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WORKSHOP REPORT

WORKSHOP ON TROPHIC FACTORS IN THE PERIPHERAL NERVOUS SYSTEM. CAPRI, OCTOBER 1991

Neurotrophic factors have been shown to have important functions in the maintenance of specific properties of peripheral and central neurons. At a recent meeting [Miniworkshop on Trophic Factors in the Peripheral Nervous System, Capri, Italy, 8–9 October 1991; organized by the European Alliance of Muscular Dystrophy Associations (EAMDA), sponsored by the Italian NMD-association (UILDM)] work performed in this field by several European research groups was presented and discussed in light of its possible relevance to neurogenic neuromuscular disorders. The abstracts summarizing the presentations given at this miniworkshop will be published elsewhere [1].

Success in the co-ordination of European research efforts in the field of muscular dystrophy has encouraged EAMDA to begin to focus on basic research on neurogenic neuromuscular disorders. Several important steps have been made in this field during the last few years: the molecular cloning of several new neurotrophic factors [brain-derived neurotrophic factor (BDNF), neurotrophin-3 and -4 (NT-3 and NT-4), ciliary neurotrophic factor (CNTF)] [2–7], the identification of a gene family of receptor molecules for some of these factors (reviewed in Ref. [8]), and 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)] [9], and *in vitro* motoneuron survival (CNTF, the IGFs and basic FGF [10–12]). The intention of the organizers was to bring together European scientists working in this field in order to discuss the clinical implications of these new findings. In particular, the present state of knowledge and the insufficient transfer of these basic research findings to clinical progress were discussed. Suggestions for support of such research efforts were made by the organizers, including the co-ordination of efforts to enable investigations with autopsy and, where possible, biopsy material and the establishment of a decentralized nerve tissue bank. Such a tissue bank would be a prerequisite for investigations concerning the question whether pathophysiological findings from animal models (regulation of expression of neurotrophic factors and their receptors, effects of the application of polypeptide factors on neuronal and muscle differentiation and regeneration under experimental conditions) can be correlated with defined pathological changes in humans. The presentations focused on several topics:

(1) the reaction of bulbar and spinal motoneurons after injury (G. W. Kreutzberg, Martinsried, F.R.G.);

(2) the molecular identification of new members of the nerve growth factor gene family, their expression in different regions of the central nervous system both during development and under pathophysiological conditions in the adult (H. Persson, Stockholm, Sweden);

(3) the identification of neurotrophic factors for embryonic spinal motoneurons (C. Mettling, Montpellier, France) and the characterization of the *in vitro* and *in vivo* survival effects of CNTF on spinal and facial motoneurons in comparison with other neurotrophic molecules and cytokines (M. Sendtner, Martinsried, F.R.G.);

(4) the identification of the *trk* oncogene as the signal transducing unit acting together with P75 as the high-affinity receptor for NGF (R. Martin-Zanca, Salamanca, Spain);

(5) the action, expression and regulation of IGF-I and IGF-II in the neuromuscular system (P. Caroni, Basel, Switzerland);

(6) the actions of synthetic peptide homologues of ACTH and αMSH on axonal regeneration and protection of neurons from toxic neuropathy caused by cisplatin (F. J. Jennekens, Utrecht, The Netherlands); and

(7) methods of culturing Schwann cells from adult rodents and humans were presented in order to analyse specific properties of these cells, to identify possible species differences and to obtain sufficient quantities of cultured Schwann cells for transplantation purposes (E. Scarpini, Milano, Italy).

The presentations on these topics were complemented by a report from M. C. Lagrange on research efforts in these fields performed by groups in France supported by the French Muscular Dystrophy Association (AFM). AFM supports a broad spectrum of research activities in the field of motoneuron diseases, which includes the development of new monoclonal antibodies for spinal motoneurons, the establishment of motoneuron cell lines and the grafting of motoneurons. Furthermore, Mrs Lagrange reported on research efforts of French groups on the creation of new mouse models for neuromuscular disease using genetic manipulation techniques, and the molecular analysis of the genetic defect of the "wobbler mouse", a well-known mouse mutant, which suffers from a yet unknown genetic defect leading to the degeneration of bulbar and spinal motoneurons [13–15].

RETROGRADE REACTIONS OF MOTONEURONS AFTER INJURY

G. W. Kreutzberg gave a summary of the cellular and molecular reactions observed in motoneurons after injury and during regeneration. The ultrastructural equivalent of the morphological changes known as chromatolysis and retrograde or axon reaction is an enormous increase in the number of ribosomes, and a redistribution of the granular endoplasmic reticulum, which is characterized by a disintegration of the cisternal arrangement. During this phase motoneurons show an overall increase in protein synthesis, glucose uptake, hexose monophosphate metabolism, in addition to enhanced iron metabolism and upregulation of transferrin receptor expression. Whereas enzyme activities such as ornithine decarboxylase are dramatically upregulated, others involved in neurotransmitter metabolism, such as acetylcholinesterase, apparently are downregulated. The changes observed in the motoneurons themselves are paralleled by accompanying reactions of the glial environment. Glial reactions include the local proliferation of microglial cells, which are involved in the displacement of presynaptic terminals from dendrites and cell somata of lesioned motoneurons (synaptic stripping), and reactions of surrounding astrocytes (induction of GFAP, formation of lamellar stacks around regenerating neurons). These morphological changes seem to be the basis for the chronic reduction of inhibitory inputs to the cell bodies detectable in lesioned motoneurons, and might therefore explain the well-known phenomenon that regenerating or chromatolytic motoneurons become hyperexcitable.

Present research focuses on the signals involved in these complex cellular reactions. One signalling molecule candidate is the calcitonin gene related peptide (CGRP). CGRP is known to affect distinct properties of muscle fibres such as cAMP synthesis [16] and acetylcholine receptor expression [17]. The expression of CGRP is increased rapidly in axotomized motoneurons [18], and several groups are presently investigating a possible role for this molecule in neuron–glia signalling.

THE NERVE-GROWTH-FACTOR GENE FAMILY: UNRESOLVED FUNCTIONS FOR MOTONEURONS

Nerve-growth-factor 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. 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) [19, 20] and be retrogradely transported in motoneurons [21]. 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 downregulated, but can be induced again in motoneurons after axonal lesion. Recently other NGF related molecules such as BDNF, NT-3 and NT-4, have been identified and cloned [2–5]. In their biologically active form, these molecules share a significant portion of amino acid identity (about 50%). It has been shown that BDNF also binds to the low-affinity NGF receptor P75 molecule with the same affinity as NGF itself [22], namely 10^{-9} M, and it seems very likely that NT-3 and NT-4 share this property. Present investigations are focusing on the question whether there might be a member of the NGF gene family which could serve as a functional, survival-promoting molecule for spinal motoneurons. At least *in vitro*, NT-3 seems to be unable to support the survival of embryonic chick spinal motoneurons [10], but not much is known yet about the physiological role of this molecule *in vivo*. In the case of NT-4 [5], nothing is known so far about

the distribution of this factor *in vivo*, and which neurons of the central nervous system respond to it.

The highest levels of NGF, BDNF and NT-3 mRNA within the brain are found in the hippocampus, and *in situ* hybridization data indicate that the expression patterns for these molecules are regionally different. NGF and BDNF mRNA expression in the adult rat brain is regulated by neuronal activity [23, 24], and Hakan Persson reported on results obtained by his group showing the upregulation of these factors following epileptic seizures and the regulation during kindling associated neural plasticity [25].

Recently, it has been reported by the same group that NT-3 mRNA is expressed by spinal motoneurons during embryonic development [26]. Highest levels were found from embryonic days 13–16. After that period NT-3 expression in the adult spinal cord decreased up to postnatal day 1 and could not be detected in the adult rat spinal cord. The physiological role of these data is not yet clear. The authors hypothesize that NT-3 synthesized by spinal motoneurons could function *in vivo* as target- derived trophic support for proprioceptive sensory neurons of the dorsal root ganglion. Indeed, these neurons have been shown to respond to NT-3, at least *in vitro* [3].

THE MOLECULAR IDENTIFICATION OF MOTONEURON SURVIVAL FACTORS

Improved isolation and culture techniques for embryonic chick spinal motoneurons have led to the identification and characterization of survival activities and molecules for these neurons. Mettling reported on a culture technique based on the enrichment of embryonic day 4 chick spinal motoneurons by panning with the monoclonal antibody SC1 [11]. 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 transforming-growth-factor-beta (TGFβ), which showed similar effects to serum in the cultures for time periods of at least 3 days. Mettling also reported on a strategy for the expression cloning of motoneuron survival molecules based on *Xenopus* oocyte injection. The authors used mRNA from denervated muscle and could show that a mRNA molecule contained in this preparation might encode for a secreted protein capable of supporting the survival and promoting neurite outgrowth of spinal motoneurons. They are working presently on the molecular identification and characterization of this activity.

Another technique for the enrichment of embryonic day 6 chick spinal motoneurons has been reported [10]. The physiological period of naturally occurring motoneuron cell death in the chick begins at the same time and it is known that the cells are dependent on survival factors at this stage. After retrograde labelling *in ovo* with a fluorescent dye the ventral parts of the spinal cord were prepared, dissociated with 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 1.5 ng ml^{-1}, supported maximally 64% of the initially plated spinal motoneurons after 3 days in culture. Similar results were obtained in single cell cultures, demonstrating that CNTF acts directly on motoneurons [27]. Whereas NGF, NT-3 and BDNF did not support the survival of motoneurons in these cultures, bFGF and aFGF showed significant effects, which were additive to that of CNTF resulting in a survival of actually 100% of cultured motoneurons in the presence of CNTF and bFGF. Of a variety of other molecules (including PDGF, EGF, TGFα, 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 with 5% survival without the addition of specific survival factors).

The cloning of CNTF [6, 7] has revealed that this factor does not belong to the NGF gene family and differs significantly from these molecules. The absence of a hydrophobic leader sequence, typical for secretory proteins, and its non-release from transfected HeLa cells indicate that CNTF is a cytosolic molecule which might be released only after cell death or by as yet unidentified, specific release mechanisms. High levels of CNTF mRNA and protein are found in Schwann cells of peripheral nerves and in astrocytes within restricted regions of the CNS, such

as the optic nerve and olfactory bulb [28]. However, in typical target tissues of responsive neurons, such as adult rat skeletal muscle or skin, CNTF mRNA cannot be detected. In the rat, CNTF expression cannot be detected by Northern blot or PCR analysis, either during embryonic development or shortly after birth, but during the following postnatal weeks a more than 30-fold increase of CNTF-mRNA and protein can be observed in the sciatic nerve and distinct areas within the CNS. Since the postnatal period of low CNTF levels in peripheral nerves coincides with the time of high vulnerability of motoneurons after lesion, 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 transection of the facial nerve can be prevented by local CNTF administration [29]. CNTF might therefore play a physiological role in the adult as a lesion factor for injured spinal motoneurons.

trk PROTO-ONCOGENES: FUNCTION AS RECEPTORS FOR NEUROTROPHIC FACTORS

The cloning of the low-affinity NGF receptor (P75) [30, 31] revealed that the molecule is a 75–80 kDa intrinsic membrane molecule with a relatively short cytoplasmic domain without typical structural domains known to be involved in signal transduction. Evidence for a different protein involved in the high-affinity binding of NGF has already been obtained by cross-linking experiments which reveal a second, 160 kDa complex (consisting of NGF and a presumptive 140 kDa protein) on NGF responsive cells [32]. Martin-Zanca *et al.* [33] have shown that the human *trk* proto-oncogene encodes a 140 kDa transmembrane tyrosine kinase molecule. Subsequent *in situ* hybridization experiments by the same authors have shown that this molecule in embryonic mice is predominantly expressed in neural crest-derived neuronal cells. Based on these studies NGF has been identified as a ligand able to bind to *trk* and to induce its tyrosine autophosphorylation [34–36] suggesting that this molecular event might be the first step in a cascade resulting in the cellular responses evoked by NGF in responsive cells. In addition, it has been shown that there are other molecules of a gene family which share significant sequence homologies with *trk* (for recent reviews see Refs [8, 37]). Binding analysis has revealed that *trk*B might serve as a receptor for BDNF and/or NT-3 [38]. It is not yet known whether spinal motoneurons express transmembrane molecules of the *trk* gene family. The identification of such a molecule and appropriate ligand would be a promising step towards the characterization of the mechanisms involved in the regulation of motoneuron survival during development.

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 [39]. During the last few years evidence has increased suggesting that these factors could play a physiological role in the neuromuscular system: they have been shown to promote neurite outgrowth from sympathetic and sensory neurons [39] and IGF-I is retrogradely transported by fast axonal transport in the sciatic nerve of the adult rat [40]. Pico Caroni reported work performed by his group on the analysis of IGF-I and IGF-II as candidates for the diffusible sprouting activity for motoneurons in adult inactivated muscle. They have found that embryonic chick motoneurons in culture can survive and grow neurites in the presence of IGF-I and IGF-II, and that they express high-affinity binding sites for these molecules on their cellular processes [9]. They also have found that injection of IGF-II on the surface of the gluteus muscle of the adult rat induces nodal sprouting of innervating nerve fibres and terminal sprouting at the endplates. During development both IGF-I and IGF-II mRNA expression in muscle are sharply decreased 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 α 1 expression in motoneurons, which are also both decreased during synapse elimination, can be maintained at significant levels by the local addition of exogenous IGF-I during the period of synapse elimination. The increase of IGF-I mRNA after muscle denervation in adult animals appears to occur more rapidly than that of IGF-II, and both the muscle fibres and interstitial cells might be the source of these factors 1 week after muscular denervation. In addition to the increased IGF expression in muscle, IGF-I receptor mRNA expression is induced in the corresponding spinal

motoneurons. These results could indicate that elevated levels of IGFs in denervated muscle trigger co-ordinate regenerative reactions which lead to nerve sprouting under these pathophysiological conditions.

CLINICAL EXPERIENCES WITH ACTH ANALOGUES IN THE TREATMENT OF NEUROMUSCULAR DISEASE

In contrast to the neurotrophic factors and cytokines described earlier, peptides derived from the amino acid sequence of ACTH have already been used for clinical applications in degenerative diseases such as Alzheimer's disease and in neurotoxic lesions. Such studies are based on earlier work on the effects of melanocortins on axonal regeneration. In particular, a synthetic analogue of amino acids 4–9 of the ACTH peptide sequence has been shown to increase sprouting after a crush lesion of the sciatic nerve in the adult rat [41]. In the unlesioned neuromuscular system, a synthetic analogue of this peptide (ORG 2766) can induce sprouting of intramuscular nerves [42]. However, it is not yet clear how this peptide exerts its effects at a cellular basis. In particular, it is not known whether the effects are caused directly after binding to specific receptors on motoneurons, or indirectly by inducing the synthesis of other factors such as the IGFs known to affect the regeneration and/or sprouting of motoneurons.

The same synthetic analogue of ACTH 4–9 has been used in animal studies with rats and also in patients suffering from cisplatin-induced neuropathy [43, 44]. Treatment of rats with cisplatin lowers the increase of conduction velocity of sensory nerve fibres detectable during normal postnatal development. In the presence of the ACTH 4–9 analogue ORG 2766 this neurotoxic effect of cisplatin can be completely abolished. Promising effects were also seen in patients suffering from cisplatin neuropathy during chemotherapy of ovarian carcinoma. Cisplatin-treated patients showed a marked increase in the vibration sense threshold, which the simultaneous application of ORG 2766 could antagonize. The effect of ORG 2766 was dose dependent [44]. These results might encourage further studies on the mechanisms of action for these peptides, which could give deeper insights into the physiological role of melanocortins in the function of spinal motoneurons.

HUMAN SCHWANN CELLS IN CULTURE: PROPERTIES AND PROSPECT FOR CLINICAL APPLICATION

Schwann cells play an important role in the maintenance of motoneuron function *in vivo*. Indeed, the integrity of the myelin sheath is necessary for neuronal signal transduction. Under pathophysiological conditions, such as nerve lesion, Schwann cells present cell surface molecules and extracellular matrix components as well as being the source of neurotrophic factors such as NGF, BDNF and CNTF, molecules which have been shown to exert distinct effects on the regeneration of lesioned nerve fibres. In order to study both the molecular events leading to differentiation/myelination and to obtain cells for transplantation purposes, several techniques have been developed for the isolation and culture of Schwann cells. In most cases tissues from newborn rats, nerves or spinal ganglia are used. Techniques for the purification of Schwann cells from adult rat sciatic nerves have also been described which allow the specific properties of these cells after postnatal differentiation to be studied [45]. This method includes steps to overcome the technical problems caused by the abundance of connective tissue and myelin in nerves at that stage, and more than 80% of the cells obtained by this method were estimated to be Schwann cells by S-100 staining during the first 3 days in culture. Scarpini and his group have used a similar method to prepare human Schwann cells from normal adult and foetal nerves. These Schwann cells showed immunological properties similar to the cells prepared from rat sciatic nerves, such as positive staining for S-100, HNK-1, P0, Gal-C and P75. In the presence of platelet-derived growth factor (PDGF) and forskolin a significant proliferative response could be observed in the Schwann cell cultures, a prerequisite for obtaining large numbers of these cells for transplantation purposes suitable for the treatment of damage to the peripheral nervous system.

CONCLUSIONS AND FUTURE PROSPECTS

Most of the findings reported have not yet led to direct clinical applications. Indeed, only the synthetic ACTH peptide analogues have reached the clinical study stage. The reasons for the absence thus far of clinical applications of neurotrophic factors are obvious: CNTF, BDNF, NT-3 and NT-4 have been cloned during the last

3 yr, and the physiological role played by these factors is far from being fully understood. In addition, problems in the production of high quantities of recombinant factors in active and pure form, a prerequisite for future clinical studies, have not yet been fully solved. CNTF is the only neurotrophic factor to date for which it seems that sufficient amounts of the recombinant human factor necessary for clinical studies might be available in the near future.

Clinical studies with neurotrophic factors should be of particular value in cases where the degeneration of the spinal motoneurons is in total or in part due to the restricted availability of a neurotrophic factor: substitution of the missing factor should then prevent progression or even alleviate the symptoms of patients suffering from such diseases. However, nothing is yet known as to whether such cases of neurogenic muscular dystrophy which are caused by reduced expression of neurotrophic molecules exist. Therefore the identification of neurotrophic factors and other survival factors for spinal motoneurons is only the first step towards treatment of spinal muscular atrophy. The characterization of the molecular mechanisms leading to the disease state is a second prerequisite for rational therapeutic steps. The establishment of an organized nerve tissue bank might be an important step towards addressing such questions during the coming years.

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