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Neurotrophin-4: The Odd One Out in the Neurotrophin Family*

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Neurotrophin-4 (NT-4) is a member of a family of neurotrophic factors, the neurotrophins, that control survival and differentiation of vertebrate neurons (2–4). Besides being the most recently discovered neurotrophin in mammals, and the least well understood, several aspects distinguish NT-4 from other members of the neurotrophin family. It is the most divergent member and, in contrast to the other neurotrophins, its expression is ubiquitous and appears to be less influenced by environmental signals. NT-4 seems to have the unique requirement of binding to the low-affinity neurotrophin receptor ($p75^{LNGFR}$) for efficient signalling and retrograde transport in neurons. Moreover, while all other neurotrophin knock-outs have proven lethal during early postnatal development, mice deficient in NT-4 have so far only shown minor cellular deficits and develop normally to adulthood. Is NT-4 a recent addition to the neurotrophic factor repertoire in search of a crucial function, or is it an evolutionary relic, a kind of wisdom tooth of the neurotrophin family?

KEY WORDS: Development; muscle; nervous system; nerve growth factor; site-directed mutagenesis.

INTRODUCTION

Numerous growth factors and receptors (and at least half of all known human genes) (1) belong to families of structurally and functionally related genes, suggesting that gene duplication, divergence and selection play a major role in the generation and expansion of repertoires of signalling molecules. Although ligand-receptor systems are obviously still under evolutionary constraints as we study them, the continuous dynamics of this process is often unintentionally neglected. We implicitly assume that a given set of molecules under study have already reached their structural and functional optimum by the time we begin our investigations. Unfortunately, neither our patience nor life-span currently allow us to study systems of ligands and receptors during evolutionary time-scales. It is nevertheless important to realize that the molecular interactions and functions we today ascribe to a particular substance are only static snapshots of the evolutionary journey of that molecule. Although we can only guess about the magnitudes of time involved in the evolution of a particular ligand-receptor system, it is conceivable, if we are lucky, that in the course of our inquires we encounter a molecule at a relatively early stage of its evolution. Some of these molecules may never make it and later be discarded, some may turn into indispensable signals for normal development and survival.

Neurotrophin-4 (NT-4) is a member of a family of neurotrophic factors, the neurotrophins, that control survival and differentiation of vertebrate neurons (2-4). Other members of this family include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). Besides being the most recently discovered neurotrophin in mammals, and the least well understood, several aspects distinguish NT-4 from other members of the neurotrophin family. It is the most divergent member (5,6) and, in contrast to the other

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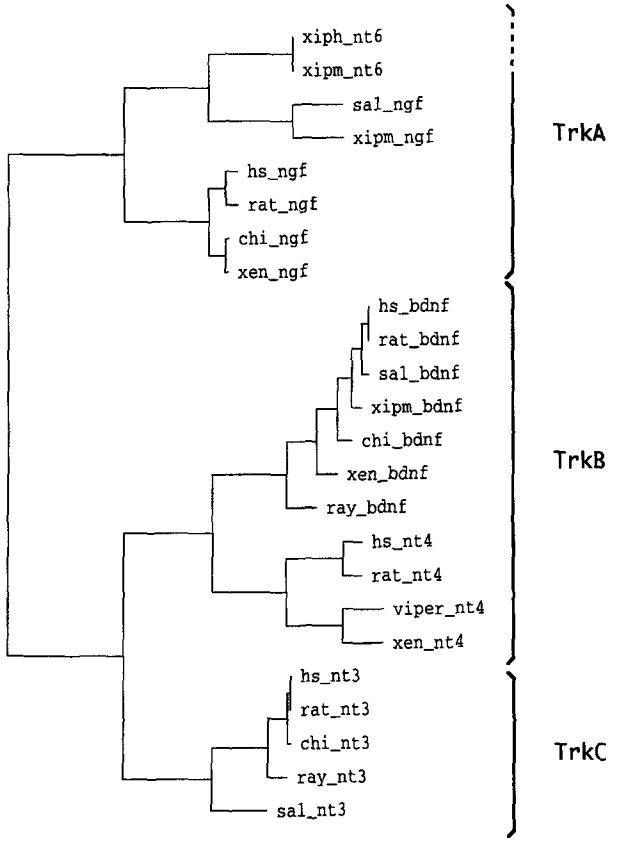

Fig. 1. Evolutionary relationships in the neurotrophin family. Alignments of protein sequences of the mature part of the indicated neurotrophins were made with the program PILEUP and manually edited with LINEUP. Phylogenetic relationships were calculated with DISTANCES (Jukes-Cantor Distance algorithm) and the tree was made with GROWTREE (UPGMA algorithm). All programs are from the University of Winsconsin Genetics Computer Group. All sequences were derived from the GenBank database or from ref. (5). Interactions with members of the Trk receptor family are indicated on the right. *xiph*, *Xiphophorus helleri*; *xipm*, *Xiphpophorus maculatus*; sal, salmon; hs, human; chi, chicken; xen, xenopus.

neurotrophins, its expression is ubiquitous and less influenced by environmental signals (7). A possible exception to the latter is the expression of NT-4 in skeletal muscle (see below), where stimuli which normally upregulate expression of other neurotrophins, cause down regulation of NT-4 expression, and vice versa (8,9). NT-4 shares its two receptors (TrkB and $p75^{LNGFR}$) with other members of the family, although it appears to have the unique requirement of binding to the low-affinity neurotrophin receptor ($p75^{LNGFR}$) for efficient signalling and retrograde transport in neurons (10,11). Moreover, while all other neurotrophin knock-outs have proven lethal during early postnatal development, mice deficient in NT-4 have so far only shown minor cellular deficits and develop normally to adulthood (12,13). Is NT-4 a recent addition to the neurotrophic factor repertoire in search of a crucial function, or is it an evolutionary relic, a kind of wisdom tooth of the neurotrophin family? This rather speculative essay does not pretend to be a comprehensive account of all the available data on NT-4, but to bring attention to some intriguing aspects of this molecule from an evolutionary perspective.

Mammalian NT-4: A Species Homologue or a Novel Family Member? Neurotrophin-4 was originally cloned from Xenopus and viper by PCR using degenerate primers derived from regions conserved between NGF, BDNF and NT-3 (5). A genomic clone containing the complete open reading frame of NT-4 was then isolated from Xenopus and was shown to contain all the structural features that characterized previous neurotrophins, namely a signal peptide, a prohormone region and a mature region of 123 amino acid residues with six cysteine residues spaced at conserved intervals (5). In Xenopus, NT-4 mRNA is highly expressed in the ovary, particularly in early stage oocytes (14), suggesting a possible role during oogenesis. Xenopus NT-4 was shown to promote outgrowth from explants of chick dorsal root and nodose sensory ganglia but not from sympathetic ganglia (5). Apart from the regions conserved among all the neurotrophins, viper and Xenopus NT-4 share a short stretch of amino acid residues in a variable β-strand which are not present in other members of the family. This sequence motif (KAKQS) was used by Ip et al. (6) to design degenerate primers for the search of the mammalian homologue of amphibian NT-4 by PCR. A novel mammalian neurotrophin was cloned in this way which resembled Xenopus and viper NT-4 more closely than any other neurotrophin (Figure 1) (6). Given the strategy followed by these authors, the assignment of this new neurotrophin as the mammalian homologue of Xenopus NT-4 was rather obvious. However, mammalian NT-4 was unexpectedly much more divergent from its amphibian counterpart than other members of the family. So much so, that Berkemeier et al., who had also isolated the same gene but using a different approach, called it instead neurotrophin-5 (15). This began a small controversy over the identity of this novel mammalian neurotrophin, until subsequent studies demonstrated a functional correspondence between amphibian and mammalian NT-4s (16,17). Some laboratories subsequently adopted the name of NT-4/5 to denote the mammalian counterpart of Xenopus NT-4, others kept the NT-4 nomenclature (which is the one used throughout this paper), and still others use the NT-5 name.

As can be seen in Fig. 1, mammalian NT-4s do segregate together with Xenopus and viper NT-4 in evolutionary comparisons of all known neurotrophins.

Closest to the NT-4 cluster are the BDNF sequences. Interestingly, these comparisons based upon structural properties of the neurotrophins parallel some aspects of their functional behaviour. Thus, neurotrophins sequences appear segregated in three major groups, each corresponding to ligands that interact with the same member of the Trk tyrosine kinase neurotrophin receptor family (Fig. 1). In agreement with its functional relatedness to NGF, the recently discovered NT-6 (so far only found in the platy fish *Xiphophorus sp* (18)) appears grouped together with a subset of fish NGF sequences, suggesting it may signal through a fish TrkA homologue. In addition, this comparison suggests that NT-6 may reflect gene duplications in the NGF lineage occurring after the divergence of fishes from a common vertebrate ancestor.

Interestingly, human chromosome 19 contains, in addition to the NT-4 gene, three related genes which are 95% identical to each other and 75% identical to human NT-4 (6,19). Their sequences are, however, incapable of encoding a functional neurotrophin—they contain multiple frame shifts, an internal stop codon, and they lack some of the conserved cysteines—suggesting that they correspond to human NT-4 pseudogenes. No NT-4 pseudogenes could be identified in the mouse (19). Although NT-4 homologues have not yet been found in birds or fishes, sequence comparisons from the available species indicate a greater variability in NT-4 compared to the other neurotrophins. Indeed, in contrast to Xenopus NT-4, mammalian NT-4 does not appear to be active on avian neurons (20)—the only reported case of a lack of interspecies cross-reactivity among the neurotrophins. BDNF, on the other hand, is the most highly conserved neurotrophin (5,21), suggesting it may resemble a common ancestral neurotrophin more closely than any other family member. This can be interpreted to mean that BDNF has reached an ''optimized'' structure earlier than other neurotrophins (22), and therefore it may be the oldest known member. Following this argument, the greater variability of NT-4 could be interpreted as an indication of a less conserved function and suggests that NT-4 could be the latest addition to the neurotrophin family.

Ubiquitous Expression and Distinct Regulation of NT-4 mRNA. NT-4 mRNA is expressed at much lower levels than any other neurotrophin, only very limited information has been possible to obtain using Northern blotting (6,15) and, with the exception of a few peripheral tissues (9,23), it has not been possible to map the cellular distribution of NT-4 mRNA by in situ hybridization. Using a sensitive RNAse protection assay (RPA), Timmusk et al. (1993) found rat NT-4 mRNA expression in every tissue and brain region examined, with the possible exception of bone-marrow (7). The RPA technique allowed these investigators to quantify the absolute levels of NT-4 mRNA in different tissues and brain regions and to compare them with those of other neurotrophins. Despite its almost ubiquitous expression, levels of NT-4 mRNA varied over a 50-fold range in different tissues; postnatal day 1 (P1) testis was shown to contain the highest level of NT-4 mRNA in the rat. Apart from changes during normal development, expression of NT-4 appears to be less influenced by environmental signals which affect the expression of other neurotrophins. Many stimuli which regulate the expression of other neurotrophins in the brain do not affect NT-4, perhaps due to its relatively low levels of expression. Among the few cases reported of NT-4 regulation are effects of mitogens and mediators of inflammation on immunocompetent cells (24), the effect of vitamin D3 on cultured astrocytes (25) and the regulation of NT-4 expression in skeletal muscle. The latter is perhaps one of the best characterized sites of NT-4 expression (8,9,26), where an unexpected form of NT-4 regulation has recently been found.

It has been known for sometime that denervated muscle produces factors which promote the re-establishment of the neuromuscular junction after axotomy by inducing axonal sprouting (27). BDNF, for example, is upregulated in muscle after axotomy, presumably reflecting an underlying lesion-induced mechanism involved in nerve repair (8). In contrast to BDNF, NT-4 mRNA virtually disappears from skeletal muscle upon nerve transsection (8). The levels of NT-4 in muscle are under direct control of acetylcholine, since blockade of neuromuscular transmission with the competitive antagonist α-bungarotoxin also results in down-regulation of NT-4 mRNA expression. On the other hand, electrical stimulation of either nerve or muscle dramatically increases NT-4 mRNA and protein, but decreases expression of BDNF and NT-3. Moreover, NT-4 mRNA expression in muscle increases during post-natal development, while expression of BDNF and NT-3 decrease. Thus, the pattern of NT-4 mRNA expression after denervation, blockade of neuromuscular transmission, electrical stimulation, and during postnatal development indicates that, unlike the other neurotrophins, the level of NT-4 mRNA in skeletal muscle is controlled by muscle activity (9). Interestingly, intramuscular administration of NT-4 induced sprouting of intact adult motor nerves. This work suggests that muscle-derived NT-4 is an activity-dependent neurotrophic signal for growth and remodelling of adult motor neuron innervation, and may thus be partly responsible for the effects of exercise and

electrical stimulation on neuromuscular performance. Is this activity of NT-4 essential for survival? Assuming no compensatory mechanisms are at work, initial analysis of mice deficient in NT-4 indicate this neurotrophin is not required for the normal development of motoneurons (12,13). However, differences in neuromuscular performance during repose, exercise and fatigue in young and adult animals have still not been investigated in these mice. Although difficult to show in the laboratory, a deficiency in any of these parameters would be likely to result in the difference between life and death for an animal in the wild.

Role of p75^{LNGFR} in NT-4 Signalling and Retrograde Transport. Although both BDNF and NT-4 bind to TrkB, NT-4 shows no similarity with BDNF in regions thought to interact with this receptor (28). Moreover, NT-4 is apparently incapable of activating a point-mutated TrkB (C345S) that is efficiently activated by BDNF (16,17), suggesting that BDNF and NT-4 may be utilizing partially distinct binding epitopes within the TrkB receptor. Further work on the recently identified TrkB domains involved in BDNF and NT-4 binding should clarify this issue (29).

Using site-directed mutagenesis, positively charged surfaces mediating the binding of neurotrophins to p75^{LNGFR} have been identified (10). In NT-4, Arg34 and Arg36, located in an exposed hairpin loop, were found to be essential for binding of this neurotrophin to p75^{LNGFR}. Disruption of this positively charged interface abolished binding of NT-4 to p75^{LNGFR}but not activation of cognate TrkB receptors or biological activity in TrkB-expressing fibroblasts. Similarly, mutant NGF, BDNF and NT-3 deficient in binding to p75^{LNGFR} also retain binding to and biological activity mediated by cognate Trk receptors (10,30). Unexpectedly, however, loss of low-affinity binding in NT-4, but not in NGF, BDNF or NT-3, affected TrkB receptor activation and biological activity in neuronal cells coexpressing p75^{LNGFR} and TrkB, suggesting a role for p75^{LNGFR} in regulating biological responsiveness to this neurotrophin. These results suggest this receptor may selectively modulate the biological actions of specific neurotrophin family members. Interestingly, recent studies have also shown that retrograde transport of NT-4 to DRG is selectively dependent upon p75^{LNGFR} (11). Of all the neurotrophins, NT-4 transport was most sensitive to inhibition by co-injected p75^{LNGFR} blocking antibodies or soluble p75^{LNGFR} extracellular domain, and was most severely reduced in mice with a targeted disruption of the p75^{LNGFR} gene (11). Furthermore, NT-4, in contrast to BDNF, was only transported to motor neurons after axotomy, coincident with increased expression of p75^{LNGFR} in these cells. Together with the data obtained from the functional analysis of neurotrophin mutants, these results indicate a distinct interaction between NT-4 and p75^{LNGFR} that appears to be required for effective TrkB activation and biological activity in cells coexpressing both receptors. Indeed, given that NT-4 can actually bind and activate chicken TrkB as efficiently as BDNF (G. Yancopoulos, personal communication), the relative inability of mammalian NT-4 to stimulate avian neurons is puzzling, and suggests that the poor responsiveness of chick neurons to mammalian NT-4 may be due to an inefficient interaction of this neurotrophin with the chicken low-affinity receptor.

Overlapping and Distinct Actions of NT-4 and BDNF: Evidence for a Novel Trk Receptor? Biological actions of NT-4 have been studied in vitro using primary cultures of different peripheral and central neuronal subpopulations, and in vivo, by directly injecting NT-4 protein in the brain or by grafting cells expressing high levels of NT-4. Numerous examples of biological actions elicited by NT-4 have recently appeared in the literature, and include effects in vitro on cultured peripheral neurons (23,31,32), as well as hippocampal (33), noradrenergic (34), dopaminergic (35,36), cerebellar (37), and striatal (38) central neurons. On the other hand, effects of NT-4 in vivo have been shown in motoneurons (9,26), visual cortex (39), substantia nigra (40) and striatum (41,42). Although most if not all of these activities can also be elicited by BDNF, there are a few examples of biological actions in which the effects of NT-4 do not parallel those of BDNF. For instance, BDNF, but not NT-4, has been shown to induce dopamine uptake and glutamic-acid-decarboxylase (GAD) activity in cultures of neurons of the ventral mesencephalon (35). On the other hand, NT-4, but not BDNF, has been reported to promote survival of striatal neurons in organotypic cultures (43), and to reverse spatial memory impairments in aged rats after intraventricular injection (44). These differences are intriguing, given that both NT-4 and BDNF are believed to act through the same receptors, i.e. p75^{LNGFR} and TrkB.

Can the biological differences between BDNF and NT-4 be taken as evidence for the existence of a novel, NT-4-specific Trk receptor? Detailed comparison of the expression pattern of TrkB mRNA and ^{125}I-NT-4 high-affinity binding sites in the forebrain indicated a one-to-one correlation (45), arguing against this possibility. However, given the widespread expression of trkB mRNA in the rat brain, these data do not formally rule out the existence of putative NT-4-specific receptors in a subset of TrkB-positive neurons. On the other hand, mice deficient in both the NT-4 and BDNF genes do not appear to have additional deficits compared to TrkB -/-

mice which, together with the in situ hybridization and binding data, strongly argues against the existence of additional receptors for NT-4.

NT-4 Is Not Essential for Normal Development and Short-Term Viability. During the last three years, the knock-out of all neurotrophin and their receptor genes were reported. Consistent with its modulatory role in neurotrophin function, knock-out of the low affinity neurotrophin receptor gene was not lethal, but caused sensory abnormalities and deficits in sympathetic innervation (46,47). NGF, BDNF and NT-3 gene knock-outs, as well as knock-outs of their corresponding Trk receptors, resulted in profound developmental defects, including drastic cell losses in the peripheral nervous system, which resulted in early postnatal death (48). Given the dramatic phenotype of neurotrophin-deficient mice, the fact that disruption of the NT-4 gene did not affect survival or fertility was unexpected. The only reported defects of NT-4 knock-out mice were a 50% cell loss in the nodose-petrosal and geniculate ganglia (12,13). Disruption of the BDNF gene also resulted in 50% neuron loss in these ganglia (13,49), and NT-4/BDNF double knock-outs have shown the effects of these two mutations to be nearly additive. These data indicate the existence of two distinct subpopulations of neurons in these ganglia, most likely representing two sets of TrkB-expressing cells projecting to different targets that express either NT-4 or BDNF, respectively. Interestingly, the loss of neurons in the nodose-petrosal ganglion appears to be dependent on BDNF, but not NT-4, gene dosage (D. Katz, personal communication), suggesting that, in contrast to BDNF, NT-4 is not expressed in limiting amounts in the targets of nodose-petrosal neurons.

NT-4 Evolution: Thumbs Up or Thumbs Down? The relatively mild phenotype of NT-4 knock-out mice is in marked contrast with those of other neurotrophin gene knock-outs. Together with the greater variability of NT-4 sequences and other evidence described above, these observations strongly suggest that something is fundamentally different between NT-4 and the other members of the family. The presence of NT-4 in amphibians and mammals indicates this protein must have been around for at least 360 million years, suggesting that some selective pressure is acting to maintain an NT-4 in vertebrates, whatever its actual function may be. The high interspecies variability observed in NT-4 sequences suggests that this neurotrophin could be under different types of evolutionary constraints in different species. These may, however, be totally unrelated to the original forces that allowed the evolution of NT-4. If the absence of detectable NT-4 sequences in fish is taken as evidence that a NT-4 gene is not present in these organisms, the NT-4 gene might have first appeared in a common ancestor of amphibians and mammals or, alternatively, it might have been lost in fishes after this lineage split from the amphibian/mammalian ancestor. The presence of multiple neurotrophins structurally related to NGF appears to be unique to fishes (18); P. Falck, M. Fainzilber and C. F. I., unpublished), suggesting these molecules could be occupying the space left by the apparent absence of NT-4 from the arsenal of neurotrophic factors of these organisms.

NT-4 may be the latest addition to the neurotrophin family. It may have arisen in an early tetrapode, a common ancestor of today's amphibians and mammals. Its spectrum of biological actions may have subsequently diverged, concomitant with the further diversification of tetrapodes, perhaps due to redundancy with other factors, or because its original function became superfluous. New roles for NT-4 may have appeared in some lineages as the vertebrate nervous system got more and more complex. In other organisms, like birds, NT-4 may not have been able to find a functional niche important enough to survive the effects of deleterious mutations. Today's mammalian NT-4 does not appear to be essential for development, although it remains to be seen whether it is required for long-term viability. It may someday acquire a crucial function or disappear altogether. It will be the task of future generations of "gene knockers" to find that out.

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