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Pharmacological Evidence for the Involvement of Endogenous α-MSH-Like Peptides in Peripheral Nerve Regeneration

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PLANTINGA, L. C., J. VERHAAGEN, P. M. EDWARDS, M. HALI, J. H. BRAKKEE AND W. H. GISPEN. *Pharmacological evidence for the involvement of endogenous α-MSH-like peptides in peripheral nerve regeneration.* PEPTIDES **16**(2) 319–324, 1995.—The possible involvement of α-MSH-like peptides in the regenerative response of peripheral nerves was investigated with a competitive antagonist of α-MSH, the synthetic hexapeptide [D-Trp^7,Ala^8,D-Phe^{10})α-MSH(6–11)-amide. Subcutaneous administration of the α-MSH antagonist during the first 10 days following sciatic nerve crush significantly decreased functional recovery as measured by the foot flick withdrawal test and the walking pattern analysis. Hypophysectomy delayed both the initial sprouting response and the outgrowth rate after major caudal nerve crush. When hypophysectomized rats were treated with the α-MSH antagonist, a further delay in initial sprouting was observed, whereas the outgrowth rate of nerve fibers was not affected. These results suggest that 1) endogenous α-MSH-like peptides stimulate nerve outgrowth following peripheral nerve injury and 2) α-MSH-like peptides derived from a source other than the pituitary may contribute to the physiological stimulus leading to sprouting.

α-MSH Melanocortins Regeneration Trophic action

ACTH- and α-MSH-like peptides, also refered to as melanocortins, exert a trophic effect on the peripheral nervous system of the rat. The trophic influence is apparent both during development and regeneration [reviewed by (1,21,29)]. During development, melanocortins improve muscle function (20,27) and promote synaptogenesis at the neuromuscular junction (14,22). In the adult rat, fragments and analogues of ACTH/α-MSH-like peptides stimulate peripheral nerve regeneration (2,4,9,32).

ACTH/α-MSH-like peptides are believed to enhance nerve regeneration by stimulating the initial process of sprout formation rather than affecting the growth speed of newly formed sprouts (3,29,31). Studies on reinnervation after major caudal nerve crush demonstrated a beneficial effect of melanocortins on sprout initiation. Treatment with the ACTH(4–9) analogue Org 2766 decreased the time necessary to reinnervate the tail by 2–3 days, but did not affect the outgrowth rate (15). In addition, an immunocytochemical study demonstrated that Org 2766 increased the number of regenerating sprouts within 48 h after nerve injury (33).

Melanocortins only stimulate nerve regeneration when administered during the first 8 days following nerve crush (13). If treatment is delayed until the second week after injury, no effect of melanocortins is observed. This critical treatment period also suggests an effect of melanocortins on processes that are set in motion early following injury, such as sprout formation.

The ability of exogenous melanocortins to stimulate peripheral nerve repair may reflect possible involvement of similar endogenous peptides in the physiological regenerative response following nerve injury. Endogenous melanocortins are predominantly synthesized in the pituitary gland from the prohormone proopiomelanocortin (POMC) by proteolytic cleavage. It has been suggested that α-MSH/ACTH-like peptides are also formed in traumatized nerves in response to nerve injury as one of a cocktail of neurotrophic substances synthesized. Using a bioassay for α-MSH, the presence of biologically active α-MSH-like peptides has been demonstrated in extracts of degenerating nerves, whereas no activity was detected in extracts of control nerves (13). Others have demonstrated increased α-MSH- and β-endorphin-like immunoreactivity in the transected sciatic nerve compared to the intact control nerve (23).

Two different hypotheses regarding the formation of α-MSH/ACTH-like peptides in traumatized nerves have been proposed. α-MSH-like peptides may be formed in the degenerating distal nerve stump by specific proteolysis of the 150-kDa neurofilament

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protein (13), or may derive from reexpression of POMC mRNA in the cell bodies of the damaged neurons (25).

In this study, the role of endogenous α-MSH-like peptides in peripheral nerve regeneration was investigated. To this end, a selective antagonist of α-MSH, the synthetic hexapeptide [D-Trp^7,Ala^8,D-Phe^{10}]α-MSH(6–11)-amide (11,26) was used to block the activity of endogenous α-MSH-like peptides. First, the role of melanocortins, irrespective of their origin, was investigated after sciatic nerve injury. Second, the effect of melanocortins secreted by the pituitary was studied by removing the pituitary. Third, the putative effect of melanocortins derived from a source other than the pituitary on nerve regeneration was investigated with the α-MSH antagonist in hypophysectomized rats.

To estimate the efficacy of the α-MSH antagonist in our animal models, the ability of this compound to counteract excessive grooming behavior was tested. Grooming behavior is a characteristic movement pattern observed in rodents that is displayed in situations in which novel or conflicting environmental stimuli are presented (28). Excessive grooming in rats can be induced by intraventricular administration of α-MSH and can thus serve as a bioassay to measure α-MSH activity (17).

METHOD

Animals and Surgery

Male rats of an inbred Wistar strain (TNO, Zeist, The Netherlands) weighing approximately 120–140 g were used for sciatic nerve or major caudal nerve crush experiments. Rats were anesthetized using Hypnorm (Duphar, Weesp, The Netherlands) at a dose of 0.08 ml/kg body weight. Crush lesioning of the right sciatic nerve was performed as described previously (9). The major caudal nerve crush was performed essentially as described by Gerritsen van der Hoop et al. (15). A 2-cm-long incision in the ventral and dorsal site of the tail was made 1.5 cm distal to the base of the tail. Both minor caudal nerves located in the dorsal part of the nerve were transected and a part of at least 0.5 cm was removed. After exposing the major caudal nerves by making an incision in the middle of the ventral tendon sheet, both major caudal nerves were crushed for 30 s. An ink line was drawn on the skin to indicate the precise crush position. The wound was closed by gluing the wound sites with cyano acrylate ester (Deltafix). Hypophysectomy was performed under light anesthesia by the transauricular route (16). The efficacy of the surgery was monitored by body weight and plasma concentration of corticosterone. Animals that increased more than 10% in body weight during the experiments were excluded. Corticosterone levels in hypophysectomized animals were less than 0.5 μg/100 ml plasma, compared to 10.5 μg/100 ml plasma in sham-operated rats.

Peptides

α-MSH was synthesized and donated by Organon International B.V. (Oss, The Netherlands). α-MSH antagonist [D-Trp^7,Ala^8,D-Phe^{10}]α-MSH(6–11)-amide was purchased from Bachem (Bubendorf, Switzerland). For grooming experiments, α-MSH (0.3 μg/3 μl) and α-MSH antagonist (0.75 μg/3 μl) were freshly dissolved in saline. For sciatic or the major caudal nerve crush experiments, stock concentrations of α-MSH antagonist were prepared, aliquoted per injection and kept frozen at −20°C until use. Animals were injected subcutaneously with α-MSH antagonist (25 μg/0.5 ml/rat) or with saline at 0900, 1700, and 2400 h for 10 days, with the first injection immediately following sciatic or major caudal nerve crush operation.

Functional Recovery of Sciatic Nerve

The return of the sensorimotor function was measured by the foot reflex withdrawal test, described previously (9). In short, the rat was immobilized and two stimulation poles were placed at different points on the skin of the foot sole. Different current strengths ranging from 0.1 to 0.6 mA were applied to the foot sole and the lowest current at which the rat retracted its paw from the stimulus was registered. No retraction from a 0.6-mA current stimulus indicates 0% recovery, whereas retraction of the paw from a stimulation of 0.1 mA is considered as 100% recovery.

Recovery of motor function was assessed by means of an analysis of walking patterns (12). The hind feet of rats were dipped in photographic developer. Subsequently rats walked over photographic paper placed on the bottom of a long confined walkway. From the footprints of the rat the sciatic functional index (SFI) was calculated (10).

Functional Recovery of the Major Caudal Nerve

Return of sensory function was measured by placing a cathode at the end of the tail, and touching the tail with an anode at fixed intervals of 1 cm from the marked lesion site (15). The rat was immobilized and the tail was put against a board with an inclination of about 60° on which the testing points were indicated. The most proximal testing point was at 2 cm distal to the crush site, the most distal testing point at 6 cm. Upon touching the tail with the anode, an instant stimulus of 0.5 mA is presented to the rat. If the regenerating axons have reached the point on the tail where the anode is placed, sensation of the noxious stimulus is indicated by vocalization. Assessment was performed by proceeding from the most distal testing point and progressing proximally until a positive response was obtained. Rats were tested daily between 0900 and 1100 h.

Grooming Behavior

Male Wistar rats, weighing between 130–140 g, were housed individually in makrolon cages. Two to three days prior to peptide treatment, a cannula was implanted into the ventricle of the rat brains (5). α-MSH (0.3 μg/3 μl), the α-MSH antagonist (0.75 μg/3 μl), saline, or a combination of saline followed by an injection of α-MSH or a combination of the α-MSH antagonist followed by α-MSH was administered by means of an intracerebroventricular (ICV) injection. Following injection, the cages with the animals were placed on shelves in a cabinet containing a mirror at the back, allowing close observation of the animals from every angle. Fifteen minutes after administration of the last injection grooming behavior was recorded every 15 s for a period of 55 min by an independent observer (17). After each experiment, the position of each cannula was checked by ICV injection of methylene blue followed by dissection of the brain.

Statistics

The data were analyzed using different statistical methods as described in the figure legends. In all experiments, the investigator was not aware of the nature of the treatment given. Upon completion of the data analysis the treatment code was broken.

RESULTS

The α-MSH Antagonist Reduces α-MSH-Induced Grooming Behavior

The synthetic hexapeptide [D-Trp^7,Ala^8,D-Phe^{10}]α-MSH(6–11)-amide has been recognized as a competitive anatagonist of α-MSH activity in the frog skin bioassay (11,26). To characterize

the α-MSH antagonistic activity in one of our rat animal models, the inhibitory effect of the α-MSH antagonist on α-MSH-induced excessive grooming behavior was measured (17). Intracerebroventricular administration of α-MSH is known to induce excessive grooming in rats and can be used as a bioassay to measure α-MSH bioactivity.

The ICV injection of α-MSH (0.3 μg/3 μl) induced excessive grooming as reported previously (Fig. 1). Injection of the α-MSH antagonist (0.75 μg/3 μl) or saline was without effect. ICV injection of the α-MSH antagonist followed by an injection of α-MSH significantly reduced α-MSH-induced grooming. When α-MSH was injected ICV prior to the antagonist, similar results were obtained (data not shown).

In all nerve crush experiments, the α-MSH antagonist is administered subcutaneously. The efficacy of the α-MSH antagonist to counteract grooming when administered subcutaneously is shown in Fig. 2. Subcutaneous injection of the α-MSH antagonist (25 μg/0.5 ml) followed immediately by ICV administration of α-MSH inhibited the grooming response significantly (Fig. 2). Although the effect was most profound when grooming was induced directly after administration of the antagonist, a significant reduction in grooming behavior was still observed when grooming behavior was induced 2 or 4 h after administration of the α-MSH antagonist. No reduction in grooming was observed when the α-MSH antagonist was administered 8 h in advance.

Effect of α-MSH Antagonist on Functional Recovery After Sciatic Nerve Crush

After sciatic nerve crush, rats were injected subcutaneously with the α-MSH antagonist (25 μg/0.5 ml/rat) or saline three times a day for 10 days, the first injection administered immediately following the operation. The α-MSH antagonist significantly delayed functional recovery as measured by the foot flick withdrawal test and by walking pattern analysis (Fig. 3).

In this experiment the α-MSH antagonist blocks the activity of melanocortins on functional recovery irrespective of the source of the melanocortins. To study the activity of melanocortins produced at sites other than the pituitary, pituitary-derived melanocortins were removed by hypophysectomy.

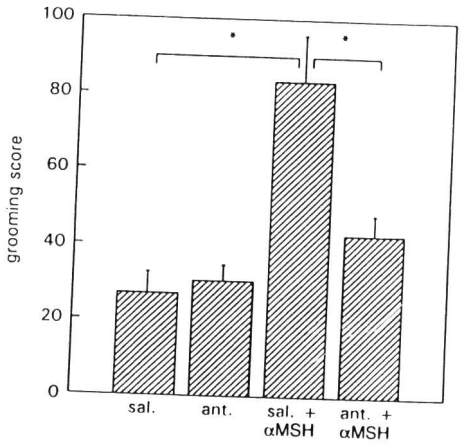

FIG. 1. The effect of the α-MSH antagonist on α-MSH-induced grooming behavior. Rats were injected ICV with saline (sal), the α-MSH antagonist (0.75 μg/3 μl) (ant), saline (0.3 μl) followed by α-MSH (0.3 μg/3 μl) (sal + α-MSH), or the α-MSH antagonist followed by an injection of α-MSH after 5 min (ant + α-MSH). Every group consisted of $n = 9$. Values are given as means ± SEM. Administration of α-MSH significantly induced grooming behavior compared to saline-treated rats, $*t(13) = 3.15$, $p < 0.005$ (Student's t-test). When the α-MSH antagonist was administered prior to α-MSH, the mean grooming score was significantly reduced, $*t(18) = 3.02$, $p < 0.005$ (Student's t-test).

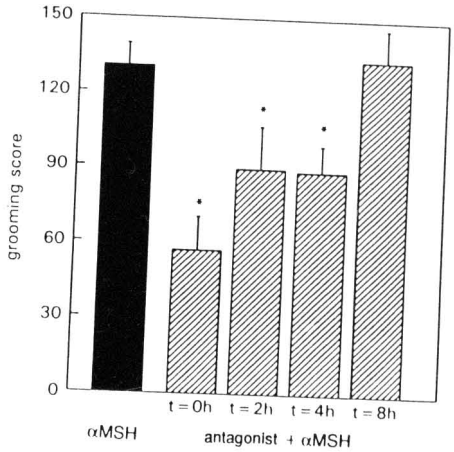

FIG. 2. Efficacy of the SC-administered α-MSH antagonist to reduce grooming behavior when grooming behavior is induced immediately or several hours after injection of the α-MSH antagonist. Control animals were injected SC with saline followed by ICV injection of α-MSH (0.3 μg) (α-MSH, black bar). In the experimental groups (hatched bars), α-MSH was administered ICV immediately ($t = 0$ h), 2 h ($t = 2$ h), 4 h ($t = 4$ h), or 8 h ($t = 8$ h) after SC administration of the α-MSH antagonist (25 μg/0.5 ml). Every group consisted of $n = 9$. Values are given as means ± SEM. A significant reduction in grooming score was obtained when the α-MSH antagonist was administered 0, 2, or 4 h prior to α-MSH (*significantly different from controls at $p < 0.05$, SKN-test).

Effect of Hypophysectomy on Functional Recovery After Sciatic or Major Caudal Nerve Crush

Seven days after hypophysectomy or sham operation, the right sciatic nerve was subjected to crush lesioning. Subsequently, the return of nerve function was monitored with the foot reflex withdrawal test and walking pattern analysis. Our results demonstrated that hypophysectomy did not affect functional recovery after sciatic nerve crush (data not shown). However, the knowledge that hypophysectomized rats do not gain weight in contrast to sham-hypophysectomized rats prompted us to measure the length of the sciatic nerve of both groups. We observed that during the experiment a difference in length of the sciatic nerve of 14.5% had developed [sciatic nerve length of sham-operated rats was 47.8 ± 0.7 mm ($n = 11$) vs. hypophysectomized rats 40.8 ± 0.3 mm ($n = 13$)]. Because differences in sciatic nerve length affects the time necessary to reinnervate the paw, we concluded that the foot flick withdrawal test and the walking pattern analysis are not adequate methods to compare differences in return of function between these two groups. Therefore, the effect of hypophysectomy on reinnervation was investigated using the major caudal nerve crush. In the major caudal nerve crush model, the outgrowth of nerve sprouts is measured at fixed distances from the crush site and thus independent of target reinnervation. Seven days after hypophysectomy or sham operation, the major caudal nerves were crushed. The effect of hypophysectomy on fiber outgrowth after a major caudal nerve crush is shown in Fig. 4. The graph demonstrates that the initiation of outgrowth is significantly retarded in hypophysectomized rats compared to sham-operated animals. A positive response monitored at the 2-cm testing point was registered 2.4 days later in hypophysectomized compared to sham-hypophysectomized animals. The difference in outgrowth increased in time because a positive vocalization

response at the 5-cm testing point was measured at day 25 after caudal nerve crush in hypophysectomized and at day 19.5 in sham-operated rats, a delay in outgrowth rate of 5.5 days.

Effect of α-MSH Antagonist on Functional Recovery After Major Caudal Nerve Crush in Hypophysectomized Rats

In hypophysectomized rats, the major source of endogenous circulating melanocortins is removed. If the α-MSH antagonist is still effective in reducing the regeneration in hypophysectomized rats, this might reflect a neurotrophic action of melanocortins synthesized at a site other than the pituitary. The effect of treatment with the α-MSH antagonist in hypophysectomized rats was investigated after major caudal nerve crush. Seven days after hypophysectomy, the major caudal nerves were subjected to crush lesioning. The animals were treated subcutaneously with the α-MSH antagonist (25 μg/0.5 ml/rat) or with saline three times a day for 10 days, with the first injection administered directly after nerve injury. Treatment with the α-MSH antagonist significantly retarded the initiation of outgrowth in hypophysectomized rats. A positive response at the 2-cm testing point in α-MSH antagonist-treated animals was obtained at day 13.5 postinjury, whereas saline-treated hypophysectomized rats responded at day 9.5 (Fig. 5). No significant difference was observed at other testing points. This indicates that the initial sprouting response was delayed in hypophysectomized rats treated with the α-MSH-antagonist, whereas the elongation rate of the sprouts was not affected.

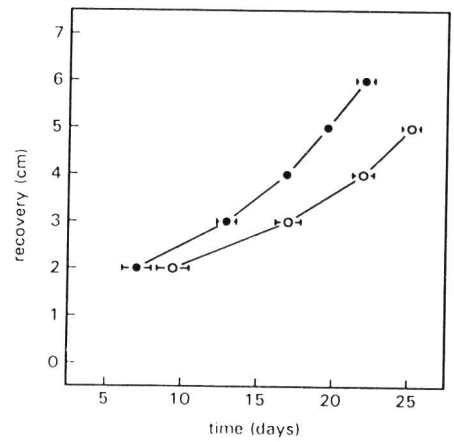

FIG. 4. Recovery of sensory nerve function after major caudal nerve crush in sham-hypophysectomized (●, $n = 12$) and hypophysectomized rats (○, $n = 12$). Values are given as mean number of days ± SEM necessary for the recovery of the sensory function at the various testing points. Hypophysectomy significantly retarded the return of sensory function compared to sham-operated animals [2–5 cm: MANOVA, $F(1, 18) = 24.80$, $p < 0.001$].

DISCUSSION

It has been suggested that exogenous administration of MSH/ACTH-like peptide fragments stimulates peripheral nerve regeneration by mimicking or amplifying endogenous melanocortin-like peptides. The present study was performed to examine the putative neurotrophic effect of endogenous melanocortins on peripheral nerve regeneration in the rat.

To assess the role of endogenous melanocortins in peripheral nerve regeneration, a competitive α-MSH antagonist was used. The α-MSH antagonist has been characterized in the frog skin bioassay and is a potent antagonist of α-MSH (11,26). We characterized the antagonistic activity of this compound in α-MSH-induced grooming behavior and demonstrated that SC injection resulted in a significant reduction of grooming behavior. The antagonist was still active 4 h after administration.

Because pharmacologically administered α-MSH/ACTH peptide fragments exert their beneficial action within a short critical period immediately following nerve injury, the α-MSH antagonist was administered only during the first 10 days in all nerve

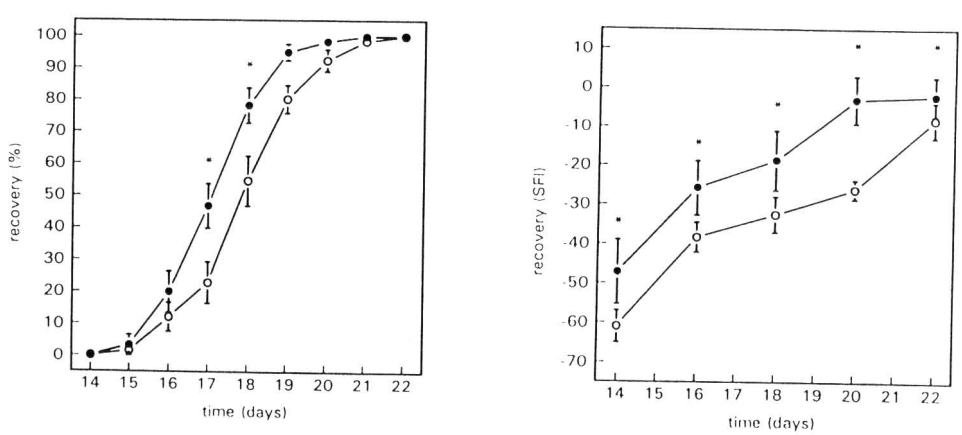

FIG. 3. Recovery of function following sciatic nerve crush in rats treated with the α-MSH antagonist. Rats subjected to sciatic nerve crush were treated three times a day with the α-MSH antagonist(25 μg/0.5 ml/rat) during the first 10 days after nerve lesion (○, $n = 12$) or with saline (●, $n = 12$). The return of sensorimotor function was determined with the foot flick withdrawal test after foot sole stimulation with a range of six current strengths of 0.1–0.6 mA (A). Values are given as mean percentage (%) recovery per day ± SEM. The return of the motor function was determined with the walking pattern analysis (B). Values are given as the mean index of sciatic nerve function (SFI) ± SEM. In the foot flick withdrawal test a significant difference between the α-MSH antagonist- and the saline-treated groups is observed at day 18 (Mann–Whitney U-test, $U = 25.5$, $p < 0.05$) and day 19 (Mann–Witney U-test, $U = 22$, $p < 0.05$). In the walking pattern analysis, both groups differ significantly from day 14 to day 22 [ANOVAR, $F(1, 19) = 10.93$, $p < 0.05$].

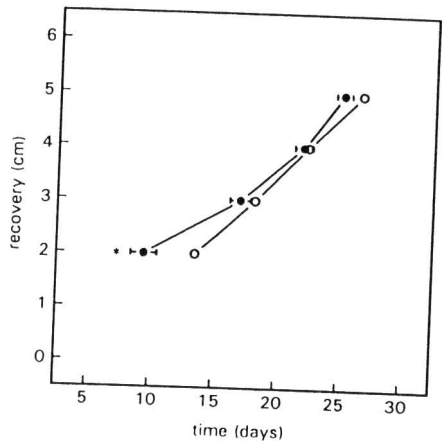

FIG. 5. Recovery of sensory nerve function after caudal nerve crush in hypophysectomized rats treated with the α-MSH antagonist (25 μg/0.5 ml/rat) (●, $n = 12$) or saline three times a day during the first 10 days after nerve injury (○, $n = 12$). Values are given as mean number of days ± SEM necessary for the recovery of the sensory function at the various testing points. A significant delay in return of sensory function is observed at the 2-cm testing point in the α-MSH antagonist-treated group compared to the saline-treated group [2 cm: $t(22) = 3.35$, $p < 0.005$, Student's t-test].

crush experiments (13). Treatment with the α-MSH antagonist significantly impaired recovery after sciatic nerve crush as assessed by the foot flick test and walking pattern analysis. This indicates that endogenous melanocortins exert effects similar to therapeutically administered peptides. The antagonistic effect of the α-MSH antagonist is evident despite the fact that the antagonist is known to stimulate growth hormone, which would be predicted to enhance regeneration (6,18,34,19).

The effect of hypophysectomy on nerve regeneration was investigated in the major caudal nerve crush model. The caudal nerve crush model enables us to measure both the initial sprouting response and the outgrowth rate following injury. Regeneration after major caudal nerve crush was severely impaired in hypophysectomized rats. A delay in the initial sprouting response was observed, implying that peptides secreted by the pituitary are involved in this process. This is in line with the notion that α-MSH/ACTH-related peptides facilitate the formation of sprouts following damage (33). The rate of outgrowth was also reduced in the hypophysectomized rats. A similar reduction in elongation rate has been demonstrated by others using the pinch test (24). Because exogenously administered melanocortins do not affect outgrowth rate (7), this effect may be mediated by pituitary hormones other that melanocortins. Hypophysectomy affects serum levels of a number of hormones, including those that have been claimed to affect nerve regeneration such as insulin growth factor 1 (IGF-1) and T4 (8,19). Thus, the decrease in regeneration of the caudal nerve in hypophysectomized rats may be the result of the removal of a number of growth-stimulating hormones, and caution in the interpretation of the effects of removal of the pituitary is necessary.

When nerve recovery after sciatic nerve crush was measured in hypophysectomized animals with the foot flick response or walking pattern, hypophysectomy did not seem to alter the recovery (30). We found that differences in sciatic nerve lengths develop between the hypophysectomized and control rats over the period of the experiment. Thus, the period of recovery after sciatic nerve crush is confounded with differences in the length of outgrowth necessary to achieve return of function.

In the last experiment, we investigated whether administration of the α-MSH antagonist in hypophysectomized animals did result in a further delay of nerve recovery. Because hypophysectomized animals are devoid of pituitary derived melanocortin, the α-MSH antagonist can only inhibit melanocortins produced at a site other than the pituitary. Administration of the α-MSH antagonist resulted in a further delay in initial sprouting response in hypophysectomized rats after caudal nerve crush. No additional effect of treatment on the rate of outgrowth was detected. Therefore, the present data underscore the hypothesis that nonpituitary-derived melanocortins are involved in the initial sprouting response following crush injury. Because immunoreactivity for ACTH/MSH-like peptides has been demonstrated after nerve transection (23), we believe that melanocortins produced in the traumatized nerve are involved in the physiological response that mediate neurite outgrowth following peripheral nerve damage.

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