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Beneficial effect of an $ACTH_{4-9}$ analogue on experimentally induced diabetic autonomic neuropathy in the eye of the rat under general anaesthesia

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Abstract

While peripheral polyneuropathy is a well-known complication in diabetes mellitus, and the subject of a great deal of study, the clinical importance of autonomic diabetic neuropathy is increasingly recognised. Using an animal model, where the pupil diameter of the eye serves as a parameter of autonomic function, we produced an age and weight curve of pupil diameter and studied the development of autonomic neuropathy in rats with streptozotocin-induced diabetes. We show that diabetic rats develop significantly ($P < 0.009$) smaller pupils compared with controls, most probably due to a defective sympathetic input, caused by sympathetic neuropathy. Treatment with the neurotrophic peptide Org 2766, a synthetic $ACTH_{4-9}$ analogue, prevents the occurrence of this sympathetic neuropathy, as the pupil diameters in the $ACTH_{4-9}$ analogue-treated group are significantly ($P < 0.05$) larger than the pupils of placebo-treated rats, and are comparable to the pupil diameters of the rats in the control group.

Keywords: Autonomic neuropathy; Diabetic neuropathy; Neuropeptide; $ACTH_{4-9}$ analogue

1. Introduction

The development of polyneuropathy is a common complication of diabetes mellitus. When peripheral nerves are affected, sensori-motor neuropathy is often the result [33]. The autonomic nervous system may also be affected, giving rise to autonomic neuropathy (sudden death, postural hypotension, resting tachycardia, impotence and delayed gastric emptying) [4,9,17]. Frequently, autonomic neuropathy is asymptomatic and can only be diagnosed with autonomic function tests. More and more attention has been drawn to the clinical importance of the possible presence of autonomic neuropathy [4,17]. While the regenerative capacity of somatic nerves has

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been the subject of intensive study, very little is known about the regenerative capacity of the autonomic nervous system.

In recent years it has been extensively documented that (1) melanocortins (peptides related to ACTH and MSH) improve peripheral nerve regeneration following mechanical damage to the rat sciatic nerve [15,29]; (2) prevent the neurotoxic side effects of cisplatin (an anti-tumour drug) in rats and humans [12,13]; (3) protect from or reverse a peripheral neuropathy in streptozotocin-induced diabetic rats [30] and (4) improve recovery from acrylamide-induced peripheral neuropathy in rats [24,25]. Most of the above-mentioned experiments have been performed on the rat sciatic nerve, a mixed sensory and motor nerve, using the peptide Org 2766, a synthetic $ACTH_{4-9}$ analogue [2,16,31].

In order to study the influence of neurotrophic peptides on autonomic neuropathies, we used a model where the pupil diameter of the eye in the rat serves as a parameter of autonomic function [10,26,27]. As the parasympathetically controlled sphincter system dominates the light reflex, the varying size of the normal pupil under different circumstances is primarily an expression of a varying tonic influence by the parasympathetic system, whilst the sympathetic system plays a supporting role [32].

The most frequently found abnormalities in human diabetics are reduced resting pupillary diameter, a delayed or absent reflex response to light, and diminished hippus [23]. These abnormal pupillary reflexes are thought to be caused by defective functioning of the sympathetic nerve supply, and have been reported as a late manifestation of diabetic neuropathy [11].

The aim of the present study was first to study the maturation of pupil diameter in newborn rats and to produce an age and weight curve (*experiment 1*). Secondly, we tried to document the development of autonomic neuropathy in the pupil of rats with streptozotocin-induced diabetes, expressed as the development of smaller pupils compared with normal controls (*experiment 2*). Thirdly, we studied whether treatment with an $ACTH_{4-9}$ analogue could prevent the development of this autonomic neuropathy (*experiment 3*). Fourthly, we examined whether the $ACTH_{4-9}$ analogue had any effect on the pupils of normal rats

2. Materials and methods

2.1. Experiment 1: pupil diameter maturation

Twenty newborn rats (19 male, 1 female) of an inbred Wistar strain (TNO, Zeist, The Netherlands), born on the same day in two litters, were used. As soon as the eyes opened, the pupils of the left eye were photographed, and thereafter once every 5–7 days until the age of 97 days. The first few weeks the animals were kept with their mother, until they were old enough to be separated into cages of four rats each.

2.2. Experiment 2: controls vs. diabetic rats

Male adult Wistar rats (age 13–14 weeks) weighing approx. 220 g at the onset of the experiment, were used. The animals were housed in Makrolon cages on sawdust and on a 12:12 hour light/dark cycle, with food and water ad libitum. The rats were randomised into two groups.

One group of animals ($n = 18$) received a single intravenous injection of streptozotocin (Zanosar^R, The Upjohn Company, Kalamazoo, MI, USA) in a dose of 50 mg/kg body weight (b.w.). After 1 week, blood glucose levels were determined by Haemo-Glukotest^R strips (Boehringer, Mannheim, Germany). Rats with a glucose level higher than 14 mmol/l were considered diabetic [6]. Diabetic rats received no insulin. A second group ($n = 10$) consisted of weight-matched non-diabetic rats and served as controls.

This experiment was repeated, yielding the same results and leading to a total group size of 36 and 19 rats, respectively.

2.3. Experiment 3: controls vs. untreated and peptide-treated diabetic rats

The rats were randomised into three groups. Two groups were made diabetic, as described

above. Again, diabetic rats received no insulin. One of these two diabetic groups ($n = 18$) was treated with the $ACTH_{4-9}$ analogue Org 2766 (Organon, Oss, The Netherlands). The peptide was administered subcutaneously in a dose of 10 μg/rat/48 h in 0.5 ml saline, beginning immediately following the injection of streptozotocin and lasting for the duration of the experiment. The other diabetic group ($n = 36$) was treated with subcutaneous saline only. A third group ($n = 19$) of weight-matched non-diabetic rats served as controls.

2.4. Experiment 4: synthetic $ACTH_{4-9}$ analogue in controls

Male adult Wistar rats were randomised into two groups. One group of animals ($n = 8$) received the $ACTH_{4-9}$ analogue in a subcutaneous dose of 10 μg/rat per 48 h in 0.5 ml saline. The second group ($n = 8$) consisted of weight-matched controls and received 0.5 ml saline only/rat per 48 h.

2.5. Pupil diameter measurements

All measurements were carried out under general anaesthesia (Hypnorm^R, Duphar, Weesp, The Netherlands, containing fluanisone 10 mg/ml and fentanyl citrate 0.315 mg/ml, dose 0.05 ml/rat, administered subcutaneously (*experiment 1*) or 0.1 ml/rat, administered intramuscularly (*experiments 2, 3 and 4*).

The rats were placed under an operating microscope (Zeiss OpMi-1; lens focal distance 200 mm; magnification ratio 16×). A 35 mm reflex camera (Canon EOS-650) with electronically-controlled automatic exposure was side-mounted to the microscope. The left eye of each rat was brought into focus in a plane parallel with the microscope lens, after which a supramaximal light stimulus was directed at that eye with an electronic flashlight. Using the camera self-timer, the flash was given after 8 s, and the shutter was released after 10 s, i.e., at the point of maximal pupillary constriction. Kodak Ektachrome 160T and 320T professional colour reversal film was used.

Prior to all measurements the focal distance of the microscope lens was tested by taking a photograph of calibrated millimeter paper. For *experiments 2 & 3* the baseline measurements were performed before the animals received their single dose of streptozotocin. Thereafter, all animals were photographed at 1- or 2-week intervals.

After developing the exposed films, all slides were mounted and projected onto a white screen. By first projecting the slide of calibrated millimeter paper, a magnification ratio could be calculated. Then the diameters of the contracted pupils were measured on the white screen and calculated using the magnification ratio. All slides were measured independently by two investigators on separate occasions (limits of agreement −0.06 to + 0.06 mm, [3]).

All measurements were carried out in a blind fashion. The codes, disclosing which animals were treated or untreated diabetics and which were controls, were broken only after analysis of all data had been performed.

2.6. Data analysis

All the pupil diameter measurements were analysed by an analysis of variance for repeated measurements (MANOVA), followed by post hoc tests (*t*-test for 2 groups; Student-Newman-Keuls Procedure for 3 groups), using the Statistical Package for the Social Sciences (SPSS) computer program. Data obtained from rats that died during the course of the experiment were excluded from statistical analysis. The cause of death was usually suffocation during anaesthesia for the pupil diameter measurements.

3. Results

3.1. Experiment 1: pupil diameter maturation

During the course of the experiment two rats died. At the age of 17 days the eyes opened in 3 rats, at the age of 18 days, in 10 more, and after 20 days in the remaining 5 rats. At the age of 18 days, the mean pupil diameter was 0.32 mm (SE 0.04) (average b.w. 26 g). The pupil size steadily

increased to 1.00 mm (SE 0.07) up till the age of 80 days (average b.w. 300–350 g), after which period a steady state settled in and the pupil diameter showed no further increase (Fig. 1a and b).

3.2. Experiment 2: controls vs. diabetic rats

Twelve rats in the untreated diabetic group and nine rats in the control group were used for final analysis. Streptozotocin-treated rats rapidly developed high blood glucose levels which, after 1 week, were 26–45 mmol/l as indicated by the Haemo-Glukotest^{R} strips.

At the onset of the experiment the mean diameter of the contracted pupil was 1.20 mm (SE 0.11) for the control group and 1.24 mm (SE 0.12) for the diabetic group (t-test, $t = 0.22$, df = 19, $P < 0.83$). Whilst the mean pupil diameter in the control group increased over a period of 3 weeks to 1.61 mm (SE 0.17), that of the diabetic group became significantly smaller: 1.00 mm (SE 0.07)(MANOVA for complete period $F_{1,19} = 8.41$, $P < 0.009$) (Fig. 2). This difference in pupil diameter was statistically significant already after 2 weeks (t-test, $t = 5.45$, df = 19, $P < 0.001$). After the instillation of phenylephrine 0.125% eyedrops the mean pupil diameter in the control group did not change significantly, whereas in the diabetic group the pupil diameter increased significantly by 40% (difference between means: 0.27 mm; 95% CI 0.16 to 0.38). These results imply the development of sympathetic neuropathy in diabetic rats, early in the course of diabetes mellitus.

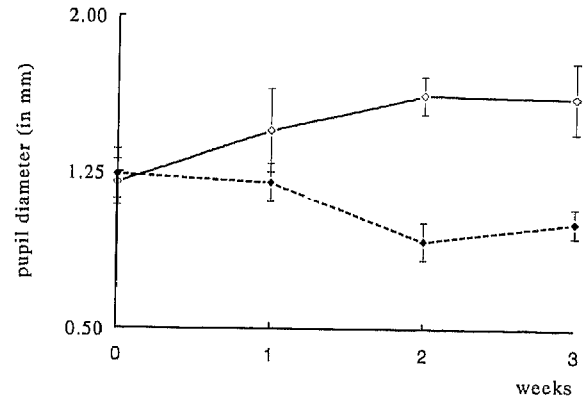
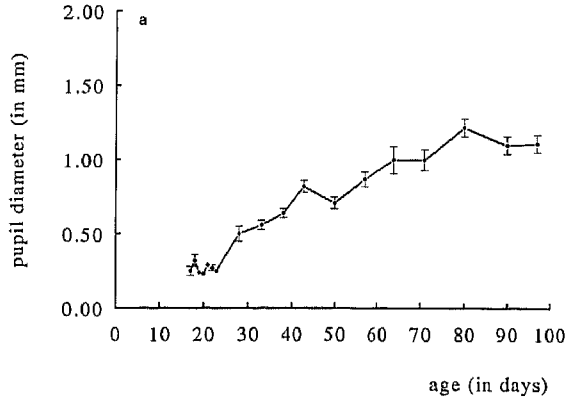


Fig. 2. *Experiment 2*: Development in time of pupil diameter (mean with SE) in weight-matched non-diabetic control rats (◇; $n = 9$, open diamonds), compared with streptozotocin-induced diabetic rats (◆; $n = 12$, filled diamonds), showing significantly smaller pupils in the diabetic rats ($P < 0.009$), implying the development of sympathetic neuropathy.

3.3. Experiment 3: controls vs. untreated and peptide-treated diabetic rats

Sixteen rats in the $ACTH_{4-9}$ analogue-treated diabetic group, 26 rats in the placebo-treated



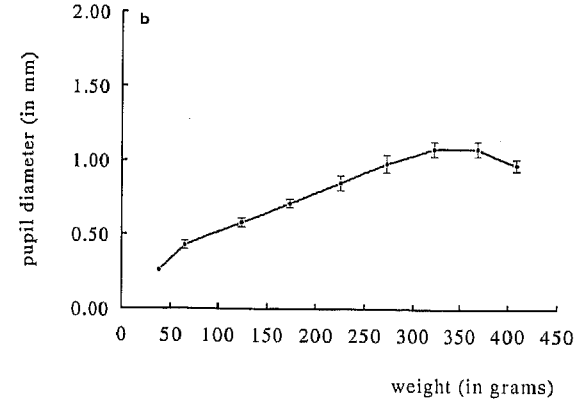


Fig. 1a. *Experiment 1*: Development of pupil diameter (mean with SE) in 18 newborn Wistar rats from the time they opened their eyes (approx. day 18), up till the age of 97 days. A gradual maturation can be seen up to approx. 80 days. Fig. 1b. *Experiment 1*: Pupil diameter compared to body weight in 18 newborn Wistar rats from the day they opened their eyes up till the age of 97 days. A gradual maturation can be seen up to approx. 300–350 g.

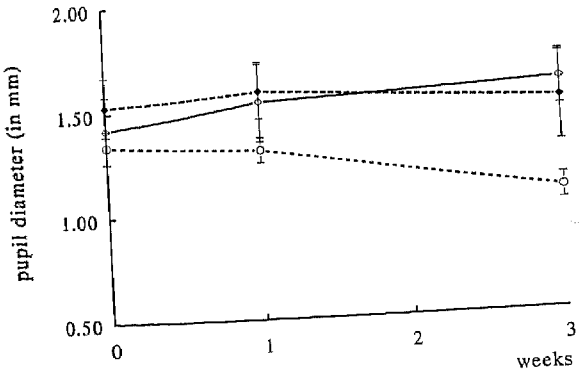


Fig. 3. *Experiment 3*: Development in time of pupil diameter (mean with SE) in weight-matched non-diabetic control rats (◇; $n = 17$, open diamonds), compared with placebo-treated streptozotocin-induced diabetic rats (○; $n = 26$, open circles) and the synthetic $ACTH_{4-9}$ analogue Org 2766-treated streptozotocin-induced diabetic rats (♦; $n = 16$, filled diamonds). The placebo-treated diabetic rats develop significantly smaller pupils ($P < 0.05$), implying the development of sympathetic neuropathy. Treatment with the synthetic $ACTH_{4-9}$ analogue prevents the occurrence of reduced pupil diameter in the treated diabetic group as they do not significantly differ from the control group ($P > 0.05$).

diabetic group and 17 rats in the control group were used for final analysis.

At the onset of this experiment the mean diameter of the contracted pupil was 1.42 mm (SE 0.16) for the control group, 1.34 mm (SE 0.08) for the placebo-treated diabetic group and 1.53 mm (SE 0.14) for the peptide-treated diabetic group (ANOVA, $F_{2,56} = 0.64$, $P < 0.53$). During the treatment period group differences developed (MANOVA for complete period, $F_{2,56} = 3.66$, $P < 0.032$). The mean pupil diameter in the control group increased over a period of 3 weeks to 1.60 mm (SE 0.13), whereas the pupil in the placebo-treated diabetic group became significantly smaller: 1.08 mm (SE 0.06) (ANOVA week 3, $F_{2,56} = 5.38$, $P < 0.0073$; Student-Newman-Keuls Procedure for controls vs. diabetics $P < 0.05$) (Fig. 3).

After 3 weeks the pupil diameter in the group that received the $ACTH_{4-9}$ analogue became only slightly smaller than the control group (1.51 vs. 1.60 mm, SE 0.21 and 0.13, respectively), but remained significantly larger than the placebo-treated diabetic group (1.51 vs. 1.08 mm, SE 0.21 and 0.06, respectively) (ANOVA week 3, $F_{2,56} = 5.38$, $P < 0.0073$; Student-Newman-Keuls Procedure for $ACTH_{4-9}$ analogue vs. placebo-treated diabetics $P < 0.05$, for Org 2766 vs. controls $P > 0.05$) (Fig. 3). These results imply that the $ACTH_{4-9}$ analogue Org 2766 prevents the occurrence of sympathetic neuropathy.

3.4. Experiment 4: synthetic $ACTH_{4-9}$ analogue in controls

Eight rats in the $ACTH_{4-9}$ analogue-treated group and seven rats in the placebo-treated group were used for final analysis.

At the onset of the experiment the mean diameter of the contracted pupil was 1.57 mm (SE 0.16) for the peptide-treated group, and 1.54 mm (SE 0.19) for the control group (*t*-test, $t = 0.11$, $df = 13$, $P < .91$). In the course of 6 weeks, there was a slight increase in mean pupil diameters in both groups, with no statistically significant difference (MANOVA for complete period, $F_{1,12} = 0.30$, $P < .59$) (Fig. 4). This result confirms the supposition that the $ACTH_{4-9}$ analogue alone has no effect on pupil diameter.

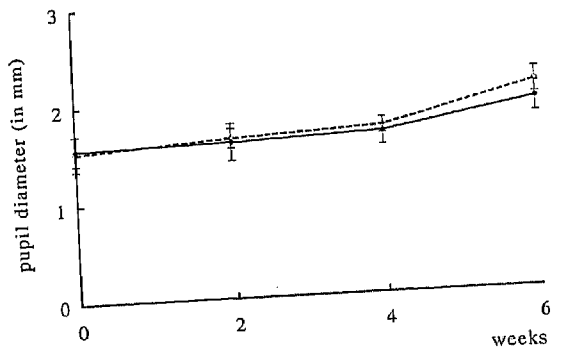


Fig. 4. *Experiment 4*: Development in time of pupil diameter (mean with SE) in weight-matched control rats (◇; $n = 7$, open diamonds), compared with $ACTH_{4-9}$-treated normal rats (♦; $n = 8$, filled diamonds). There is no influence of the neuropeptide on pupil diameter.

4. Discussion

Simply by photographing the rat's pupil under standardised conditions, the absolute pupil diameter can be used as a parameter of autonomic nervous system function [26,27]. The present study shows that rats with streptozotocin-induced diabetes develop significantly smaller pupils compared with controls. This smaller mean diameter of the contracted pupil reflects a dominant expression of parasympathetic tone in the diabetic group, most probably due to a defective sympathetic input. Using the concept of denervation hypersensitivity, instillation of phenylephrine 0.125% eyedrops, a direct acting sympathomimetic agent, has no effect in normal control rats, but leads to mydriasis in diabetic rats, proving that the smaller pupils in diabetic rats are indeed caused by sympathetic neuropathy.

Furthermore, treatment of streptozotocin-induced diabetic rats with a synthetic $ACTH_{4-9}$ analogue prevents the occurrence of sympathetic neuropathy, as the pupil diameters in the $ACTH_{4-9}$ analogue-treated group are significantly larger than the pupils of the placebo-treated rats, and are comparable to the pupil diameters of the rats in the control group. The administration of the $ACTH_{4-9}$ analogue to normal control rats has no effect on pupil size, as would be expected.

General anaesthesia partially blocks central sympathetic nervous system outflow. This will lead to small, miotic pupils in normal subjects. All measured pupil sizes were thus primarily a resultant of relatively uninhibited parasympathetic function. As the control group had significantly larger pupils, more sympathetic counter-action was present in this group. Lesser sympathetic counter-action in the diabetic group would explain the significantly smaller pupils, which can thus be regarded as the result of sympathetic neuropathy.

The continuously varying balance between sympathetic and parasympathetic activity ultimately determines the pupil diameter. The smaller pupils in diabetic rats are thus caused by either a diminished activity of the sympathetic nervous system or by an increased activity of the parasympathetic nervous system, or a combination of both. It can not be excluded that parasympathetic activity was increased in the diabetic rats leading to reduced pupil diameters. The possibility that the pupils of the diabetic rats are affected by the anaesthetic to a greater extent than the controls can not be the only explanation for the smaller pupils though, as the denervation hypersensitivity to phenylephrine shows the presence also of sympathetic neuropathy.

In our second experiment we found evidence of autonomic sympathetic neuropathy of the iris, occurring quite early in the course of the diabetes mellitus. Lanting [17] found that parasympathetic pupillary dysfunction precedes sympathetic dysfunction in diabetic autonomic neuropathy in humans. It can not be ruled out that parasympathetic neuropathy developed concurrently in our diabetic rats as our model does not differentiate between a sympathetic and a parasympathetic dysfunction. Yet we conclude that the dysfunction of the sympathetic innervation must have been more outspoken than any possible parasympathetic dysfunction as there was no increase in pupil diameter, pointing to a defective functioning of the parasympathetically controlled sphincter system.

The rats in our control group showed an initial, gradual increase of pupil diameter. This may be seen as the result of maturation through 'rat-adolescence'. We found a steady increase in pupil diameter from the day newborn rats open their eyes, up till the age of 80 days, after which period a steady-state settles in and the pupil diameter shows no further increase (Fig. 1a and b). This correlates well with the age curve of the pupil diameter in humans [18]. In infancy the pupil is small; during the first 6 months of life, the pupil begins to widen, and in adolescence it attains its widest diameter [32]. Whether this increase in size is related to an incompletely developed iris sympathetic system at birth, to growth of the anterior segment of the eye, or both, remains unclear.

All rats were anesthetised using Hypnorm^{R}, containing fentanyl, a morphine-like opioid agonist acting primarily at the μ receptors. Most μ (and κ) opioid agonists cause constriction of the

pupil due to an excitatory action on the autonomic segment of the nucleus of the oculomotor nerve (Edinger-Westphal)[14]. Some tolerance to this miotic effect develops however, and this could also explain the slight increase in pupil size that occurred in the control group. This tolerance to the agonistic action of fentanyl at the μ receptor sites probably also occurs in the diabetic rats. However, pupil size did not increase in this group. Again this can be explained by lesser sympathetic counter-action in the diabetic group as the result of sympathetic neuropathy.

In experimental diabetes mellitus there are alterations in autonomic cardiovascular function. These involve both the parasympathetic and sympathetic systems and appear to be time dependent [19]. The blood pressure response to intravenously administered tyramine and phenylephrine has been shown to be severely reduced in streptozotocin-induced diabetic rats, indicating an impaired function of the sympathetic system. Treatment of diabetic rats with the $ACTH_{4-9}$ analogue does not alter the vasopressor responsiveness to phenylephrine compared with untreated diabetic rats, whereas the vasopressor response to tyramine is restored to normal values [28]. This implies a protective action of the $ACTH_{4-9}$ analogue on the presynaptic sympathetic nerve fibres (response to tyramine), while the changes at the (vascular) postsynaptic α_1-receptor site are not affected (response to phenylephrine). Possibly, this protective action of the $ACTH_{4-9}$ analogue can also explain the prevention of sympathetic neuropathy in the iris of the rat, as indicated by our results.

The regenerative capacity of the peripheral nervous system is limited. Several humoral and structural factors of neuronal, glial or target cell origin, most of which are active during development, appear to facilitate nerve repair. The mechanism by which melanocortins (peptides derived from ACTH and MSH) exert their beneficial effect is still largely unknown. Studies demonstrating an increase at the damaged nerve site in peptides derived from proopiomelanocortin (POMC), the large precursor peptide from which ACTH and α-MSH derive, are in line with the idea of a physiological role of endogenous ACTH/MSH-like peptides in the process of nerve regeneration [1].

Two different working hypotheses have been put forward to explain the source and the nature of the naturally occurring endogenous melanocortins after nerve damage [8]. First, mature neurons might re-express proopiomelanocortin in their cell bodies after peripheral nerve damage. POMC mRNA is expressed in the developing spinal cord of rat embryos and is subsequently downregulated. However, no increase in the expression of POMC mRNA has been detected in dorsal root ganglia, spinal cord or in the damaged nerve after sciatic nerve crush [20]. Therefore, it is still not clear whether POMC mRNA expression in the nerve cell bodies and axons contribute to the physiological response leading to regeneration following injury.

The second hypothesis suggests that ACTH/MSH-like peptides are derived from the degenerating distal nerve stump [7,8]. Immunoblotting and immunohistochemistry have demonstrated epitopes shared by α-MSH and the 150-kDa neurofilament protein (NF 150). Since after injury NF150 is rapidly degraded it was suggested that breakdown of NF150 results in the formation of an α-MSH-like peptide. Current research is aimed to further characterise that peptide.

The work on the mechanism of action of melanocortins has been hampered by the fact that no receptor for the $ACTH_{4-9}$ analogue has been identified, as yet [21]. Identification of this receptor for melanocortins involved in the neurotrophic action of these peptides will have a significant impact on advances in understanding the molecular mechanisms underlying the stimulatory effect of ACTH/MSH-like peptides on nerve regeneration.

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