animals (Kucera *et al.* 1995). This intriguing result suggests that NT-3 may be required early on for sensory neurons to acquire a muscle afferent identity in the first place. If this interpretation is correct then NT-3 may be a phenotypic differentiation factor for these neurons and the amount of NT-3 present before target innervation might determine how many neurons become committed to the muscle afferent lineage (see model 2).

The interpretation that NT-3 is a differentiation factor for muscle afferents before target innervation is complicated by the fact that many sensory neurons are also lost before target innervation in the absence of NT-3 (Gaese *et al.* 1994; Tessarollo *et al.* 1994). Thus cell death (of neuroblasts or newly differentiated neurons) could also account for the loss of the muscle afferents early on. One argument against this is provided by recent evidence that certain cutaneous sensory neurons differentiate normally in animals lacking NT-3, even though up to 70% of the sensory neurons are missing (Airaksinen *et al.* 1996). Also if one decreases the number of neurons generated by giving excess NT-3 during neurogenesis (see paper by M. Ockel *et al.* this volume) there are still muscle afferents present (Ockel *et al.* 1996). The presumptive role of NT-3 as a differentiation factor does not exclude it acting as a more classical survival factor for subsets of sensory neurons later in development during and after target innervation (see below).

(*c*) *Cutaneous mechanoreceptors*

Cutaneous sensory neurons are far more numerous than those innervating muscle and in rodents many of these neurons express Trk C receptors (McMahon *et al.* 1994 see also papers by W. B. Snider & H. S. Phillips, this volume). In adult mice heterozygous for a null mutation of the NT-3 gene it was found that around 25% of the myelinated axons in the purely cutaneous saphenous nerve were missing. Using electrophysiologi-

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cal techniques we recorded from sensory receptors in this nerve *in vitro* (Carroll *et al.* 1994; Airaksinen *et al.* 1996). Using this technique it is possible to reliably classify and quantify the number functionally defined myelinated afferents in the nerve. In the mouse saphenous nerve these can be divided into four types (outlined in table 1 and figure 2) that is, mechanonociceptors (AM), D-hair afferents, rapidly adapting mechanoreceptors (RA) and slowly adapting mechanoreceptors (SA).

Analysis of heterozygote NT-3 animals showed that the axon loss seen in the nerve could be accounted for by the loss of only two functional types, D-hair receptors (reduced by 50%) and SA mechanoreceptors (reduced by over 70%) (Airaksinen *et al.* 1996). The latter mechanoreceptor type proved easy to analyse at the morphological level as these afferents have readily identifiable end-organs, the Merkel cells in the touch dome complex of hairy skin (Perl & Burgess 1973). Examination of these cells (which depend on their innervation for survival; see Nurse *et al.* 1984) together with their innervating axons (SA afferent endings) showed that unlike muscle afferents the end-organ complex develops normally in the absence of NT-3. However, in the first few post-natal weeks of life these afferents are lost together with their end-organs the Merkel cells. Thus these sensory neurons appear to be unique in that they become dependent on NT-3 for survival in post-natal life. This is quite different than for NGF dependent sensory neurons which appear to cease their NGF dependence after post-natal day 2 in the rat (Lewin *et al.* 1992).

4. BRAIN DERIVED NEUROTROPHIC FACTOR

Compared to NT-3 and NGF the interaction between BDNF and sensory neurons has been somewhat obscure. Analysis of knockout mice failed to show subtypes of sensory neurons to be selectively lost in these mice despite claims that up to 30% of the DRG neurons are missing (Ernfors *et al.* 1994*b*; Jones *et al.* 1994). One clear exception to this is the clear dependence of vestibular afferents and a subset of cochleal afferents innervating outer hair cells on BDNF during development (Ernfors *et al.* 1995). As there is no obvious loss of proprioceptive neurons in the BDNF knockout animals it has been presumed that a subset of cutaneous afferents may be missing in these animals. We sought to test this idea by recording from sensory neurons in the saphenous nerve of BDNF heterozygote animals. The assumption was that if BDNF is absolutely required for the survival or phenotypic differentiation of a subset of cutaneous neurons this should be obvious in heterozygotes as it was in NT-3 heterozygote animals. To our surprise the incidence of the four types of myelinated cutaneous afferents (outlined above) was not changed. However, the functional integrity of two of these afferent types, mechanonociceptors and SA mechanoreceptors, was severely impaired. The latter type had mechanical thresholds around 100-fold higher than normal. This effect on the neurons mechanical threshold appeared not to be accompanied by any other changes in the their physiology. Thus axonal conduction velocities and the integrity of the Merkel cells in the touch dome complex were all normal (Carroll *et al.* 1994; S. L. Carroll *et al.*, unpublished data). These result raise the interesting possibility that BDNFs' primary *in vivo* function might be to regulate specific aspects of sensory neuron function and perhaps not necessarily survival (but see paper by A. L. Acheson, this volume).

5. DO NEUROTROPHINS REGULATE PHENOTYPE AS WELL AS CELL NUMBER?

The evidence presented in this review suggest the following general hypothesis. The availability of the three major neurotrophins, NGF, NT-3, and BDNF regulates the number of sensory neurons that develop to subserve different functional modalities. The fact that neurotrophins also regulate cell number must, however be incorporated. One variant of the hypothesis which is outlined in model 1 of figure 1 is more easily reconciled with the hypothesis. It says that sensory neurons which become dependent on a neurotrophin for survival during or sometime after target innervation will develop into one or more of a limited subset of functional types. Thus the neurotrophin receptor expression in this case (presuming the receptor in question mediates survival) will define a limited set of possible functional phenotypes that the surviving neuron can express. One example of such an instance is that virtually all Trk A expressing sensory neurons during embryonic development depend on NGF for survival (Carroll *et al.* 1992; Smeyne *et al.* 1994). Because nociceptors are missing or reduced in number after NGF signalling is disrupted these embryonic neurons appear to be committed by virtue of their reliance on NGF. A more complex example is that of NT-3. It would appear that at least three functionally distinct types of sensory neurons are missing after NT-3 withdrawal during target innervation, muscle spindle afferents (Group Ia), cutaneous slowly adapting mechanoreceptors and D-hair receptors. The latter two afferent types only require NT-3 in post-natal life well after peripheral target innervation is complete. In any case, the results imply that the amount of NT-3 present can regulate the number of these three afferent types generated.

The second more radical formulation of the hypothesis is that neurotrophin availability can determine the number of neurons that become committed to a particular phenotype independent of its survival effects. This is a much more difficult hypothesis to test as to obtain unequivocal evidence one would ideally require that changes in neuronal phenotype occurred in the absence of changes in neuronal number. We believe that in the case of neonatal anti-NGF administration leading to a conversion of mechanonociceptors to D-hair afferents these conditions were met (see Lewin *et al.* 1992). I here reviewed two more putative examples of regulation of neuronal phenotype independent of cell death. First by giving excess NT-3 throughout the period when sensory neurons innervate their central and peripheral targets in chick it was

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found that unmyelinated afferents appeared to take on some characteristics of low threshold afferents (Lewin *et al.* 1994). These effects were seen in the absence of changes in total neuron number (M. Ockel, unpublished observations). However, the experiments do not necessarily prove that the endogenous level of NT-3 functions to regulate the number of neurons adopting a low threshold phenotype. The third example regards the differentiation of muscle afferents in general. Thus in NT-3 knockout animals these afferents apparently fail to differentiate as virtually no muscle afferents innervate the muscle and those that do are incapable of inducing muscle spindles (Kucera *et al.* 1995). However, this example must be qualified by the fact that there are major changes in cell number in the absence of NT-3 even before target innervation commences (Tessarollo *et al.* 1994).

6. COMBINATORIAL EFFECTS OF NEUROTROPHINS

Studies on tyrosine kinase receptor localization in sensory neurons of the DRG has indicated that at least in adult animals there may be a substantial number of neurons which could express more than one receptor. This is especially true of the putative BDNF receptor Trk B which has been claimed to be co-localized with Trk A in some neurons and perhaps even with Trk C (McMahon *et al.* 1994). The results of our electrophysiological analysis provide some potential insights into the possible physiological significance of such observations. Thus as outlined in figure 2 it is possible for functionally characterized cutaneous afferents to define them in terms of overlapping neurotrophin responsiveness. For example, slowly adapting mechanoreceptors require NT-3 for post-natal survival but BDNF for normal mechano-transduction function. The data so far indicates that D-hair afferents only require NT-3 for survival, another afferent type rapidly adapting mechanoreceptors so far seem not to be affected by the loss of NGF, BDNF or NT-3.

Therefore, on a general level it is possible to define functionally characterized cutaneous afferents in terms of overlapping or non-overlapping neurotrophic requirements. This is of course at the present time a speculative model as we do not know the spatial and temporal characteristics of these overlapping neurotrophic requirements. Nevertheless, these data so far indicate that we can add one more characteristic to the description of sensory neuron phenotype outlined at the beginning of this article. Thus, as well as neurochemistry, modality and connectivity we may add that its neurotrophic responsiveness is highly characteristic of the afferent type.

7. CONCLUSIONS

Under certain circumstances the functional phenotype of sensory neurons may be defined by its neurotrophic requirement. This may happen independent of survival effects of the neurotrophins during development. Furthermore, single functionally defined neurons can respond to more than one neurotrophin with very different consequences (survival versus regulation of mechano-transduction). The combination of neurotrophin responsiveness could in some sense define the identity of a given neuron. These principles may turn out to be usefully applied to neurons of the central nervous system which may not be so dependent on neurotrophins for survival during development (Snider 1994; Lewin & Barde 1996). Instead the functional properties or phenotypic fate of these central neurons could be regulated by neurotrophin availability.

I would like to thank all my colleagues with whom I have worked on the projects reviewed in this article. They include Matti Airaksinen, Yves-Alain Barde, Patrick Carroll, Annelill Eide, Martin Koltzenburg, Yasuo Masu, Lorne Mendell, Michael Meyer, Amy Ritter, Michaela Ockel and Hans Thoenen.

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