The Potential of Melanotropins in the Treatment of Nervous System Diseases

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INTRODUCTION

Peripheral nervous system disorders still present a major problem to the clinicians. Often the pathogenesis of the disease is poorly understood, and in general no effective pharmacotherapy is at hand to counteract the disease process. There are numerous different polyneuropathies leading to disturbed sensorimotor function of the extremities, and thus the search for insight into the disease mechanisms and possible effective pharmacotherapy is carried out in university and industry laboratories worldwide. Current thinking about the development of new pharmacotherapies is influenced by the notion that neurotrophic factors, which are developmentally active, may also play a role in the maintenance and repair of mature neurons and neuronal networks and connections. In order to screen neurotrophic efficacy of compounds, in general, tissue culture experiments are performed that are aimed to reveal neuronal survival or neurite-promoting activities. A problem with this approach is that once neurotrophic activity has been established, the experimenters are confronted with the question of how to test the effect in the animal or human. Additional problems are encountered when one wants to screen potential therapeutic efficacy in a given nerve disorder of the human. What model system should be used prior to pilot clinical tests? In order to cope with these serious experimental problems, experimental neurology often employs animal models that are more or less directly applicable to human peripheral nerve disorders, such as mechanical trauma (crush lesions or transections), or models that by metabolic or toxic manipulation of the animal result in histological or functional deficits that resemble aspects of the human condition. Examples of the latter category are streptozocin treatment of rats resulting in a form of insulin-dependent neuropathy or acrylamide intoxication leading to a diseased state resembling a neuropathy of the so-called dying back type. More recently, one can discern a strategy that is based on the notion that compounds that are shown to be effective in enhancing recovery of function following traumatic nerve injury are also effective in protection or healing of the nerve during or after intoxication. In this context, the development of gangliosides and melanocortins (ACTH/MSH-like peptides) as new leads in peripheral nerve pharmacotherapy provide examples of the success of this approach.

NEUROTROPHIC EFFICACY OF MELANOCORTINS

Melanocortins are peptides related to the pituitary hormones ACTH and MSH (melanocyte-stimulating hormone), and as hormones, they mediate a variety of

adaptive responses. The pioneering studies of David de Wied suggested that pituitary peptides may directly modulate nervous system function and behavior. In considering whether neural tissue is a target for circulating peptides, various investigators have studied the possibility that melanocortins may exert effects on the nervous system analogous to their known trophic effects on their target organs.

Neurons in Culture

In a series of experiments, the putative neurotrophic effect of these peptides was studied on fetal neurons in culture. Daval *et al.* treated chick cerebral neurons with $ACTH_{1-24}$ and observed an enhancement of cell metabolism in general, and an increased neurite formation.[1] The effect appeared to be dose dependent, with a maximum at 10-nM $ACTH_{1-24}$. Azmitia and de Kloet used cultures of dissociated raphe and hippocampal cells and were able to show an effect of melanocortins on serotonergic maturation.[2] It was concluded that α-MSH may affect the ability of certain cells to produce a specific neurotrophic factor without having a trophic effect itself. Richter-Landsberg *et al.* showed that $ACTH_{4-10}$ and $ACTH_{1-24}$ in the nM to μM range increased the density of the network of rat central neurons, the formation of neuronal aggregates, and the activity of acetylcholine-esterase.[3] In none of these studies were quantitative data concerning the effect of the peptides on neurite formation reported. In our initial studies, slices of fetal spinal cords in culture were used to establish possible trophic effects of α-MSH and $ACTH_{4-10}$ on the outgrowth of neurites from spinal neurons.[4] The spinal cord slices were treated with peptides over a wide concentration range. Using monoclonal antibodies against (subunits of) neurofilament (NF) followed by immunofluorescence, we could show that the extension consisted mainly of axons. After 5 and 7 days, outgrowth was quantified with two different techniques, namely, by visual scoring under phase contrast and by means of an ELISA for NF. The maximal effect of the peptides (0.1–0.01 nM) was 30–40% as compared with controls. It was concluded that α-MSH and $ACTH_{4-10}$ stimulate axonal outgrowth from fetal spinal cord slices *in vitro* in a dose-dependent way.[4] In a subsequent study, the effect of melanocortins was tested on neurite outgrowth from dissociated rat spinal and sensory neurons.[5] The outgrowth was quantified by using ELISAs for the growth-associated protein B-50/GAP43 and the NF protein. Dissociated fetal rat spinal cord neurones, or neonatal rat dorsal root ganglion (DRG) cells, were cultured with peptide and assayed after 24, 48, or 96 h. In spinal neurones, α-MSH and $ACTH_{1-24}$ induced the expression of B-50 dose dependently. After 24 h, α-MSH had a stimulatory effect (from 10 nM on), with a maximum at 100 μM (36% increase). After 96 h, the maximal effect of α-MSH (100 μM) was 19%. $ACTH_{1-24}$ (100 μM) stimulated B-50/GAP43 by 19%. NF levels (96 h) were elevated maximally by 64% at 100-μM α-MSH. In DRG neurones, a bell-shaped dose-response curve was found for α-MSH, the maximal effect being observed after 48 h at 100 nM: 54% for B-50/GAP43 and 22% for NF. In both culture systems, neither $ACTH_{4-10}$ nor the $ACTH_{4-9}$ analogue ORG2766 [H-Met(O)$_1$-Glu-His-Phe-D-Lys-Phe-OH] was effective. More recently Bar *et al.* reported that in contrast to dissociated fetal DRG cells, cultured intact rat fetal DRGs do respond to the $ACTH_{4-9}$ analogue in a dose-dependent manner.[6] Neurite outgrowth was quantified by the neurofilament ELISA. ORG2766 enhanced NF production after 48 h in culture (optimum of 40% increase at 10-nM peptide). Collectively, the data show that depending on the experimental conditions used, peptides derived from ACTH or MSH may facilitate neurite outgrowth from motor and sensory fetal neurons.

Postlesion Repair

The regenerative capacity of the peripheral nervous system is limited, but regeneration and reinnervation are possible if the damage is restricted to the neuronal processes (dendrites and axons). Numerous humoral and structural factors of neuronal, glial, or target origin appear to facilitate nerve repair. The first study on the neurotrophic effect of peptides on postlesion repair in the peripheral nervous system was that by Strand and Kung, who reported that adrenalectomized rats subjected to sciatic nerve denervation recovered sooner when treated with $ACTH_{1\text{-}39}$ than when given saline solution.[7]

Using a foot reflex withdrawal test and a free-walking pattern analysis test to monitor return of sensorimotor function following crush lesion of the sciatic nerve in rats, we have found that neurotrophic fragments and analogues of melanocortins, including the $ACTH_{4\text{-}9}$ analogue ORG2766, exhibit a U-shaped dose-response relationship and are maximally active in a dose range of 7.5–75 $\mu g\ kg^{-1}$ when given daily or every other day subcutaneously (sc).[8,9] Oral doses of 125 $mg\ kg^{-1}$ are ineffective,[9,10] but it is possible to facilitate recovery of function by delivery of the peptide by slow release from osmotic minipumps implanted sc, or from biodegradable microspheres.[11] Peptide treatment must begin within a short period of induction of the lesion—there is a "critial period" of one week[12]—but its beneficial effects on histological and neurophysiological parameters are still apparent after several months.[13,14] The precise location of the amino acid sequence that confers neurotrophic activity on the peptides is uncertain. It is clear however that, as for many of the other effects of melanocortins on the nervous system, the critical information for stimulating neurite outgrowth is contained in the amino acid sequence between positions 4 and 10; the peptide ORG2766 is frequently used in studies of these effects.[15]

Histological and functional studies strongly support the notion that the melanocortins do not enhance the rate of outgrowth but rather increase the number of newly formed sprouts at the site of lesion.[16] It has been suggested that the peptides mimic or amplify an endogenous signal that operates early in the regenerative response of the damaged neuron (reactivation of expression of proopiomelanocortin or neurofilament breakdown).[16]

Edwards *et al.* have shown with a bioassay for α-MSH that α-MSH-like bioactive material is present in degenerating nerve stumps.[12] During the development of the rat spinal cord POMC (proopiomelanocortin) mRNA and POMC-derived neuropeptides were shown to be expressed transiently from E13–E15 days of gestation,[17] suggesting a specific role for POMC in the development of the peripheral nervous system. Furthermore, immunocytochemical evidence of enhanced amounts of α-MSH in the motoneurones of mice with inherited motoneurone disease[18] or muscular dystrophy,[19] conditions in which the maturation of the end plates is delayed into adulthood and continued formation of new neuromuscular junctions is an ongoing process, suggest that enhanced production of melanocortins is associated with stages of active axonal growth. However, in a recent study, we were unable to establish that up regulation of POMC gene expression does occur in the neurons of the damaged sciatic nerve or locally in proliferating Schwann cells or in infiltrating mononuclear blood cells at the site of the nerve crush.[20] Thus the previously demonstrated induction of POMC-related immunoreactivity in damaged nerves is not due to increased POMC mRNA levels. Although we were not able to show an increase in POMC gene expression, we cannot exclude the possibility that there is increased production of biologically active peptides caused by (1) a more efficient translation of the low levels of POMC mRNA present in

both neuronal cell bodies and nonneuronal cells at the crush site after crush or (2) altered precursor processing. However Verhaagen *et al.* have not been able to detect α-MSH in crushed peripheral nerve with a radioimmunoassay.[21] Therefore we favor the view that the previously shown immunoreactive α-MSH-like material in peripheral nerve is unrelated to POMC and could represent cross-reactivity of α-MSH antibodies with as yet unidentified molecules.

DIABETIC NEUROPATHY

Diabetic neuropathy is a frequently diagnosed and underestimated complication in patients with type 1 and type 2 diabetes mellitus. Prevalence figures vary between 5 and 80%, depending on the duration of the disease, screening methods, and heterogeneous criteria.[22,23] Neuropathy is a syndrome that presents itself in different ways: the most common neuropathy is the combined sensorimotor-autonomic neuropathy.[24,25] Frequently occurring symptoms are burning pain, paresthesia, and numbness, usually beginning in the distal parts of the lower extremities. Muscle weakness sometimes develops in the distal parts of the leg. The combination of sensory loss and muscle weakness can cause problems with walking.[22,26]

There is a considerable amount of literature on the pathogenesis of diabetic neuropathy. One can roughly divide the various theories into three categories. First, there is the so-called metabolic theory.[27–30] Chronic hyperglycemia causes an increased activation of the polyol pathway by which glucose is converted into sorbitol by the enzyme aldose reductase, and subsequently into fructose. The accumulation of sorbitol causes a depletion of the glycolipid myoinositol.[31,32] Myoinositol is an important glycolipid for normal neuronal functioning. Depletion of myoinositol causes a decreased activity of Na^+, K^+, ATP-ase and thereby a decrease in impulse conduction. Inhibition of aldose reductase and supplementation of myoinositol at an early state of experimental diabetic neuropathy (EDN) have proven that functional and morphological changes in diabetic nerves can be postponed.[33,34] The second theory is the so-called vascular hypothesis. Chronic hyperglycemia causes structural changes (thickening of basement membranes) in the endoneurial vessels, resulting in decrease in blood flow to the peripheral axons and Schwann cells. This leads to chronic hypoxia and a decrease in all oxygen-dependent processes, resulting in nerve degeneration.[35–38]

A third theory is based on the glycosylation of neural proteins, which results in neuropathy. Analogous to the formation of glycosylated hemoglobin (HbA1), glucose can irreversibly attach itself to functionally important neuronal proteins. This can ultimately lead to a loss of function of these proteins. It has been reported that glycosylated myelin can increase the activation of macrophages, thereby inducing an excessive turnover of myelin.[39,40] It is likely that not one single mechanism is involved in the pathogenesis of diabetic neuropathy, but rather that a combination of various factors is involved.

Animal Studies

A simple and effective treatment of diabetic neuropathy does not exist, although it is generally assumed that proper metabolic control may prevent or ameliorate the diabetic peripheral neuropathy. In two experimental models of diabetic neurop-

athy, we tested the putative efficacy of chronic treatment with the synthetic $ACTH_{4-9}$ analogue. In the first model, rats received a single intravenous (iv) injection of streptozocin. This cytostatic drug is known to kill the β-cells in the islands of Langerhans. As a consequence, these animals developed high blood glucose levels, and as no insulin was injected, these animals showed pathology much the same as seen in human diabetes mellitus. Measurement of peripheral nerve conduction velocity is often used as a tool to longitudinally monitor the onset and development of a peripheral neuropathy. In the first series of experiments, immediately following the streptozocin injection, rats received either saline or peptide treatment (7.5 or 75 mg/kg sc three times a week) that lasted throughout the experimental period of seven weeks. At various intervals, the functionality of motor and sensory nerve fibers was measured by means of transcutaneous stimulation of the sciatic nerve and recording of the M- and H-responses.[41] Three weeks after the induction of the diabetes mellitus, all streptozocin-saline-treated rats displayed a significant reduction in both sensory nerve conduction velocity (SNCV) and motor nerve conduction velocity (MNCV). This decrement gradually increased until the end of the experimental period. If however ORG2766 (75 mg/kg) instead of saline was given, following the initial decrease, a gradual restorement of both SNCV and MNCV was observed, indicative of a neuroprotective effect of the high-dose peptide regime.[42] Rats of each treatment group were sacrificed seven weeks following the streptozocin injection. Histological analysis of cross sections of the sural nerve showed no difference in the total number of nerve fibers in saline- or peptide-treated diabetic rats. In contrast, a difference in fiber size distribution was demonstrated: the sural nerves of diabetic rats contained fewer thick myelinated fibers. Treatment with ORG2766 resulted in a normal distribution. Apparently, the peptide ORG2766 has a protective action on nerve fibers and nerve function during streptozocin-induced diabetes.[42]

In a next series of experiments, we examined the effect of treatment of an already existing neuropathy in streptozocin-diabetic rats with the $ACTH_{4-9}$ analogue ORG2766. Four groups of rats were studied: group 1 consisted of age-matched, nondiabetic controls and groups 2, 3, and 4 of diabetic rats. Four weeks following the streptozocin injection, the SNCV and MNCV were decreased and treatment was then started. Group 2 was treated with placebo, group 3 with low-dosage (7.5 μg/kg) ORG2766 sc every 48 h, and group 4 with high-dosage (75 μg/kg) ORG2766 sc every 48 h. The animals that were treated with the high peptide dosage showed a significant improvement in both SNCV and MNCV from week 6 onwards, whereas this beneficial effect was not found in rats treated with the low dosage. Thus we concluded that the $ACTH_{4-9}$ analogue ORG2766 can ameliorate existing diabetic neuropathy in streptozocin-induced diabetic rats.

Prior to clinical testing of the putative efficacy of peptide treatment in patients suffering from diabetic neuropathy, the neuroprotective efficacy in EDN was studied using another animal model of diabetic neuropathy in which, as in the clinic, insulin treatment is standard. This model concerns the use of the BB/WOR rat first described in 1974. Diabetes mellitus develops between 55 and 120 days of age in approximately 80% of the rats.[43,44] The onset of the diabetic syndrome is acute and characterized by hyperglycemia, hypoinsulinemia, polyuria, and glycosuria.[43,44] Autoantibodies are produced against the 64-kDa islet cell protein preceding the onset of spontaneous diabetes.[45]

We studied the effect of ORG2766 treatment in already existing neuropathy in the BB/Wor rat. Thirty BB/Wor rats were used: after the development of diabetes, insulin treatment was started immediately using a single injection of long-acting Zn-insulin every 24 h. Insulin treatment was adjusted according to individual

weight and glucose levels. SNCV and MNCV were measured at weeks 0, 2, 4, 6, 8, 10, and 12. The BB/Wor rats that did not develop diabetes were kept as age-matched controls. After 6 weeks, when a significant slowing of SNCV and MNCV had developed as compared with group 1, the diabetic animals were divided into two treatment groups: group 2 was treated with saline 0.5 ml sc every 48 h, and group 3 was treated with 75 μg/kg ORG2766 sc every 48 h. The level of metabolic control, as assessed by measurement of glucose and HbA1 levels, was not different for groups 2 and 3. After the start of treatment at week 6, the rats in group 3 showed a significant improvement in SNCV and MNCV as compared with group 2. Apparently ORG2766, when given in a high dosage, can also improve existing neuropathy in the BB/Wor rat.

Clinical Studies

Recently, the first clinical study on the putative efficacy of the $ACTH_{4-9}$ analogue in diabetic neuropathy was completed.[46] It concerns a randomized double-blind, placebo-controlled study in 62 patients suffering from type 1 diabetes mellitus. The mean age of the patients was 46.3 ± 11.4 (standard deviation) years. The duration of the diabetes was 24.8 ± 11.3 and HbA1 9.4 ± 2.4%. Inclusion criteria: vibratory threshold (VT) > p95 and/or warm thermal threshold (TTw) in the hand > p95. Patients were randomly divided into 2 groups: group 1 (n = 32) received placebo and group 2 (n = 30) was treated with ORG2766 3 mg sc every 24 h. At 0, 4, 8, and 12 months, the following neurophysiological parameters were measured: VT, TTw, cold thermal threshold, sensory nerve conduction velocity (NCV) in ulnar and sural nerve, motor NCV in ulnar and tibial nerve. Hoffmann reflex latency, cardiovascular autonomic score, darkness adapted pupil size, and pupil constriction latency.

The peptide treatment was only effective in lowering the vibration threshold in patients with diabetic neuropathy. There were no differences in any other variables studied. Based on this first study in which Org2766 improved vibration perception in patients suffering from diabetic neuropathy, further and larger studies are required to ascertain effects of the treatment on other neurophysiological parameters.

CISPLATIN NEUROPATHY

The cytotoxic drug cisplatin (*cis*-diaminedichloroplatinum) has shown particular efficacy against bladder, testicular, and ovarian cancer. However, the acute nephrotoxicity but especially the neurotoxic side effects that occur after several cycles of administration limit its therapeutic use. The neurotoxicity manifests itself as a primarily sensory neuropathy; diminishment of vibratory sensibility probably indicating an impairment of the thickest afferent nerve fibers, followed by a prolongation of the distal sensory latency and slowing of the sensory conduction velocity. Signs of an involvement of motor nerve fibers are seldom observed.

Animal Studies

In order to test the notion that peptides might stimulate nervous recovery from damage induced by toxins, De Koning developed a rat model for cisplatin

neuropathy:[41] by biweekly injections of cisplatin [1 mg per kg per injection intraperitoneally (ip)] a sensory neuropathy, as evidenced by a decrease in the SNCV, was induced. This decrease became significant from 6 weeks (12 mg cisplatin/kg cumulative) onwards. The conduction velocity is dependent on the thickest myelinated sensory nerve fibers. This correlates rather well with the human clinic in which it is also the thickest myelinated sensory nerve fibers that degenerate in cisplatin-treated patients. Using this model, we were able to show that concomitant administration of the neurotrophic peptide ORG2766 in a dose of 75 μg/kg every other day could protect from the cisplatin-induced decrease in nerve conduction velocity observed in rats cotreated with saline.[41] Subsequent reports on the protecting effects of ORG2766 in this animal model confirmed and extended these data.[47,48]

As one of the rationales of peptide treatment in this particular neuropathy is to increase cisplatin dose intensity, we have recently performed experiments employing a twofold higher cisplatin dose per time unit, in combination with ORG2766. Our results indicate that the peptide prevents the neuropathy even in a high-dose-intensity cisplatin treatment.[49] Since in our animal model for cisplatin neuropathy we employ young adult rats, it could be argued that the cisplatin neurotoxicity observed is related to a slowing of the maturation of the peripheral nerve and thus does not closely resemble the clinical situation. However, in a recent study with full grown adult rats (7–10 months of age), we observed a cisplatin-induced slowing of the SNCV which could be counteracted by concomitant peptide treatment (Hamers, in preparation).

Clinical Studies

Based on the experimental evidence as outlined above, a randomized, double-blind, placebo-controlled study was initiated to assess the efficacy of ORG2766 in the prevention of cisplatin neurotoxicity in women with ovarian cancer. The peptide was administered sc in doses of respectively 0.25 mg (low dose) and 1.0 mg (high dose) per m^2 of body-surface area before and after each cisplatin and cyclophosphamide dose (75 and 750 mg/m^2 every 3 weeks). The principal measure of neurotoxicity was the vibration perception threshold. After four cycles of chemotherapy, the mean (standard error) placebo group increased from 0.67 (0.12) to 1.61 (0.43) microns of skin displacement. In the high-dose treatment group, there was no increase in the threshold value after four cycles [from 0.54 (0.12) to 0.50 (0.06) microns]. After six cycles of chemotherapy, the threshold value was 5.87 (1.97) microns in the placebo group (a > eightfold increase from base line), as compared with 0.88 (0.17) microns (a < two-fold increase) in the high-dose group; fewer neurological signs and symptoms were recorded than in the placebo group. With the lower dose of the peptide, the effects were less prominent. No side effects were seen after treatment with ORG2766. The rates of clinical response to chemotherapy were the same in all three groups.[50] These results suggest that ORG2766 can prevent or attenuate cisplatin neuropathy in the human clinic.

CONCLUDING REMARKS

In this brief review, I have discussed some of the data, mainly obtained from our own group, that point to the potential of ACTH and MSH in the treatment of peripheral nerve disorders. One major drawback of these studies is that up until

now, there is no clear insight into the mechanism of action of these peptides or what neural cells are their primary target. It is hoped that the recent cloning of the melanocortin receptor family[51] will further our search for the molecular neurobiological mechanism of action. Many of the nervous system effects of peptides derived from ACTH and MSH are consistent with receptor-mediated effects as seen in the nonneural target cells of these peptides. With respect to the neurotrophic effects, preliminary evidence points to a signal transduction pathway involving cAMP stimulation and c-fos-expression.[52,53] It remains to be demonstrated whether the primary target *in vivo* is the neuron or the nonneuronal nerve elements. Hence the terms neurotrophic and neuroprotection were used instead of the more precise neuronotrophic.

If the peptides mimic or amplify an endogenous signal in the natural trophic or protective repertoire of the nerve, then it may be assumed that the peptides are of benefit under a variety of conditions that compromise nerve function. The data reviewed in this paper are in line with that notion; for ACTH- and MSH-like peptides are restoring function after various types of damage (mechanic trauma, neurointoxication by drugs or metabolic dysfunction). In fact, there are other examples not discussed in this paper, such as the efficacy of the peptide in animal models of pyridoxine or acrylamide neurotoxicity and of allergic neuritis.[54–56]

At first glance, it may seem unlikely that a given factor may show efficacy under such a variety of experimental conditions, for certainly the pathogenesis of these nerve traumas is different. At present we can only assume that there is (in part) a common adaptive response of the nerve that is triggered by a variety of compromising conditions, and as peptide treatment enhances such response, efficacy of peptide treatment of a variety of peripheral nerve disease can be expected.

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