

The Neurotrophic Analogue of ACTH(4-9) Reduces the Perineuronal Microglial Reaction After Rat Facial Nerve Crush

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ABSTRACT Following peripheral nerve crush, microglial cells proliferate and migrate to motoneuron cell bodies of the injured nerves. Newly formed glial processes displace nerve terminals from the cell bodies. This process is known as synaptic stripping. In animal models of peripheral nerve diseases, the ACTH(4-9) analogue, ORG2766, was shown to facilitate axonal regeneration and to protect against experimental neuropathy. In the present study we examined the effect of ORG2766 on the microglial reaction. After facial nerve crush, rats were treated with either ORG2766 (75 µg/kg/48 h) or saline and were killed on day 2–6 after operation. Blind counting of the number of perineuronal glial cells in the facial nucleus was used to assess the effect of ORG2766 treatment on the microglial reaction. In the saline-treated animals the number of perineuronal glial cells per motoneuron cell body on the crushed side increased significantly. This number increased up to day 5 after operation and decreased significantly from day 5 to 6. After an initial increase in the peptide-treated animals, however, the number of perineuronal glial cells remained constant from day 3 onwards. Hence, on post-operation days 4 and 5, this number was significantly less than that seen in saline-treated animals. Microglial cells proliferate, presumably through signalling by injured motoneurons. It is suggested that the decrease in the number of perineuronal glial cells in the ORG2766-treated animals is the result of a peptide-induced reduction in the release of mediating signals/cytokines or, alternatively, increased protection of motoneurons by stress proteins. Further research should address the mechanism of action of ORG2766 in animal models of motoneuron disease. © 1993 Wiley-Liss, Inc.

INTRODUCTION

Peripheral nerve injury results in a series of structural and cytochemical changes. Distal to the nerve injury these events are briefly referred to as Wallerian degeneration (Seckel, 1990). Proximal to the lesion site, in and around neuronal cell bodies of the injured nerves, the characteristic succession of events is collectively referred to as the retrograde reaction (Kreutzberg et al., 1988; Selzer, 1980).

Following a sublethal peripheral facial nerve injury, resting microglial cells in the facial nucleus become activated, but not phagocytotic, i.e., they proliferate and

migrate to the affected neurons: perineuronal satellitosis (Streit and Kreutzberg, 1988). Astrocytes and oligodendrocytes do not show any increase in mitotic activity (Graeber et al., 1988; Kreutzberg, 1966). However, the

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Abbreviations: ACTH, adrenocorticotrophic hormone; ANOVA, analysis of variance; CGRP, calcitonin gene-related peptide; CNS, central nervous system; NMDA, N-methyl-D-aspartate; SEM, standard error of mean; SNK, Student Newman Keuls.

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resident astrocytes become hypertrophic, while glial filaments and astrocytic processes increase (Graeber and Kreutzberg, 1986; Tetzlaff et al., 1988). Blinzinger and Kreutzberg (1968) demonstrated in the rat that pseudopods of perineuronal and peridendritic microglial cells intrude into the synaptic clefts and simply push aside the morphologically intact afferent synaptic nerve terminals from the motoneuron perikaryon and its main dendrites. This process is called synaptic stripping or deafferentation. Microglial cells migrate again to locations within the neuropil from 1 week after nerve lesioning (Blinzinger and Kreutzberg, 1968; Kreutzberg, 1968). Their place on the motoneuron cell body is taken by processes of reactive, mostly fibrous, astrocytes. These astrocytes maintain the synaptic terminal isolation of the motoneurons over a longer period (Graeber and Kreutzberg, 1988; Kreutzberg et al., 1989; Streit and Kreutzberg, 1988). It seems likely that species differences (cat, rabbit, mouse, and rat), differences in ages (adult, newborn), areas, and lesions (crush and transection) may exist with regard to the glial reaction after axon lesion as discussed by Torvik and Skörten (1971) and by Torvik and Söreide (1972). However, when motoneurons are lethally injured and degenerate, the microglial cells change morphologically and transform into phagocytotic amoeboid brain macrophages (Streit et al., 1988; Streit and Kreutzberg, 1988).

During the last decade, several effects of melanocortins on peripheral nerve regeneration have been reported (Bär et al., 1990). Treatment with the peptide ORG2766, a stable ACTH(4-9) analogue, was shown to facilitate i) axonal regeneration following sciatic nerve crush in healthy rats (de Koning and Gispen, 1987), and ii) collateral sprouting in conditions of partial denervation (de Koning et al., 1989). The peptide decreases degeneration and prevents neurotoxicity induced by cisplatin (Mollman, 1990) both in rats (de Koning et al., 1987; Gerritsen van der Hoop et al., 1988) and in humans (Gerritsen van der Hoop et al., 1990). The mechanism underlying these effects is still unknown. Effects of ACTH derivatives on B-50/GAP-43 expression or tubulin/actin synthesis have been searched for, but so far to no avail (Edwards et al., 1985; van der Zee et al., 1989).

In the present study we investigated whether the peptide, ORG2766, affected the microglial reaction following peripheral rat facial nerve crush.

MATERIALS AND METHODS

Peptide and Treatment

ORG2766, a degradation resistant ACTH(4-9)-analogue, (H-Met(O₂)-Glu-His-Phe-D-Lys-Phe-OH), was a gift from Organon International B.V. (Oss, The Netherlands). It was dissolved in 0.9% NaCl (saline) and stored at -75°C in a concentration of 160 µg/50 µl. The peptide was administered subcutaneously in the neck region in a dosage of 75 µg/kg/48 h in 0.5 ml saline, beginning immediately after the operation. The control animals received 0.5 ml saline at the same time points.

Animal Treatment and Surgery

Female Wistar rats (120–140 g) were obtained from HSD-E (Zeist, The Netherlands). Both the saline- and peptide-treated groups consisted of 25 animals. The rats were housed randomly, five in a cage at 21–23°C and 60–70% relative humidity. They were fed Hopefarm pellet food and water ad libitum. Rats (n = 50) were deeply anaesthetized with Hypnorm™, containing 10 mg flunixin and 0.315 mg fentanyl citrate per ml, obtained from Janssen Pharmaceutica B.V. (Tilburg, The Netherlands). The right facial nerve was crushed 3 mm distal from the stylomastoid foramen, for 30 s, by closing haemostatic forceps with grooved jaws three clicks (de Koning et al., 1986; Kreutzberg et al., 1988). On the basis of preliminary experiments and the literature (Kreutzberg, 1966; Kreutzberg, 1968), we expected the greatest difference between the peptide- and saline-treated animals to occur at the time points of the greatest proliferation of the glial cells, namely, about day 4 after surgery. Therefore, we decided to take eight animals on days 4 and 5, and three animals on days 2, 3, and 6.

Animals from the ORG2766-treated group and the saline-treated group were killed daily from day 2 up to day 6 after surgery: under deep Nembutal® anaesthesia, these rats were heparinized and perfused transcardially with 250 ml fixative warmed to 37°C and consisting of 2% paraformaldehyde, 0.1 M lysine, and 0.2% periodate in 50 mM phosphate buffer at pH 7.4 (McLean and Nakane, 1974). The brainstem, containing the facial nuclei, was dissected, postfixed for 1 h in the same fixative, and incubated in 0.05 M phosphate-buffered solutions of increasing sucrose gradient (7½%, 15%, and 25%, pH 7.3, 3 h each). After cryoprotection, the tissue was quickly frozen in liquid N₂-cooled *iso*-pentane and stored at -75°C.

Light Microscopy

The facial nucleus was cut away approximately 150 µm from the cranial site. Then, brainstem cryostat sections 8 µm in thickness containing the right and left facial nuclei were cut. Every third section was fixed on a 0.5% formol-gelatin coated cover glass until five sections per animal had been obtained. Brain tissue lipid was removed by de- and re-hydrating the sections in *iso*-propanol. The sections were stained for 10 min in a haematoxylin solution (Papanicolaous solution 1b, Merck) with the addition of 4% HAc. After rinsing for 10 min under a constant flow of tapwater, the sections were shortly incubated in a 1% NH₃ solution and again rinsed for 10 min. The sections were dehydrated and mounted in DePeX. Finally, the sections were randomized, blinded, and renumbered by an independent investigator.

To demonstrate synaptic stripping at light microscopic level we stained the synapses in some sections with anti-synaptophysin antibodies. Synaptophysin is an integral membrane-glycoprotein of small presynaptic ves-

icles and neuroendocrine granules (Wiedenmann and Franke, 1985); in CNS, it occurs predominantly in presynaptic boutons. The sections were incubated overnight with monoclonal mouse anti-bovine synaptophysin antibodies (1:80; Dakopatts) in a humid box at room temperature. After washing in PBS (3×5 min), the sections were incubated for 1 h with biotin-conjugated horse anti-mouse IgG(H + L) antibodies (1:220; Vector Labs, Burlingame, CA). After washing, the sections were incubated for 1 h with the standard peroxidase-conjugated Vectastain[®] ABC kit. Finally, the peroxidase reaction was performed for 15 min according to Adams (1981). All antibodies were diluted in 0.2% bovine serum albumin in PBS. Immunohistochemical controls were carried out by omitting the primary antibodies and continuing the staining sequence further.

Electron Microscopy

Under deep Nembutal[®] anaesthesia two rats were heparinized and perfused transcardially with 4% paraformaldehyde in 0.1 mM phosphate buffer (pH 7.4; 2 min; 37°C) and subsequently 200 ml 2% glutaraldehyde in 0.1 mM phosphate buffer, on day 4 after facial nerve crush. The part of the brainstem containing the facial nucleus was dissected and divided into tissue sections about 1 mm in thickness. After postfixation for 1 h in the glutaraldehyde, the tissue was subsequently fixed in 2% osmium tetroxide for 2 h (Palade, 1952), dehydrated in a graded acetone sequence, incubated overnight at room temperature in Epon812:acetone absolute (1:1), and finally embedded in Epon812 and polymerized at 60°C (Luft, 1961). Ultrathin sections were cut on an LKB ultratome and contrasted with uranyl acetate (30 min) and lead citrate (7 min) before observation with a Philips 301 electron microscope (Reynolds, 1963).

Measurements

The motoneuron cell bodies of the injured facial nerves in the right facial nucleus as well as those in the left intact facial nucleus were photographed from blinded haematoxylin stained cryostat sections. After printing, a montage of each facial nucleus was made (magnification $\times 400$). A detail of a montage is shown in Figure 2.

The motoneuron cell bodies in the facial nucleus with a visible nucleolus were marked according to Oppenheim et al. (1986). We did not intend to quantify all the microglial cells or all the motoneuron cell bodies in the facial nucleus. The perineuronal glial cells at a distance $<1.75 \mu\text{m}$ from the membrane surface of the marked cell bodies were counted by two independent observers unaware of the treatment a given rat had received. The perineuronal glial cells of 100–200 marked motoneuron cell bodies per animal were counted in five photographed sections from both facial nuclei. The mean numbers of the perineuronal glial cells per motoneuron per animal

were calculated by each observer. The mean numbers of both observers were averaged and used for statistical evaluation. To test whether there was a difference between the animals at different time points on the control side (days 2–6), the saline-treated groups were compared with each other by one way analysis of variance (ANOVA), followed by the Student-Newman-Keuls test (SNK). A *P* value of less than 0.05 was taken as indicating a significant difference. To test the effect of the peptide treatment on the control side, the peptide-treated groups were compared with the saline-treated groups at each time point using two-sided Student's *t*-test. At each time point the saline-treated groups on the crush and control sides were compared with each other by ANOVA, followed by the SNK-test. The effect of the peptide treatment on the glial reaction on the crushed side was tested by comparing the peptide-treated groups with the saline-treated groups at each time point on the crushed side using the Student's *t*-test.

RESULTS

Qualitative Light Microscopy

All the sections contained both the facial nucleus of the control side and of the crushed side. With anti-synaptophysin staining, motoneuron cell bodies on the control side showed mostly a continuous or fragmented dense ring of immunoreactive terminals around the perikarya that could not be resolved as discrete dots in $8 \mu\text{m}$ thick cryostat sections (Fig. 1a). On the crushed side, the somata of the motoneuron cell bodies were surrounded by fragmented rings or were devoid of axosomatic immunoreactive terminals (Fig. 1b). The immunohistochemical control sections without the primary antibody were negative.

On the crushed sides the characteristic degenerative changes were visible. The number of glial cells opposing the motoneuron cell bodies was clearly increased on the crushed side (Fig. 2b, arrows) compared with the control side (Fig. 2a). The glial cells contained elongated or triangular nuclei that stained deeply with haematoxylin, which is typical for microglial cells (Peters et al., 1976). A mitotic figure of a microglial cell could sometimes be observed (upper right inset Fig. 2b, arrowhead). An elevation in the number of microglial cells was also noticeable in the neuropil.

Qualitative Electron Microscopy

At the submicroscopical level, many afferent nerve terminals (arrows) appeared to occupy the membrane of the motoneuron cell bodies on the control side (Fig. 3). On the crushed side perineuronal cells were seen lying against the cell membrane of the motoneuron cell body of the injured facial nerve (Fig. 4). The perineuronal cells seemed to encompass the motoneuron cell bodies with processes (asterisks). The perineuronal cells contained a rather dark elongated nucleus (n) close to

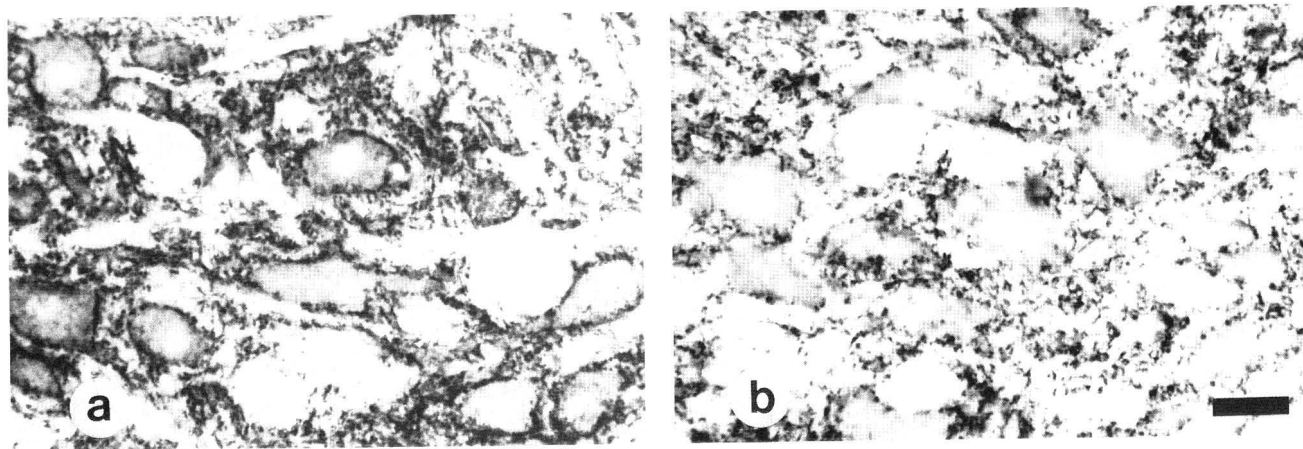


Fig. 1. Motoneuron cell bodies on the control left facial nucleus, stained with anti-synaptophysin, usually showed a continuous or fragmented dense ring of immunoreactive terminals around the perikarya that could not be resolved as discrete dots in 8 μ m thick cryostat sections (a). On the crushed side, the right facial nucleus, the somata of the motoneuron cell bodies were surrounded by fragmented rings or were devoid of axosomatic immunoreactive terminals (b). Bar, 20 μ m.

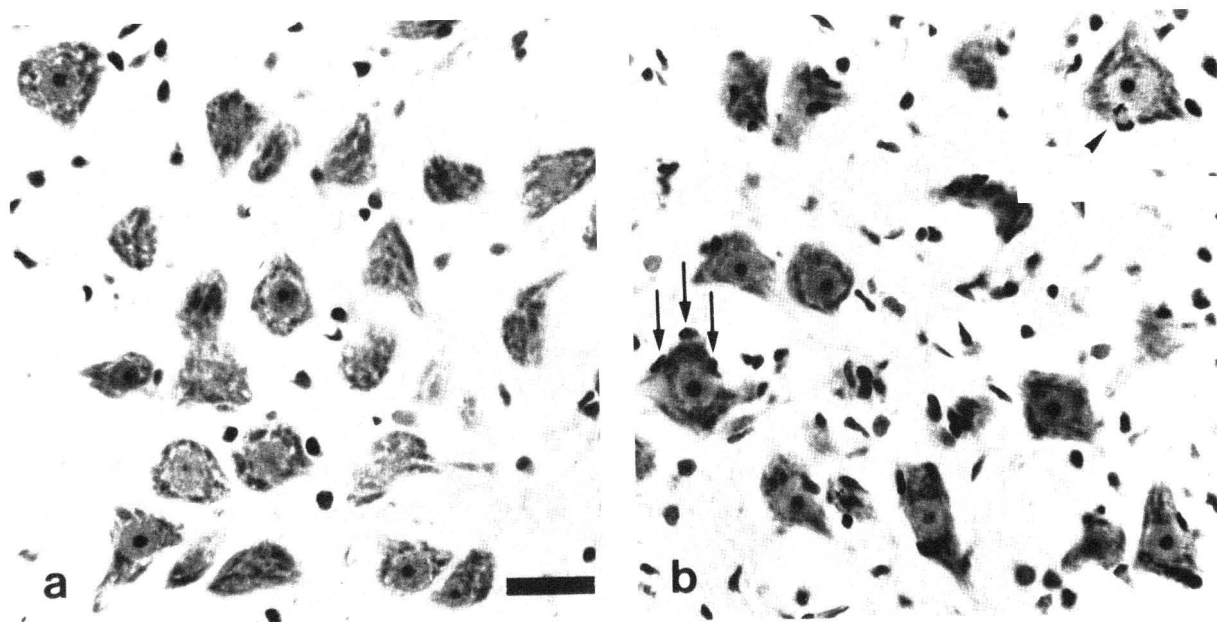


Fig. 2. Compared with the intact contralateral left facial nucleus (a) there was a clear increase in the number of perineuronal glial cells (b, arrows) in the affected right facial nucleus, 4 days after nerve injury. Sometimes a mitotic figure of a dividing microglial cell could be observed in the right facial nucleus (upper right inset, b, arrowhead), 4 days after nerve injury. In the affected neuropil an elevation in the number of microglial cells was noticeable compared with the intact left side. Bar, 20 μ m.

the chromatolytic perikaryon of a motoneuron, with clumps of chromatin beneath the nuclear envelope and throughout the nucleoplasm. Around the nucleus thin rims of cytoplasm were seen, which were rather dark and contained droplets and long cisternae of the endoplasmic reticulum (Peters et al., 1976). The cell morphology observed was characteristic for microglial cells. There were no nerve terminals between the microglial cells and the perikaryon (arrowheads).

Quantitative

There was no statistically significant difference among the five different sections in each animal on the crushed and control side. There was no significant difference among the animals in the peptide- or saline-treated group at each time point. On the control side there was no difference between the animals of the saline-treated groups at the different time points. Statistical evalua-

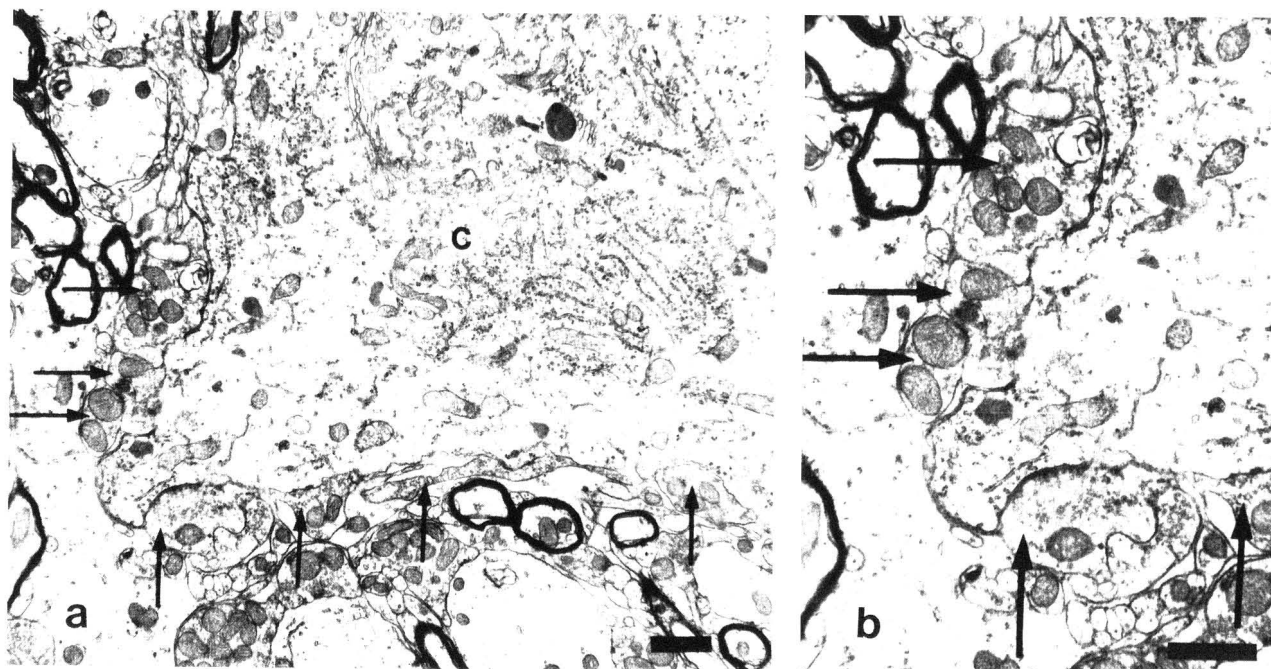


Fig. 3. a: Electron microscopic micrograph presenting a perikaryon of an intact lower motoneuron on the intact control side. Many afferent nerve terminals (arrows) occupy the membrane surface of the cell body (c). b: Higher magnification of the intact axosomatic terminals (arrows). Bar, 1 μ m.

tion showed no significant effect of the peptide treatment on the control side at each time point measured (the mean numbers of perineuronal glial cells per cell body of the saline-treated animals were between 0.26 ± 0.01 and 0.58 ± 0.08 and of the peptide-treated animals between 0.28 ± 0.06 and 0.48 ± 0.07 ; Fig. 5: \circ vs. \bullet). All values were expressed as mean numbers \pm standard error of mean (S.E.M.).

The saline-treated animals showed a marked increase in the number of perineuronal glial cells per motoneuron cell body on the crushed side compared with the control side at each time point (Fig. 5: Δ vs. \circ). From day 2 (Fig. 5: Δ , 0.73 ± 0.1) to day 3 (Δ , 1.69 ± 0.07) a clear increase in the number of perineuronal glial cells per cell body was observed. This increase continued from day 3 to 5 (Δ , 2.01 ± 0.06). From day 5 to 6 there was a marked decrease in the number of perineuronal glial cells per cell body (Δ , 1.39 ± 0.14).

In the peptide-treated animals there was a clear increase in the number of perineuronal glial cells per motoneuron cell body on the crushed side compared with the control side at each time point (Fig. 5: \blacktriangle vs. \bullet). From day 2 (Fig. 5: \blacktriangle , 0.83 ± 0.05) to 3 (\blacktriangle , 1.65 ± 0.09) the number of perineuronal glial cells per body was clearly increased. However, from day 3 to 5, no further increase was seen in the peptide-treated animals: day 4 (\blacktriangle , 1.55 ± 0.1) and day 5 (\blacktriangle , 1.65 ± 0.09). After day 5 the number of perineuronal glial cells tended to decrease (\blacktriangle , 1.39 ± 0.04).

The peptide effect on the crushed side on days 2 and 3 was not statistically significant (Fig. 5: Δ vs. \blacktriangle ; $n = 3$).

On days 4 and 5 the peptide-treated animals showed a significantly decreased number of perineuronal glial cells per cell body ($P < 0.03$, respectively, $P < 0.007$, Student's t-test) compared with the saline-treated animals (Fig. 5: Δ vs. \blacktriangle). On day 6 there was no significant difference in the number of perineuronal glial cells per cell body between the peptide-treated group (Fig. 5: Δ , 1.39 ± 0.04) and the saline-treated group (\blacktriangle , 1.39 ± 0.14).

DISCUSSION ACTH(4-9)/ORG2766

The ACTH(4-9) analogue, ORG2766, has been proven to facilitate the functional recovery and collateral sprouting following rat sciatic nerve crush in vivo (Gerritsen van der Hoop et al., 1988) and following recovery in streptozocin-induced diabetic rats (van der Zee et al., 1989). In their report on the process of brain aging, Landfield et al. (1981) found fewer glial clusters and fewer astrocytes in the hippocampal formation of chronic ORG2766-treated rats than in control animals.

Microglial Reaction

At the light microscopic level it was demonstrated that the motoneuron cell bodies of injured facial nerve were more or less deafferented compared to the motoneuron cell bodies of intact facial nerve [cf. Neiss et al. (1992)]. We were interested in the number of perineu-

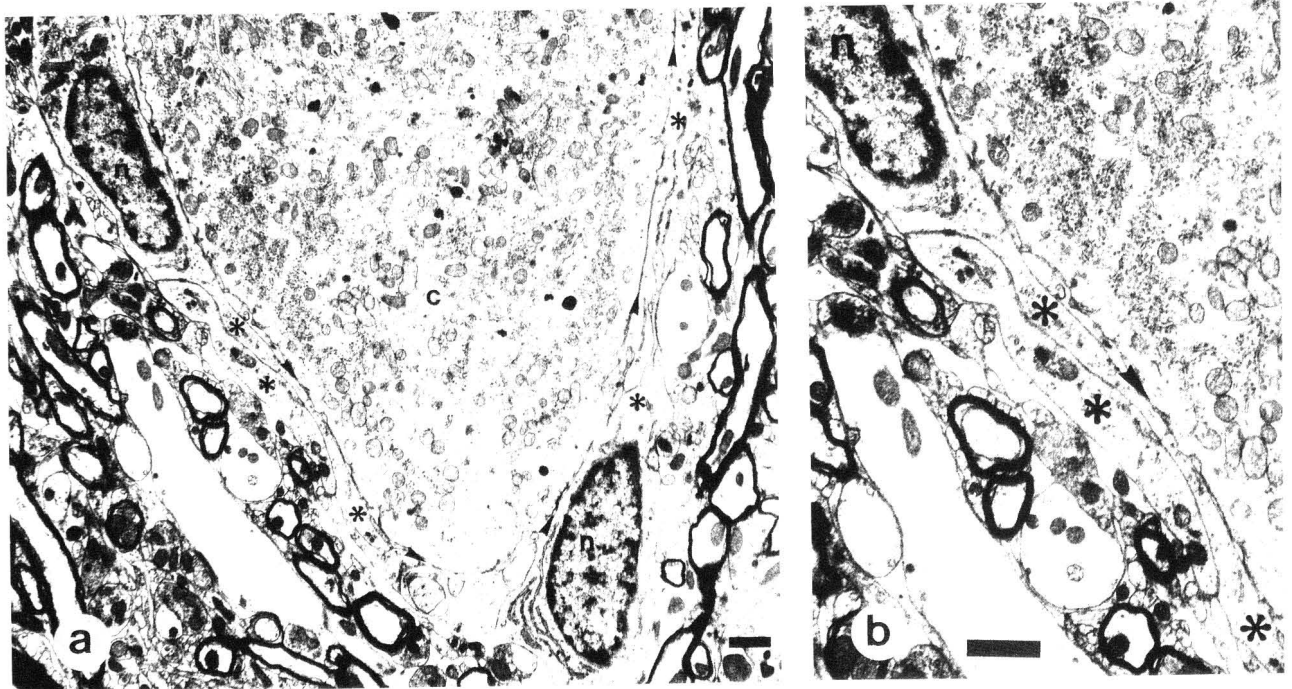


Fig. 4. a: Perineuronal microglial cells are seen lying against the membrane of a motoneuron cell body on the crushed side, 4 days after nerve injury. No nerve terminals are noticeable between the glial cell and the perikaryon (arrowheads). Note the processes (asterisks) of the microglial cells. The neuron cell body shows dispersed endoplasmic

reticulum. Note the dense nucleus and cytoplasm of the microglial cells and the long subcellular cisterns of granular endoplasmic reticulum. b: Higher magnification of the microglial processes (asterisks) and the lack of axosomatic terminals; c, cytoplasm motoneuron cell body; n, nucleus. Bar, 1 μ m.

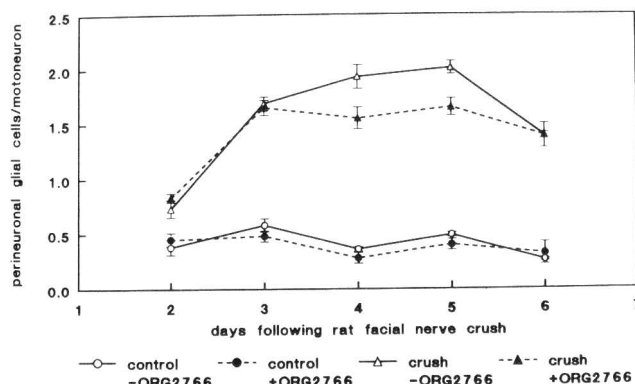


Fig. 5. On the control side, no statistically significant effect of ORG2766-treatment was present at the time points measured (○ vs. ●). On the crushed side the number of perineuronal glial cells per motoneuron cell body was markedly increased in peptide- (▲) and saline-treated (△) animals at each time point. From day 2, a clear rise in the perineuronal glial cells per cell body was noticeable in the peptide- and saline-treated animals. However, during this proliferation phase, days 2 and 3, the effect was not statistically significant (△ vs. ▲; $n = 3$). From day 3 the proliferation continued in the saline-treated group up to day 5 (△) but was stabilized in the ORG2766-treated group (▲). On days 4 and 5 the ORG2766-treated group (▲) showed a significantly decreased number of perineuronal glial cells per cell body compared with the saline treated group ($P < 0.03$ respectively $P < 0.007$ Student's *t*-test; △ vs. ▲). After day 5 the proliferation phase tends to decrease (▲). On day 6 there was no significant difference noticeable in the peptide- and the saline-treated group (△ vs. ▲). The values are expressed as mean number \pm S.E.M.

ronal microglial cells which strip the synapses with their processes. Counting these perineuronal microglial cells seemed to be a reliable and reproducible method of quantification. According to the criteria of Oppenheim et al. (1986), only those microglial cells around motoneuron cell bodies with a clear nucleolus were counted. The nucleolus diameter is small compared to the section thickness. Using this criterion it is impossible to count a motoneuron twice in different sections. We counted nucleoli as representatives of the motoneuron cell bodies. In five photographed sections per animal, 100–200 motoneuron cell bodies with a nucleolus were marked.

At the contralateral side no effect in the microglial reaction was detected at the time points measured. Neiss et al. (1992) described an effect on the contralateral side but the effect was after the initial state of retrograde reaction, namely, between the first and second week. This time period was beyond the scope of our experiment.

Blinzinger and Kreutzberg (1968) observed a high proliferation rate between days 3 and 6 after nerve injury which peaked on day 4 (Blinzinger and Kreutzberg, 1968; Kreutzberg, 1968). Our results are in line with this course of mitotic activity. The peptide-treated as well as the saline-treated animals had a high increase in the number of perineuronal glial cells per cell body during days 2 and 3. This period seemed to be the main period of proliferation. A further increase up to day 5

was measured in the saline-treated group. In the peptide-treated animals no further increase was seen from day 3 onwards. The neuronal cell bodies showed less perineuronal satellitosis. The reduction in the microglial reaction does not necessarily mean a reduction in synaptic stripping. Similar to the saline-treated rats, peptide-treated rats showed a decline in the number of perineuronal glial cells from day 5 to 6.

Signal and Hypothesis

The few preexisting microglial cells in the facial nucleus presumably proliferate through signalling by injured motoneurons (Blinzinger and Kreutzberg, 1968; Del Rio-Hortega, 1932; Kreutzberg, 1966; Streit et al., 1988). The nature of this signal is at present unknown.

Glial cells express all kind of cytokines (Kiefer et al., 1993; Lazar et al., 1991; Lindholm et al., 1992; Raivich et al., 1991) which seem to regulate the proliferation and which may be influenced by neuronal calcitonin gene related peptide (CGRP) (Reddington et al., 1992; Streit et al., 1989). In culture, microglial cells show high cytotoxic capacity; for references, see Banati et al. (1993) and Merrill (1992). This in contrast to astrocytes that secrete products that may be beneficial to neurons (for references, see Giulian, 1993). The microglial derived neurotoxic factors studied by Giulian (1993) seem to be small (<500 D), heat stable, resistant to proteinases and might be mediated via NMDA-receptors.

Also of interest are the stress proteins ("heat-shock proteins") (Gower et al., 1989) which are highly expressed by motoneuron cell bodies within 1 h after nerve injury (Brown et al., 1989; Gonzalez et al., 1989; Gower et al., 1989; New et al., 1989) and appear to be important in the protection of cells against damage. These proteins may influence the neuronal signal.

ORG2766 may decrease the neuronal signal that stimulate the glial cells or the receptor for this signal. The peptide-effect on the glial reaction, from day 3 on, was relatively late compared with the peptide-effect on the axonal outgrowth (from day 2 on) (Verhaagen et al., 1987).

In conclusion, the number of perineuronal glial cells was decreased in ORG2766-treated rats compared with saline-treated rats. This decrease in number may be the result of fewer mediating neuronal signals/glial derived cytokines, or of less damage to motoneurons through more protection by stress proteins. Further research may be of interest for ORG2766 mechanism studies and for future possibilities in treatment of motoneuron diseases by inhibiting microglial cell derived toxins near the site of injury to improve functional recovery.

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