/: Medicographia 44 (Vol. 14, No. 1), 1993

Neurotrophic factors and their action on motoneuron survival: Implications for neuromuscular disorders

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Abstract

Motoneuron diseases represent a major challenge to modern neurology, yet their clinical manifestations were first described more than hundred years ago, and despite many studies the etiology of these diseases remains obscure with no effective treatments having been reported. Although progress has been made in establishing genetic linkage in the rare inherited forms of these diseases such as familial amyotrophic lateral scleriosis[1], spinal muscular atrophy[2] and X-linked bulbo-spinal-muscular atrophy[3], this new information has not yet affected therapeutic techniques. During the last few years several important steps have been taken concerning the physiological mechanisms involved in motoneuron survival during development, after lesion and in animal models of degenerative diseases; the molecular cloning of several new neurotrophic factors (brain-derived neurotrophic factor (BDNF), neurotrophin-3 and-4 (NT-3 and NT-4) and ciliary neurotrophic factor (CNTF))[4,5,6,7,8,9]; the identification of a gene family of receptor molecules for some of these factors[10]; progress in the understanding of the effects of polypeptide growth factors on muscle cell differentiation, neuronal sprouting (insulin-like growth factor-I and -II (IGF-I and IGF-II)[11], and *in vitro* motoneuronal survival (CNTF, IGF-I and -II and basic FGF)[12,13,14]. These findings have raised new hopes in that they could lead to a better understanding of the pathophysiological processes underlying these diseases, and that the pharmacological use of some of these newly characterized neurotrophic factors could present new possibilities for the treatment of these diseases.

The nerve growth-factor gene family: A function for motoneurons?

Nerve growth factor (NGF) is a small basic secretory molecule which is known to support the survival of several neuronal populations, such as neural crest-derived sympathetic and sensory neurons and cholinergic neurons within the central nervous system. It is expressed at low levels in target fields of responsive neurons, has been shown to be taken up by the nerve endings and being retrogradly transported to the cell bodies of such neurons in order to exert its specific effects. However, NGF does not support the survival of spinal and bulbar motoneurons, although NGF has been shown to bind to these neurons via its low-affinity receptor (P75)[15,16], and to be retrogradely transported in motoneurons[17]. The demonstration that antibodies specific for P75 are also taken up and retrogradely transported in a similar manner to NGF suggests that this effect is solely mediated by this receptor molecule. The P75 molecule is expressed at high levels in rat spinal cord motoneurons at the time of naturally occurring cell death during embryonic development. After that time period the expression of this molecule is down-regulated, but can be re-induced after axonal lesion in motoneurons.

Other NGF-related molecules, such as brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 and a molecule closely related to NT-4 (referred to as NT-5) have recently been identified and cloned[4,5,6,7]. These molecules share a significant portion of amino acid identity (about 50%) in their biologically active form. It has been shown that both BDNF and NT-3 also bind to the low-affinity NGF receptor P75 molecule with the

me affinity as NGF itself[18], namely 10^{-9}M, and it seems very likely that NT-4/5 shares this property. BDNF has recently been shown to be retrogradely transported by spinal motoneurons after lesion in the adult rat[19]. NT-3 is also transported, but by significantly fewer motoneurons compared with BDNF. Present investigations question which member(s) of the NGF gene family could serve as (a) functional and survival-promoting molecule(s) for spinal motoneurons. Recent reports that BDNF, NT-3 and also NT-4 are expressed in skeletal muscle[20,21,22] make it very likely that one of these molecules might represent a physiological target-derived neurotrophic molecule for spinal motoneurons.

BDNF and NT-3 seem to be unable to support the survival of embryonic chick spinal motoneurons[4] at least *in vitro*, under conditions where muscle extract or other factors such as CNTF and bFGF are active. However, not much is yet known about the physiological role of BDNF and NT-3 *in vivo*. Only one study with BDNF has been published so far describing a significant survival effect of this molecule on nodose and neuronal crest-derived sensory neurons after application onto the chorioallantois membrane during embryonic development of the quail[23]. In the case of NT-3 and NT-4, similar studies have not been reported. However, it is expected that the question will be answered in the near future as to whether or not these members of the neurotrophin gene family might act on motoneuron survival *in vivo*.

[redacted]urons: Neurotrophic interactions with sensory and [redacted] motoneurons?

It has recently been reported that NT-3 mRNA is expressed by spinal motoneurons during embryonic development[24]. The highest levels were found from embryonic day 13-16. After that period NT-3 expression in the spinal cord decreased up to post-natal day one and could not be detected in the adult rat spinal cord. The physiological role of this data is not yet clear. The authors hypothesize that NT-3 synthesized by spinal motoneurons could function *in vivo* as a target-derived trophic factor for other neuronal cells which project to the motoneurons, such as the proprioceptive sensory neurons of the dorsal root ganglion. Indeed, these neurons have been shown to respond to NT-3, at least *in vitro*[5].

In Man, the function of the corticospinal tract seems to be essential for controlled movement. In ALS patients, the cortical neurons forming this tract are known to be affected in addition to the spinal motoneurons and undergo cell death at various rates. Thus it seems important to know whether similar trophic effects as those exerted by the targets of spinal motoneurons regulate the interactions between these cells, and the spinal neurons to which they project. However, the identification and isolation of cortical motoneurons seems to be technically difficult, and nothing is so far known about the molecular mechanisms which could underlie putative trophic interactions between these cells and the spinal motoneurons. It remains to be determined whether members of the neurotrophin gene family are involved in these interactions

the establishment of transgenic animals, where the genes of individual neurotrophins have been eliminated or over-expressed and might be a tool for the further analysis of this question.

The molecular identification of motoneuron survival factors

Improved isolation and culture techniques for spinal motoneurons have led to the identification and characterization of molecules and activities which support the *in vitro* survival of these neurons. Our laboratory has established a technique for the enrichment of embryonic day 6 chick spinal motoneurons[12]. The physiological period of naturally occurring motoneuron cell death in the chick begins at E6, and it is known that the cells are dependent on survival factors at this stage. After retrograde labelling *in vivo* with a fluorescent dye the ventral parts of the spinal cord were prepared, dissociated and the motoneurons enriched by density gradient centrifugation. The cultures consisted of at least 80% motoneurons and the survival of nearly all of these cells could be supported by muscle extract, which was chosen as a positive control. Under these conditions, CNTF, at a concentration of 1.5ng/ml, supported maximally 64% of the initially plated spinal motoneurons after three days in culture. Similar results were obtained in single cell cultures, suggesting that CNTF acts directly on motoneurons (Fig. 1)[25]. Whereas NGF, NT-3 and BDNF were inactive in supporting the survival of motoneurons in these cultures, bFGF and aFGF showed significant effects, which were additive to CNTF, and resulted in the survival of 100% of cultured motoneurons in the presence of CNTF and bFGF.

variety of other mitogens and cytokines (including PDGF, EGF, TGFβ, IL-1, IL-3, IL-6) tested in the same culture system, only IGF-I and IGF-II showed a small survival effect at high concentrations (15% of the originally plated motoneurons in comparison to 5% survival without the addition of specific survival factors.

Another technique has been reported for enrichment and culture of embryonic day 4 chick spinal motoneurons by panning with the monoclonal antibody SC1[13]. In these cultures skeletal muscle extract was able to enhance neuronal survival, whereas bFGF was active only in the presence of 10% horse serum, and not under serum free culture conditions. It was therefore concluded that serum might contain a co-factor required for bFGF action. Replacement of serum by heparin, NGF, or CNTF did not enhance neuronal survival in these cultures, indicating that none of these factors are components of the serum activity. However, the action of bFGF was significantly enhanced in the presence of TGFβ, which showed similar effects to serum in the cultures for time periods of at least three days. These results provide an example of how survival of motoneurons in culture can be supported by the interaction of several growth factors which are not active on their own. It is to be expected that such interactions between growth factors occur more generally than known so far, and that the unresponsiveness of cultured chick motoneurons to neurotrophins[12] is due to the fact that an as yet unknown co-factor is missing in these cultures.

Ciliary neurotrophic factor: A non-target-derived neurotrophic factor for spinal motoneurons

The cloning of CNTF[8,9] has revealed that this factor does not belong to the NGF gene family and differs significantly to these molecules. The absence of a hydrophobic leader sequence, typical for secretory proteins and its non-release from transfected HeLa or *cos* cells, indicate that CNTF is a cytosolic molecule which might be released only after cell death or by as yet unidentified and specific release mechanisms. High levels of CNTF-mRNA and protein are found in myelinating Schwann cells of peripheral nerves and in astrocytes within restricted regions of the CNS, such as optic nerve and olfactory bulb[26,27,28]. However, in typical target tissues of responsive neurons, such as adult rat skeletal muscle or skin, CNTF mRNA cannot be detected. Neither is CNTF expression detectable in the rat during embryonic development or shortly after birth by *Northern* blot or PCR analysis, although during the following post-natal weeks a more than 30-fold increase of CNTF mRNA and protein can be observed in the sciatic nerve and distinct areas of the brain. Since the post-natal period of low CNTF levels in peripheral nerves coincides with the time of high vulnerability of motoneurons after lesion[29], insufficient availability of CNTF may be responsible for the high rates of cell death after lesion of young neurons. This hypothesis is supported by the finding that the extensive degeneration of motoneurons in the newborn rat facial nucleus after transsection of the facial nerve can be prevented by local CNTF administration. CNTF might therefore play a physiological role in the adult as a lesion factor for injured spinal motoneurons. After lesion, significant

[illegible]tities of CNTF protein seem to persist for at least one week [illegible]the lesioned nerve[30]. Immunohistochemistry at the lesion site and the distal nerve segment has shown that CNTF is present in the extracellular space and it seems to be available to regenerating axons.

The recent observation that BDNF expression is up-regulated in the sciatic nerve of the adult rat after lesion[31] is particularly interesting when these results are compared to those of CNTF. The expression of BDNF starts with a delay of at least three days after sciatic nerve lesion, and there is a gradual increase up to the third and forth week post-lesion. Thus after nerve lesion motoneurons could be supported first by CNTF which is released from injured Schwann cells, and then by BDNF at later post-injury stages, and BDNF would then guide the regeneration of motoneurons to their targets.

The role of insulin-like growth factors in the neuromuscular system.

IGF-I and IGF-II are synthesized by a variety of tissues, including the liver, pituitary, and nervous system, and are present in relatively high concentrations in serum and cerebral fluid. During the last few years evidence has increased suggesting that these factors could play a physiological role in the neuromuscular system: embryonic chick motoneurons in culture can survive and grow neurites in the presence of IGF-I and IGF-II[12], and they express high affinity binding sites for these molecules

their cellular processes[32]. IGF-I has been shown to be retrogradely transported by fast axonal transport in the sciatic nerve of the adult rat[33]. IGF-I and IGF-II are synthesized by skeletal muscle, and therefore have been suggested as candidates for the diffusible sprouting activity for motoneurons found in adult inactivated muscle. Indeed, injection of IGF-II on the surface of the gluteus muscle of the adult rat induced nodal sprouting of innervating nerve fibers and terminal sprouting at the endplates. During development both IGF-I and IGF-II mRNA expression in muscle sharply decrease at the onset of the developmental period of synapse elimination, and the mRNAs for both factors are re-expressed in skeletal muscle after denervation. GAP-43 and tubulin-α1 expression in motoneurons, which also decrease during synapse elimination, can be maintained at significant levels by the local addition of exogenous IGF-I during the period of synapse elimination. It can be concluded from these results that elevated levels of IGFs in denervated muscle might trigger coordinate regenerative reactions which lead to nerve sprouting under these pathophysiological conditions.

CNTF prevents degeneration of motoneurons in mouse mutant progressive motor neuronopathy (pmn), an animal model for human motoneuron disease.

Little is known so far about the molecular mechanisms which are responsible for the functional loss and cell death of motoneurons in human motoneuron disease. Similarly, the genes which lead to motoneuron dysfunction in three inherited forms of motoneuron

eneration in mice (wobbler, pmn, mnd) have not been identified [35,36]. Therefore therapeutical strategies based on substitution of the putative defective gene products in these animal models are still impossible. However, these animal models are very useful for analyzing the effects of drugs which could influence the symptoms caused by the underlying gene defects. Particularly in the case of neurotrophic factors it was not known until recently whether paralysis or other symptoms typical of these diseases could be influenced.

We analyzed the effects of CNTF in the pmn mouse[37]. The progression of the disease appears more rapidly than in wobbler mice[35]: first symptoms become apparent during the third post-natal week as weakness of the hind limbs, and all animals die between the sixth and seventh post-natal week. At this stage forelimbs are also paralytic and a massive reduction of motor axons is detectable in the phrenic nerve, presumably the cause of death in these mice.

The chromosomal localization of the CNTF gene has so far been determined in human and mouse[38,39], and no linkage with known hereditary forms of neuromuscular diseases has yet been found. Particularly, in the wobbler and the pmn mice, which serve as animal models for human spinal motoneuron disease, the levels of CNTF mRNA, protein and bioactivity are primarily unchanged. Therefore an insufficient or defective expression of CNTF does not seem to be responsible for the degenerative changes observed in the affected animals. Because of technical problems with injections of the CNTF protein into these mice, we established a

e cell line by transfection of mouse D3 cells with a cDNA ding for a secreted form of CNTF. Intra-peritoneal injection of hese cells at early stages of the disease significantly prolonged the survival of these mice and greatly improved their motor function (Fig. 2). Significant levels of CNTF bioactivity could be measured in the blood of these animals, whereas sera from untreated pmn/pmn mice did not show noticeable survival activity. The number of phrenic nerve axons was significantly higher in CNTF treated animals (144 ± 22 axons) as compared to untreated pmn/pmn mice (87 ± 4 axons). In addition, a significant number of motoneurons could be rescued as judged by the number of facial motoneurons: the number of facial motoneurons in 40-50 day old pmn/pmn mice was reduced by about 40% as compared to healthy control mice. With CNTF treatment facial motoneuron loss can be reduced to less than 15%. Because the initial symptoms cannot be recognized in pmn/pmn mice before the third post-natal week, it is to be expected that the disease will already have reached a relatively advanced stage before treatment of the degeneration of motoneurons can be started. The beneficial effects of CNTF under such conditions serve as a rational basis for the use of this factor in patients.

Conclusions and future prospects

The situation for a vast number of patients with idiopathic ALS is still without hope as none of the clinical trials performed in the past have shown convincing positive effects. In spite of this depressing situation many new findings which appear relevant for

[redacted]eatment of this disease lead to clinical studies within a very short time. Also in the case of CNTF, whose structure and effects on motoneurons were only reported no longer than three years ago, first clinical studies with ALS patients are already underway[40]. This is conceivable in the light of the impressive *in vitro* and *in vivo* effects observed with CNTF.

However, objections have also been raised against an uncritical enthusiasm in the use of neurotrophic factors in motoneuron disease: for example, for aFGF and bFGF, which are very effective in supporting motoneuron survival *in vitro*, it is debated as to whether it exerts any effects on motoneurons *in vivo*[41]. IGF-I and -II are found in relatively high quantitiess in the blood[42] and it is not known whether forms of motoneuron disease exist at all which are caused by reduced expression of IGF-I or -II. It remains to be demonstrated whether the exogenous addition of IGFs can prevent motoneuron degeneration in specific animal models.

On the other hand, there is also experimental evidence (at least for CNTF) which invalidates the considerations that neurotrophic factors might only be effective in cases where the disease is caused by a lack of endogenous expression of these molecules. The endogenous expression of CNTF is primarily unaffected in pmn/pmn mice. Nevertheless, CNTF is highly effective in preventing the degeneration of motoneurons in this mouse mutant, indicating that it might not be available in abundance under physiological conditions. The same situation might also apply for members of the neurotrophin gene family, which are physiologically expressed at very low levels, so that general availability is unlikely[43]. This

finding raises high hopes in that the pharmacological use of these molecules might be beneficial in the treatment of idiopathic ALS.

It should be kept in mind that the primary goal for the molecular characterization of these neurotrophic factors was the elucidation of the physiological mechanisms which regulate motoneuron survival and function, but not the design of new drugs. Several factors have been identified as being active on motoneuron survival both *in vitro* and *in vivo*,, and it appears likely that both the survival of motoneurons during a critical period of development, and the maintenance of motoneuron function in the adult, are not regulated by a single molecule but by the interaction of different factors. Such interactions are far from being understood.

Moreover, in contrast to many hormones, neurotrophic factors, such as the neurotrophins and CNTF, are not secreted into the blood stream and transported to their target cells by this route, but are taken up by neurons directly at the sites where they are produced. Therefore careful pharmacokinetic studies are very important. In particular, the possiblity of side effects and the stability of these proteins after systemic injection should be thoroughly investigated.

The outcome of the first clinical trials with these factors is awaited with great interest, and it seems possible that the results could lead to a better understanding of motoneuron diseases and could present new perspectives for their treatment.

Figure legends:

Figure 1: The effect of CNTF on neuronal survival in cell culture: **a and b:** Ciliary neurons in the presence (a) or absence (b) of 100pg/ml of recombinant rat CNTF: The neurons were isolated from the ciliary ganglia of eight day old chick embryos and plated on culture dishes, which had previously been coated with Polyornithine and Laminin (4µg/ml). After 24 h in culture, all of the neurons were dead in the absence of CNTF, whereas in its presence virtually 100% of the isolated neurons can survive. **c-e:** Survival of isolated motoneurons in the presence (c,d) or absence (e) of CNTF: Spinal motoneurons of five day old chick embryos were retrogradely labelled by Rhodamine isothyocianate, and the motoneurons were isolated 24 h later as described[12]. The cells were plated on laminin coated culture dishes and only cultures which contained a single motoneuron were analyzed. After three days in culture, only those cells which had been kept in the presence of CNTF were alive and had grown long neurites (c). The retrograde label which clearly identifies them as motoneurons was still detectable (d). Motoneurons which had been cultured in the absence of CNTF were dead at this stage (e).

Figure 2: The effect of CNTF treatment in pmn/pmn mice. Two pmn/pmn mice at the beginning of the sixth post-natal week are shown. The mouse on the left was treated with CNTF by intraperitoneal injection of CNTF secreting D3 cells at post-natal day 20. The mouse shown on the right is an untreated litter mate. CNTF treatment significantly improved motor performance of pmn/pmn mice and prolonged their survival[37].

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