The Journal of Neuroscience, February 1, 1996, 16(3):1041–1048

Nimodipine Accelerates Axonal Sprouting after Surgical Repair of Rat Facial Nerve

Doychin N. Angelov,[1] Wolfram F. Neiss,[1] Michael Streppel,[2] Jonas Andermahr,[1] Konrad Mader,[1] and Eberhard Stennert[2]

[1]Institut I für Anatomie and [2]Klinik für Hals-, Nasen-, und Ohrenheilkunde der Universität zu Köln, Lindenthal, D-50924 Cologne, Germany

Facial–facial anastomosis (FFA), i.e., suture of transected facial nerve, was performed in adult Wistar rats. For 10–112 d post-operation (DPO), half of the animals received standard food (placebo) and half received food pellets containing 1000 ppm nimodipine, a Ca^{2+} channel blocker. The time course of mimetic reinnervation between these two groups was compared by counting all retrogradely labeled motoneurons after injection of horseradish peroxidase (HRP) into the whiskerpad. In unoperated animals, injection of HRP labeled 1280 ± 113 motoneurons. After FFA, this number dropped to zero, and the first HRP-labeled facial motoneurons reappeared in both placebo- and nimodipine-treated animals at 14 DPO. The treatment with nimodipine yielded two beneficial effects. (1) It accelerated axonal sprouting until 28 DPO. Whereas the number of HRP-labeled cells in the placebo group was 171 ± 9 (mean ± SD) at 16 DPO, 372 ± 43 at 21 DPO, and 636 ± 187 at 28 DPO, the number of sprouted motoneurons in nimodipine-treated rats was twice as high: 386 ± 34 at 16 DPO, 620 ± 28 at 21 DPO, and 756 ± 257 at 28 DPO. (2) Nimodipine reduced the polyneuronal innervation of the target muscles. Whereas the number of HRP-labeled cells in the placebo group increased to 1430 ± 36 at 56 DPO and 1600 ± 31 at 112 DPO, the number of labeled motoneurons in nimodipine-treated rats remained almost within the normal range: 1315 ± 31 at 56 DPO and 1354 ± 33 at 112 DPO.

Key words: rat; motoneuron; facial nerve; axotomy; axonal regrowth; retrograde tracing; horseradish peroxidase; nimodipine; neuron number

The outgrowth of regenerating axons of a transected peripheral motor nerve is a slow process. After an initial delay, which is necessary for the cell bodies to compensate for the retrograde effects of axonal transection, the regenerating axonal sprouts cross the site of injury, reach the distal stump, and grow down the nerve to their peripheral terminations (Thomas, 1988; Fawcett and Keynes, 1990; Liuzzi and Tedeschi, 1991). The speed of this regeneration varies from 0.6 to 4.2 mm/d (Seddon et al., 1943; Jasper, 1946; Sunderland, 1946; Isch et al., 1968; Braam and Nicolai, 1993), with a mean value of ~1 mm/d in humans (Guth, 1956; Thomas, 1988). For example, in a case with lesioned facial nerve, this regeneration rate would promote a dysfunction of the facial nerve for several months. This facial palsy causes great morbidity for the sufferer which, in combination with the grotesque disfigurement, often may lead to a psychological incapacitation (Bento and Miniti, 1993; Braam and Nicolai, 1993; Vaughan and Richardson, 1993).

Obviously, it would be desirable to accelerate the axonal sprouting. However, all pharmacological treatment strategies, including trophic factors, gangliosides, and phosphatidylserine, have not been as successful as expected (Gottfries, 1989; Pepeu et al., 1993). Today there is no clinically available pharmacological treatment that would speed up nerve regeneration. We report here for the first time experimental data from the rat that the Ca^{2+} entry blocker nimodipine accelerates the axonal resprouting after nerve suture.

Calcium ions play a crucial role in depolarization, outgrowth, excitability, aging, learning, and cell proliferation—in short, neuronal plasticity (Gispen et al., 1988). It is well known that peripheral nerve injury disrupts the permeability barrier function of the plasma membrane, allowing an influx of Ca^{2+} down a steep electrochemical gradient between the outside and the inside of the cell (Borgens, 1988). The resultant intracellular free Ca^{2+} overload triggers a wide array of chain reactions, which eventually may lead to cell death (Schanne et al., 1979; Choi, 1988). Therefore, an agent preventing the excessive influx of Ca^{2+} (e.g., Ca^{2+} channel blocker) might attenuate cellular damage caused by mechanical neuronal injury and thus improve neuronal recovery (Takimoto and Fujibayashi, 1988).

We tested the Ca^{2+} channel blocker nimodipine (1,4,-dihydropyridine, or Bay E-9736) because this drug has been shown previously (1) to reduce the age-related deterioration of motor performance (Schuurman et al., 1987; Van der Zee et al., 1990; Ingram et al., 1994), (2) to favor the functional recovery of gait after crush lesion of the rat sciatic nerve (Van der Zee et al., 1987, 1991; Gispen et al., 1988; Bär et al., 1990), (3) to attenuate neuronal damage after cerebral ischemia (Mabe et al., 1986; Choi, 1988; Uematsu et al., 1989; Nyakas et al., 1994), and (4) to provide a variable but significant neuroprotection after traumatic insult to murine cortical neurons and glia (Regan and Choi, 1994).

MATERIALS AND METHODS

The design of our experiment was simple: after transection and immediate microsurgical end-to-end suture of the facial nerve [facial–facial anastomosis (FFA)], half of the rats were treated with 1000 ppm

Received June 9, 1995; revised Sept. 25, 1995; accepted Nov. 2, 1995.

This work was supported by the Alexander von Humboldt Foundation (D.N.A.), the Bayer AG (W.F.N.), and the Jean Uhrmacher Foundation (E.S., M.S.).

Correspondence should be addressed to Dr. Doychin N. Angelov, Institut I für Anatomie der Universität zu Köln, Joseph Stelzmann Straße 9, D-50931 Cologne, Germany.

nimodipine in the food pellets and half were treated with placebo, i.e., they received standard laboratory chow. Because it has been shown previously that there is no neuronal loss in the brainstem after immediate suture of a peripheral motor nerve (Neiss et al., 1992; Angelov et al., 1994; Guntinas-Lichius et al., 1994), the postoperative survival periods were not unnecessarily extended. We counted the number of facial motoneurons that projected into the mimetic muscle of the whiskerpad, from 10 to 112 d after FFA, using the standard method of retrograde neuronal tracing with horseradish peroxidase (HRP) (Watson et al., 1982; Yu and Yu, 1983; Hinrichsen and Watson, 1984; Thomander, 1984; Friauf and Herbert, 1985; Klein and Rhoades, 1985; Aldskogius and Thomander, 1986; Semba and Egger, 1986; Angelov et al., 1993).

Animals

A total of 126 adult female Wistar rats (175–200 gm; strain HsdCpb: WU; Harlan Winkelmann, Borchen, Germany) was used for this study. Before the experiments, all rats were kept on standard laboratory food (Ssniff, Soest, Germany) and tap water *ad libitum* with an artificial light/dark cycle of 12 hr lights on/12 hr lights off.

The 126 rats were divided into 21 groups. Each group contained six rats. One group served as normal control, and 20 experimental groups were subjected to the same facial nerve suture (see Surgery). Ten of the operated groups received postoperative treatment with placebo, and 10 groups were treated with nimodipine (see Drug treatment). The rats of one placebo group and one nimodipine group were killed after 10 different treatment times, i.e., postoperative survival times, namely, at 10, 14, 16, 18, 21, 24, 28, 42, 56, or 112 d postoperation (DPO), which comprises a total of 20 different time-treatment groups of six rats each.

Surgery

Transection and immediate end-to-end suture of the facial nerve (FFA) was performed unilaterally under an operating microscope by a trained microsurgeon in 120 rats. After an intraperitoneal injection of 1.4 ml of Avertin [2.0 gm of tribrom-ethanol (Aldrich, Milwaukee, WI), 1 ml of 3-pentanol, 8 ml of absolute ethanol in 90 ml of 0.9% saline], the main trunk of the facial nerve was exposed and transected at its emergence from the foramen stylomastoideum but distal to the posterior auricular branch. The proximal stump then was sutured microsurgically to the distal stump with two 11-0 atraumatic sutures (Ethicon, Norderstedt, Germany).

Drug treatment

Control animals. Six rats were not operated on, received bilateral injections of HRP-solution (see below), and served as normal controls.

Placebo-treated animals. After FFA, 60 rats were kept on Ssniff food pellets (standard formula rat/mouse), which served as placebo treatment.

Nimodipine-treated animals. Starting immediately after surgery and ending with the perfusion fixation, 60 rats received special pellets, which were based on Ssniff standard formula for rat/mouse diet and contained 1000 ppm nimodipine supplied by Bayer (Leverkusen, Germany; pellets made by Ssniff). It is well known that most Ca^{2+} channel blockers are highly potent drugs and that their therapeutic concentrations in plasma and other biological fluids generally are within the range of high picograms to low nanograms per milliliter (Formenti et al., 1993; Mück and Bode, 1994). In addition, it has been shown previously that clinically convenient oral administration of the drug causes a virtually complete and rapid absorption in the gastrointestinal tract (Raemsch et al., 1985; Wadworth and McTavish, 1992; Herbette et al., 1994). Finally, the daily dosage chosen was in accordance with the study of Van der Zee et al. (1991), which reported acceleration of functional recovery after crush lesion of the rat sciatic nerve. To allow for retrograde transport, HRP was always applied 48 hr before fixation of the animal (e.g., for investigation at 14 DPO, HRP was administered at 12 DPO). Six rats per group were fixed by perfusion after the same postoperative survival times as after placebo treatment.

Application of HRP

The muscles of the whiskerpad were selected as representative for the mimetic musculature because the constant arrangement of the vibrissae in rows (Arvidsson, 1982) ensures identical injection sites in each animal. In addition, the motoneurons that innervate the vibrissal muscles comprise a compact group of nerve cells in the lateral subdivision of the facial nucleus (Dörfl, 1982; Klein and Rhoades, 1985; Semba and Egger, 1986). There is no other motor nerve supply to these muscles except from the lateral subdivision of the facial nucleus (Papez, 1927; Martin et al., 1977; Watson et al., 1982; Hinrichsen and Watson, 1984).

HRP was injected into the whiskerpads of all rats bilaterally, i.e., both on the operated and on the contralateral untreated control side. Because we wanted to compare the numbers of labeled nerve cells in different animals, great care was taken to ensure identical conditions of injection in each animal. Under deep ether narcosis, 2 mg of HRP (Type VI-A; Sigma, St. Louis, MO) dissolved in 0.2 ml of distilled water containing 2% dimethylsulfoxide was injected under the skin of each whiskerpad (on the operated and on the unoperated side), always at the same site, i.e., exactly in the middle between dorsal vibrissal rows A and B (Arvidsson, 1982; Angelov et al., 1993). Forty-eight hours later, the animals were fixed by perfusion (see below).

Tissue processing

Fixation. All animals were anesthetized and pericardially perfused with 0.9% NaCl in distilled water for 30 sec, followed by a mixture of 1.25% *p*-formaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 20 min. Thereafter, a postfixation rinse *in situ* with the same buffer was performed for another 20 min.

Histochemistry. The entire brain was removed, the operated side was marked, and the brainstem was cut through both facial nuclei in 50-μm-thick coronal sections on a Vibratome (FTB-vibracut; Plano, Marburg, Germany). HRP activity was revealed using the incubation chamber of Paull and King (1983) simultaneously in sections from 12 brainstems (6 from placebo-treated rats and 6 from nimodipine-treated rats) according to the tetramethylbenzidine protocol of Mesulam (1978).

Quantitative estimates

Counting of the labeled facial neurons was performed with the fractionator method (Gundersen, 1986) in every third 50 μm section through the facial nucleus both on the operated and on the untreated contralateral side of the brainstem as described previously (Neiss et al., 1992; Guntinas-Lichius et al., 1993).

For overall evaluation of the drug/time effect, two-factorial ANOVA was used on the number of HRP-labeled neurons in the facial nucleus on the operated side, testing the data of the 20 time-treatment groups ($n_{group} = 6$; $n_{total} = 120$) with treatment time (i.e., postoperative survival time) as factor A and type of treatment (placebo or nimodipine) as factor B.

To check for a drug effect on the HRP labeling of neurons without operation, we used Student's *t* test for unpaired data on the number of labeled motoneurons on the unoperated side, testing the pooled data of operated rats with placebo versus nimodipine treatment ($n_{group} = 60$; $n_{total} = 120$).

For the assessment of postoperative "hyperinnervation" (see Results), we pooled the data sets of 56 and 112 DPO. The *t* test for paired data was used separately for placebo- and nimodipine-treated rats to test the neuron numbers on the operated versus unoperated side in the same animals ($n_{test} = 12$ rats). The *t* test for unpaired data then was used for the neuron numbers on the operated side of placebo-treated rats versus nimodipine-treated rats ($n_{group} = 12$; $n_{total} = 24$).

For more detailed information on the nimodipine effect on the early phase of regeneration than is afforded by ANOVA, we used the *t* test for unpaired data with Bonferroni–Holm correction for multiple testing on the neuron numbers of the operated side in placebo-treated rats versus nimodipine-treated rats at 16, 18, 21, and 24 DPO ($n_{group} = 6$, $n_{test} = 12$; 4 parallel tests).

RESULTS

All data presented here are the mean ± SD of six rats per group unless stated otherwise.

Normal innervation of the whiskerpad

Application of HRP solution into the whiskerpads of unoperated animals labeled 1280 ± 113 ($n = 12$ facial nuclei of 6 rats) motoneurons, all of which were located exclusively in the lateral subdivision of the facial nucleus (Fig. 1). This number and location are nearly equal to our results from a previous experiment, in which we counted 1278 ± 97 ($n = 42$ facial nuclei of 42 rats) motoneurons projecting into the whiskerpad [Angelov et al. (1993); see page 216 and their Fig. 2].

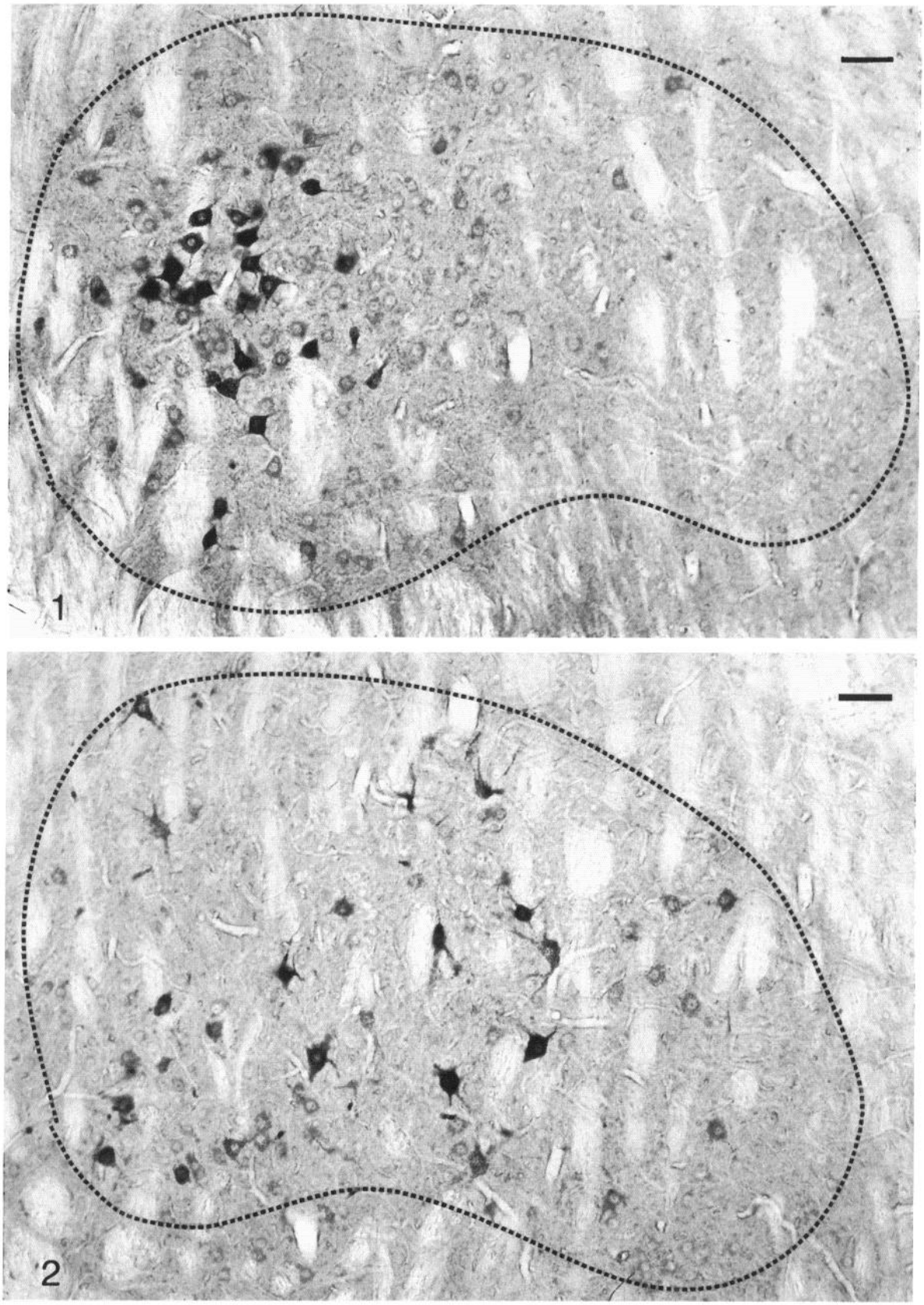

Figure 1. Facial nucleus (*outlined*) of a normal rat 48 hr after injection of 2 mg of HRP in 0.2 ml of distilled water into the whiskerpad. The retrogradely labeled motoneurons are localized only in the lateral subnucleus. Vibratome section, 50 μm. Scale bar, 65 μm.

Figure 2. Cross-cut facial nucleus (*outlined*) of a rat 42 d after FFA. The retrogradely labeled motoneurons are dispersed throughout the entire facial nucleus: this loss of somatotopy after injury of the facial nerve is the morphological correlate of misdirected resprouting (aberrant reinnervation). Vibratome section, 50 μm. Scale bar, 65 μm.

Table 1. Time course of the reneurotization of the whiskerpad after FFA in rats

	Placebo animals			Nimodipine-treated rats		
Days after FFA	Unoperated facial nucleus	Operated facial nucleus	Range for operated facial nucleus	Unoperated facial nucleus	Operated facial nucleus	Range for operated facial nucleus
10 DPO	1321 ± 45	0	0	1215 ± 29	0	0
14 DPO	1282 ± 101	3 ± 4	0–12	1180 ± 102	7 ± 4	3–12
16 DPO	1279 ± 59	171 ± 9	153–183	1287 ± 88	386 ± 34	327–438
18 DPO	1375 ± 91	362 ± 40	306–411	1394 ± 15	597 ± 120	348–738
21 DPO	1237 ± 71	372 ± 43	309–429	1324 ± 81	620 ± 28	582–660
24 DPO	1463 ± 127	367 ± 81	261–501	1368 ± 45	657 ± 194	318–843
28 DPO	1253 ± 50	636 ± 187	366–882	1203 ± 72	756 ± 257	291–1080
42 DPO	1169 ± 87	1328 ± 154	1044–1437	1149 ± 83	1291 ± 153	1077–1482
56 DPO	1269 ± 38	1430 ± 36	1389–1483	1203 ± 41	1315 ± 31	1284–1362
112 DPO	1207 ± 46	1600 ± 31	1551–1653	1208 ± 22	1354 ± 33	1303–1398

The same data were obtained on the contralateral, i.e., the unoperated side of animals with unilateral FFA. Injection of HRP into the contralateral whiskerpad labeled 1286 ± 111 (n = 60) motoneurons in the placebo group and 1253 ± 103 (n = 60) motoneurons in the nimodipine group. There was no significant difference between these groups (unpaired t test), and the number of labeled neurons remained constant throughout the experiment (Table 1); i.e., the nimodipine treatment had no effect on the number of motoneurons on the unoperated side and did not obviously affect the retrograde transport of HRP.

When we amalgamated the data sets of placebo- and nimodipine-treated animals, the normal facial lateral subnucleus of the Wistar rat contained 1269 ± 108 (n = 120) motoneurons that projected into the whiskerpad.

Reinnervation of the whiskerpad after FFA

Placebo

Injection of HRP into the whiskerpad on the side of operation did not label any neurons on the operated side of the brainstem at 10 DPO. The first HRP-marked motoneurons were detected 14 d after nerve suture (Table 1).

The number of labeled neurons gradually increased and reached 1328 ± 154 at 42 DPO. Although this number was equal to the number of facial neurons that project into the whiskerpad under normal conditions (see Table 1, Fig. 3), the labeled neurons were scattered throughout the facial nucleus, i.e., no somatotopic organization into subnuclei was evident (Fig. 2). After 42 DPO, the number of HRP-labeled motoneurons on the operated side increased further, causing a hyperinnervation, also termed "polyneuronal innervation" (Rich and Lichtman, 1989; Son and Thompson, 1995a). At 56 and 112 DPO, 12.7 and 32.6% more facial motoneurons were projecting into the mimetic muscles of the whiskerpad on the operated than on the unoperated contralateral side, respectively, of the same animals. When we pooled the data of 56 and 112 DPO and tested the neuron numbers on the operated versus unoperated side in the same animals with the t test for paired data, this hyperinnervation proved highly significant (p = 0.001).

Nimodipine

As in the placebo-treated animals, the first retrogradely labeled motoneurons in the facial nucleus appeared at 14 DPO (Table 1). In the next 2 weeks, however, the increase in number of the HRP-marked neurons, i.e., the reinnervation of the mimetic musculature, proceeded much more rapidly than in placebo-treated animals (Table 1, Fig. 3). From 16 to 24 DPO, more facial neurons were labeled after FFA followed by nimodipine than after placebo treatment (Fig. 3).

The acceleration of axonal sprouting by nimodipine occurred only during the first 3–4 postoperative weeks. At 42 DPO, the number of motoneurons that had reached the whiskerpad with nimodipine or placebo was the same as under normal conditions (Table 1, Fig. 3). Thereafter, the number of HRP-labeled motoneurons increased further. At 56 and 112 DPO, 9.3 and 12.1% more facial neurons were labeled on the operated than on the unoperated side, respectively, in the same rats. Although modest, this hyperinnervation nevertheless proved significant (p = 0.001; t test for paired data).

Drug effect

With two-factorial ANOVA, both treatment time (factor A) and type of treatment (factor B) proved significant (p = 0.001 for A, B, and A+B). These results mean that the number of motoneurons that can be labeled by injection of HRP into the denervated target muscle depends on the time for regeneration, which is obvious, and on the postoperative drug treatment of the animals, which is the new result of our study.

Figure 3 shows that 1000 ppm nimodipine administered as needed yielded two beneficial effects on facial reinnervation. (1) In the early phase, nimodipine accelerated the axonal sprouting of facial motoneurons. (2) In the final phase, nimodipine reduced the postoperative hyperinnervation. The significance of these two effects was tested separately.

Early phase. Using the t test for unpaired data, the numbers of labeled neurons differ significantly between the respective placebo and nimodipine groups from 16 to 24 DPO (see Fig. 3). Because these are multiple tests, however, the p-values (Fig. 3, *asterisks*) have been corrected according to Bonferroni–Holm. With this correction, the mean data of the nimodipine-treated rats 16–24 DPO differ with $p < 0.05$ from those of the placebo-treated animals. The Bonferroni–Holm correction for multiple testing is a very conservative procedure, and in our data at 16 and 21 DPO, even the ranges of neuron numbers did not overlap between the six placebo-treated rats and six nimodipine-treated rats at each

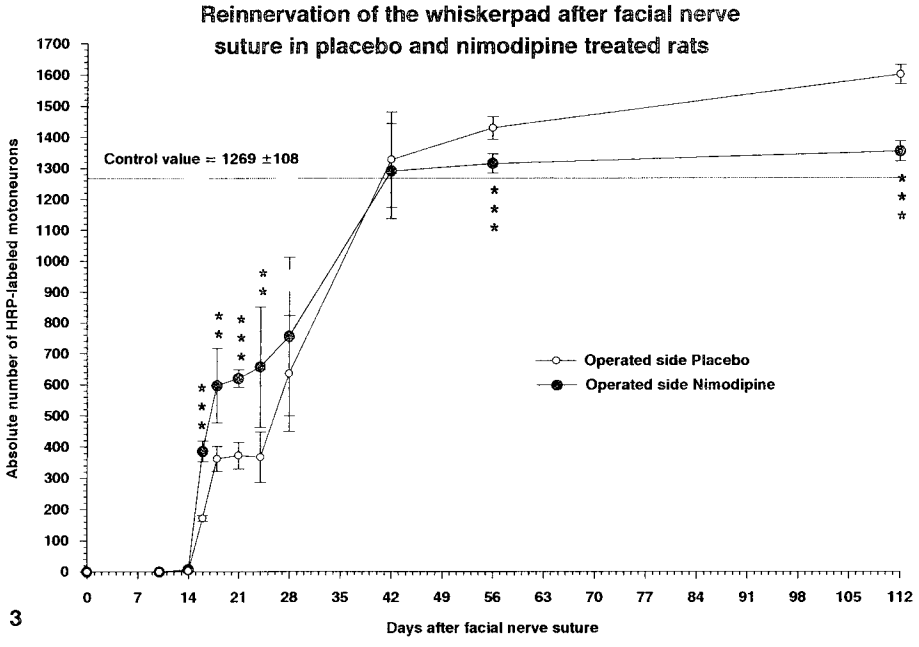

Figure 3. Time course of the changes in the number of motoneurons projecting to the mimetic muscles after FFA, placebo treatment, or nimodipine treatment, and injection of HRP into the whiskerpad. Each point of the graph represents the mean ± SD of six rats. *t* test for unpaired data: $^{**}p < 0.01$, $^{***}p < 0.001$.

time point. Thus, we feel very confident that the acceleration of axonal sprouting by nimodipine holds true.

Final phase. At 56 and 112 DPO, the *t* test for paired data independently showed in both treatment groups a significant hyperinnervation (see above) that appeared more modest after nimodipine therapy (Fig. 3, Table 1). When we used the *t* test for unpaired data, this difference between placebo- and nimodipine-treated rats was highly significant ($p = 0.001$). In conclusion, nimodipine cannot prevent fully, but does suppress greatly the pathological hyperinnervation (polyneuronal innervation) of the whiskerpad that follows transection and suture of the facial nerve.

DISCUSSION

Methodological approach

In this study, neurons were counted after axotomy and subsequent retrograde labeling with HRP. Because we did not measure the post-transectional axonal growth directly—rather, we measured the uptake of a neuronal tracer injected into the target musculature—the possible critique regarding the extent to which our counts accurately reflect sprouting should be addressed carefully. Three sound arguments support the chosen approach as reasonable and appropriate. First, a remote diffusion of the HRP solution from the whiskerpad to the proximal stump of the transected facial nerve can be ruled out completely: if such diffusion had occurred, labeling of facial motoneurons also would have resulted in experimental animals with the shorter postoperative survival period of 10 DPO. This, however, was never detected. Second, even the earliest axonal sprouts (or neuroma) that grow after axotomy are capable of incorporation and retrograde transport of HRP (Sparrow and Kiernan, 1979; Olsson, 1980). Third, there is no uptake of HRP by lethally injured, i.e., nonregenerating motoneurons (Angelov et al., 1993) and, likewise, there is no neuronal labeling from muscles with electromyographic evidence of denervation (Anonsen et al., 1986). Therefore, only those regenerated motoneurons can be labeled, the axons of which are projecting into the target muscle.

Nimodipine-accelerated sprouting of facial nerve fibers

Our quantitative estimates show that 14–28 d after FFA, the number of facial axons (or axonal branches) that have reached the target successfully is higher in nimodipine-treated rats than in placebo-treated rats (Table 1, Fig. 3). In this way, the proposed favorable effect of nimodipine on neuronal recovery (Poplawsky, 1990; Nelson et al., 1993; Neiss et al., 1993) has been confirmed morphologically under conditions of a simple but reliable comparative experiment.

We are not aware of the mechanism of nimodipine stimulation of sprouting. The precise mechanism of action of this agent is still unknown (for review, see Wadworth and McTavish, 1992). So far, our explanation concurs with the common hypothesis that a dual effect (on both perikarya and neurites) occurred. The neuroprotective effect for the perikarya has been suggested previously: as nimodipine passes the blood–brain barrier and binds to specific dihydropyridine receptors, it may prevents the influx of Ca^{2+} into the injured neuronal cell bodies (Schanne et al., 1979; Siesjo, 1981; Belleman et al., 1983; Simon et al., 1984; Kazda et al., 1985; Van den Kerckhoff and Drewes, 1985). The second, peripheral beneficial effect of nimodipine on the axonal branches involves the fine regulation of intracellular calcium in outgrowing sprouts. It is well known that most of the voltage-sensitive Ca^{2+} channels are localized on the outgrowing axonal sprouts (Anglister et al., 1982), the metabolism of which depends on a narrow range of intracellular Ca^{2+} concentrations (Meyer, 1989; Kater and Mills, 1991). Via a specific binding to the dihydropyridine receptors, the L-type Ca^{2+} channel antagonist nimodipine reduces the influx of Ca^{2+} into the injured neurons and, thus, may exert a beneficial growth promoting action.

Nimodipine suppression of post-transectional hyperinnervation

In placebo-treated animals, the continuing sprouting of the facial axons after 42 DPO causes a massive (~30%) hyperinnervation. This term was introduced earlier (Angelov et al., 1993; Neiss et al., 1993) and means that paradoxically, after peripheral nerve lesion,

the axonal branches of more motoneurons project to the target muscles than under normal conditions. The primary reason for this is the misguidance of the regenerating fibers to inappropriate peripheral targets (Thomander, 1984; Aldskogius and Thomander, 1986; Rich and Lichtman, 1989; Matsumoto, 1992; Angelov et al., 1993; Son and Thompson, 1995a,b). Despite the use of presently available microsurgical techniques for repair of injured peripheral nerves (i.e., cleaning of the wound, gentle tissue handling, good adaptation and coaption, use of a minimal number of sutures, and absence of tension) (Millesi, 1979), a substantial mismatching of motoneurons and muscles always occurs after transection and subsequent regeneration within a nerve trunk.

After nerve transection in adult animals, virtually all neurons survive, but the regenerating axons appear to grow in a relatively random manner ("escaped fibers"), causing a considerable disarray of the facial nucleus. This loss of somatotopic organization in the facial nucleus and the subsequent hyperinnervation of targets are morphological correlates of the phenomenon of "misdirected resprouting" (also termed excessive, multiple, redundant, or aberrant reinnervation, or misdirected regrowth of axons). Consequently, the coordinated activity of individual muscles is impaired (Monserrat and Benito, 1988; Wasserschaff, 1990) (for review, see Sumner, 1990), and abnormal associated movements (synkinesis), hemifacial spasms, or contractures may develop (Yagi and Nakatani, 1986).

We cannot state whether the axonal branches of all HRP-labeled neurons terminate in the mimetic muscles as functioning motor endplates—the HRP method reveals and proves only the presence of axonal branches, but not the presence of motor endplates formed by them (Flumerfelt et al., 1986). This question was not the subject of the present study and requires systematic investigation of reinnervated muscle fibers, which is presently under way.

Hyperinnervation also occurs in nimodipine-treated rats; however, it does not exceed 15%. At first sight, nimodipine appears to have a paradoxical effect after transection of a peripheral motor nerve: on one hand, it stimulates and accelerates axonal resprouting, and on the other, it suppresses the excessive neurotization (hyperinnervation, polyneuronal innervation) of the target.

In our opinion, this restriction of further sprouting is attributable not to a suppressive effect of nimodipine, but to the very rapid and functionally better reinnervation of the whiskerpad muscles induced by it.

Under normal conditions (placebo treatment), the ruptured cell membranes of the proximal and distal portions of the transected axons are resealed 5–30 min after the transection (Yawo and Kuno, 1985). Thereafter, the proximal stump gradually regrows, fostered by the neural cell surface molecule of the transmembrane glycoprotein L1, nerve cell adhesion molecule (N-CAM), myelin-associated glycoprotein P0, and the extracellular matrix components laminin and tenascin (for review, see Martini, 1994). The regenerating axons are guided by the Schwann cell processes (Son and Thompson, 1995a,b), and their growth within the Schwann cell basal lamina tubes is synchronous with the withdrawal and degeneration of the axonal remnants of the distal stump (for review, see Fawcett and Keynes, 1990). Before this, however, the distal nerve stump (together with its injured Schwann cells) and the denervated target secrete a palette of tropic and trophic factors, e.g., nerve growth factor (for review, see Seeburger and Springer, 1993), insulin-like growth factor (for review, see Lewis et al., 1993), basic fibroblast growth factor (Grothe and Unsicker, 1992), brain-derived neurotrophic factor (Hofer and Barde, 1988), and ciliary neurotrophic factor (Sendtner et al., 1990, 1992), which promote and/or enhance motor neuronal sprouting.

In our model, the growing axons and their branches reach the neuromuscular junction sites between 10 and 14 DPO. However, they still do not release neurotransmitters (Dennis and Miledi, 1974). The first miniature end-plate potentials (MEPPs) appear ~2 weeks after crush (McArdle and Albuquerque, 1973) and probably later after transection, because this is a more severe lesion of the nerve. Particularly in these early stages of reinnervation, the Ca^{2+}-dependent release mechanisms are highly efficient, i.e., the influx of Ca^{2+} increases the frequency of MEPPs. The reason for this is the low buffering capacity of the regenerating nerve terminals for free intracellular Ca^{2+}, which would allow a stronger stimulatory action of the Ca^{2+} that would enter the presynaptic ending (Kater et al., 1988). Despite this rather high efficiency, however, the frequency of the MEPPs remains far below normal (Carmignoto et al., 1983). In consequence, synchronously with the ongoing secretion of all neurotrophic factors (see above), these poorly innervated facial muscles accumulate N-CAMs, which enhance their attractiveness to axons (Covault and Sanes, 1985). This may be the cause, in part, for the well documented hyperinnervation (Thomander, 1984; Aldskogius and Thomander, 1986; Angelov et al., 1993; Neiss et al., 1993).

By reducing the amount of calcium influx into the axoplasm of the resprouting nerve fiber, the treatment with nimodipine may provide the necessary optimum level ("set-point") of Ca^{2+} influx that promotes accelerated growth cone elongation (Mattson and Kater, 1987; Kater et al., 1988; Kater and Mills, 1991; Fields et al., 1993). However, it further reduces the buffering capacity of the terminals for Ca^{2+}, which might render their responsiveness to Ca^{2+} even stronger. This would yield MEPPs with higher frequency. In this way, a much earlier and qualitatively better reinnervation of the facial muscles is achieved, which may lead to a partial reduction in the secretion of neurotrophic agents (see above) which, in turn, may reduce the postregeneration hyperinnervation.

In conclusion, we suggest that it is the acceleration of muscle innervation itself that suppresses the final stage hyperinnervation.

REFERENCES

Aldskogius H, Thomander L (1986) Selective reinnervation of somatotopically appropriate muscles after facial nerve transection and regeneration in the neonatal rat. Brain Res 375:126–134.

Angelov DN, Gunkel A, Stennert E, Neiss WF (1993) Recovery of original nerve supply after hypoglossal-facial anastomosis causes permanent motoric hyperinnervation of the whiskerpad muscles in the rat. J Comp Neurol 338:214–224.

Angelov DN, Neiss WF, Gunkel A, Guntinas-Lichius O, Stennert E (1994) Axotomy induces intranuclear immunolocalization of neuron-specific enolase in facial and hypoglossal motoneurons of the rat. J Neurocytol 23:218–233.

Anglister L, Farber JC, Shahar A, Grinvald A (1982) Localization of calcium sensitive calcium channels along developing neurites: their possible role in regulating neurite elongation. Dev Biol 94:351–365.

Anonsen CK, Trachy RE, Hibbert J, Cummings CW (1986) Assessment of facial reinnervation by use of chronic electromyographic monitoring. Otolaryngol Head Neck Surg 94:32–36.

Arvidsson J (1982) Somatotopic organization of vibrissae afferents in the trigeminal sensory nuclei of the rat studied by transganglionic transport of HRP. J Comp Neurol 211:84–92.

Bär PR, Traber J, Schuurman T, Gispen WH (1990) CNS and PNS effects of nimodipine. J Neural Transm Suppl 31:55–71.

Belleman P, Schade A, Towart R (1983) Dihydropyridine receptors in rat brain labelled with [^{3}H]nimodipine. Proc Natl Acad Sci USA 80:2356–2360.

Bento RF, Miniti AM (1993) Anastomosis of the intratemporal facial nerve using fibrin tissue adhesive. Ear Nose Throat J 72:663–672.

Borgens RB (1988) Voltage gradients and ionic currents in injured and regenerating axons. Adv Neurol 47:51–66.
Braam MJI, Nicolai JPA (1993) Axonal regeneration rate through cross-face nerve grafts. Microsurgery 14:589–591.
Carmignoto G, Finesso M, Siliprandi R, Gorio A (1983) Muscle reinnervation. I. Restoration of transmitter release mechanisms. Neuroscience 8:393–401.
Choi DW (1988) Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends Neurosci 11:465–469.
Covault J, Sanes JR (1985) Neural cell adhesion molecule (N-CAM) accumulates in denervated and paralyzed skeletal muscles. Proc Natl Acad Sci USA 82:4544–4548.
Dennis MJ, Miledi R (1974) Characteristics of transmitter release at regenerating frog neuromuscular junctions. J Physiol (Lond) 239:571–594.
Dörfl J (1982) The musculature of the mystacial vibrissae of the white mouse. J Anat 135:147–154.
Fawcett JW, Keynes RJ (1990) Peripheral nerve regeneration. Annu Rev Neurosci 13:43–60.
Fields DR, Guthrie PB, Russel JT, Kater SB, Malhotra BS, Nelson PG (1993) Accommodation of mouse DRG growth cones to electrically induced collapse: kinetic analysis of calcium transients and set-point theory. J Neurobiol 24:1080–1098.
Flumerfelt BA, Kiernan JA, Krcek JP, Sholdice J (1986) Reinnervation of skeletal muscle in the tongue by preganglionic parasympathetic vagal neurons. J Anat 146:117–130.
Formenti A, Arrigoni E, Mancia M (1993) Low-voltage activated calcium channels are differently affected by nimodipine. NeuroReport 5:145–147.
Friauf E, Herbert H (1985) Topographic organization of facial motoneurons to individual pinna muscles in rat (*Rattus rattus*) and bat (*Rousettus aegyptiacus*). J Comp Neurol 240:161–170.
Gispen WH, Schuurman T, Traber J (1988) Nimodipine and neural plasticity in the peripheral nervous system of adult and aged rats. In: The Ca^{2+} channel: structure, function and implications (Morad M, Nayler W, Kazda S, Schramm M, eds), pp 491–502. New York: Springer.
Gottfries CG (1989) Pharmacological treatment strategies in dementia disorders. Pharmacopsychiatry 22:129–134.
Grothe C, Unsicker K (1992) Basic fibroblast growth factor in the hypoglossal system: specific retrograde transport, trophic, and lesion-related responses. J Neurosci Res 32:317–328.
Gundersen HJG (1986) Stereology of arbitrary particles. J Microsc 143:3–45.
Guntinas-Lichius O, Mockenhaupt J, Stennert E, Neiss WF (1993) Simplified nerve cell counting in the rat brainstem with the physical dissector using a drawing microscope. J Microsc 172:177–180.
Guntinas-Lichius O, Neiss WF, Gunkel A, Stennert E (1994) Differences in glial, synaptic and motoneuron responses in the facial nucleus of the rat brainstem following facial nerve resection and nerve suture reanastomosis. Eur Arch Otorhinolaryngol 251:410–417.
Guth L (1956) Regeneration in the mammalian peripheral nervous system. Physiol Rev 36:441–478.
Herbette LG, Mason PE, Sweeney KR, Trumbore MW, Mason RP (1994) Favorable amphiphilicity of nimodipine facilitates its interactions with brain membranes. Neuropharmacology 33:241–249.
Hinrichsen CFL, Watson CD (1984) The facial nucleus of the rat: representation of facial muscles revealed by retrograde transport of horseradish peroxidase. Anat Rec 209:407–415.
Hofer MM, Barde Y-A (1988) Brain derived neurotrophic factor prevents neuronal death in vivo. Nature 331:261–262.
Ingram DK, Joseph JA, Spangler EL, Roberts D, Hengemihle J, Fanelli R (1994) Chronic nimodipine treatment in aged rats: analysis of motor and cognitive effects and muscarinic-induced striatal dopamine release. Neurobiol Aging 15:55–61.
Isch F, Isch-Treussard C, Jesel M (1968) EMG findings on reinnervation in peripheral nerve lesions. EEG Clin Neurophysiol 24:404–405.
Jasper HH (1946) The rate of re-innervation of muscle following nerve injuries in man as determined by the electromyogram. Trans R Soc Can 40:81–92.
Kater SB, Mills LR (1991) Regulation of growth cone behaviour by calcium. J Neurosci 11:891–899.
Kater SB, Mattson MP, Cohan C, Conner J (1988) Ca^{2+} regulation of the neural growth cone. Trends Neurosci 11:315–320.
Kazda S, Garthoff B, Luckhaus G (1985) Prevention of acute and chronic cerebrovascular damage with nimodipine in animal experiments. In: Nimodipine: pharmacological and clinical properties (Betz E, Deck K, Hoffmeister F, eds), pp 31–43. Stuttgart: Schuttauer.
Klein BG, Rhoades RW (1985) Representation of whisker follicle intrinsic musculature in the facial motor nucleus of the rat. J Comp Neurol 232:55–69.
Lewis ME, Neff NT, Contreras PC, Stong DB, Oppenheim RW, Grebow PE, Vaught JL (1993) Insulin-like growth factor-I: potential for treatment of motor neuronal disorders. Exp Neurol 124:73–88.
Liuzzi FJ, Tedeschi B (1991) Peripheral nerve regeneration. Neurosurg Clin N Am 2:31–42.
Mabe H, Nagai H, Takagi T, Umemura S, Ohno M (1986) Effect of nimodipine on cerebral functional and metabolic recovery following ischemia in the rat brain. Stroke 17:501–505.
Martin MR, Kaddy KWT, Biscoe TJ (1977) Numbers and diameters of motoneurons and myelinated axons in the facial nucleus and nerve of the albino rat. J Anat 23:579–587.
Martini R (1994) Expression and functional roles of neural cell surface molecules and extracellular matrix components during development and regeneration of peripheral nerves. J Neurocytol 23:1–28.
Matsumoto K (1992) Observation of motoneuron after recovery from experimental facial nerve paralysis. Nippon Jibiinkoka Gakkai Kaiho 95:373–380.
Mattson MP, Kater SB (1987) Calcium regulation of neurite elongation and growth cone motility. J Neurosci 7:4034–4043.
McArdle JJ, Albuquerque XE (1973) A study of the reinnervation of fast and slow mammalian muscles. J Gen Physiol 61:1–23.
Mesulam M-M (1978) Tetramethylbenzidine for horseradish peroxidase neurohistochemistry: a non-carcinogenic blue reaction product with superior sensitivity for visualizing neural afferents and efferents. J Histochem Cytochem 26:106–117.
Meyer FB (1989) Calcium, neuronal hyperexcitability and ischemic injury. Brain Res Rev 14:227–243.
Millesi H (1979) Nerve suture and grafting to restore the extratemporal facial nerve. Clin Plast Surg 6:331–341.
Monserrat L, Benito M (1988) Facial synkinesis and aberrant regeneration of the facial nerve. Adv Neurol 47:9–29.
Mück W, Bode H (1994) Bioanalytics of nimodipine: an overview of methods. Pharmazie 49:130–138.
Neiss WF, Angelov DN, Gunkel A, Stennert E (1993) Nimodipine accelerates the sprouting of axotomized motoneurons following hypoglossal-facial anastomosis in the rat (Abstr). J Neurotrauma 10:55A.
Neiss WF, Guntinas-Lichius O, Angelov DN, Gunkel A, Stennert E (1992) The hypoglossal-facial anastomosis as model of neuronal plasticity in the rat. Ann Anat 174:419–433.
Nelson C, Finger S, Simons D (1993) Effects of nimodipine on two neurologic measures sensitive to sensorimotor cortex damage. Exp Neurol 119:302–308.
Nyakas C, Buwalda B, Kramers RJK, Traber J, Luiten PGM (1994) Postnatal development of hippocampal and neocortical cholinergic and serotonergic innervation in rat: effects of nitrite-induced prenatal hypoxia and nimodipine treatment. Neuroscience 59:541–559.
Olsson T (1980) Somatopetal transport of horseradish peroxidase following incorporation into axonal sprouts and axons in neuromas after nerve transection. Acta Pathol Microbiol Scand Sect A Pathol 88:195–199.
Papez JW (1927) Subdivisions of the facial nucleus. J Comp Neurol 43:159–191.
Paull WK, King JC (1983) A rinsing and incubation chamber used for immunocytochemistry of vibratome sections. Histochemistry 78:413–416.
Pepeu G, Casamenti F, Pepeu JM, Scali C (1993) The brain cholinergic system in aged animals. J Reprod Fertil Suppl 46:155–162.
Poplawsky A (1990) Nimodipine accelerates recovery from the hyperemotionality produced by septal lesions. Behav Neural Biol 53:133–139.
Raemsch KD, Groefe KH, Sommer J (1985) Pharmacokinetics and metabolism of nimodipine. In: Nimodipine: pharmacological and clinical properties (Betz E, Deck K, Hoffmeister F, eds), pp 147–161. Stuttgart: Schuttauer.
Regan RF, Choi DW (1994) The effect of NMDA, AMPA/kainate, and calcium channel antagonists on traumatic cortical neuronal injury in culture. Brain Res 633:236–242.
Rich MM, Lichtman JW (1989) *In vivo* visualization of pre- and postsynaptic changes during synapse elimination in reinnervated mouse muscle. J Neurosci 9:1781–1805.

Schanne FAX, Kane AB, Young EE, Farber JL (1979) Calcium dependence of toxic cell death: a final common pathway. Science 206:700–702.

Schuurman T, Klein H, Beneke M, Traber J (1987) Nimodipine and motor deficits in the aged rat. Neurosci Res Commun 1:9–15.

Seddon HJ, Medawar PB, Smith H (1943) Rate of regeneration of peripheral nerves in man. J Physiol (Lond) 102:191–215.

Seeburger JL, Springer JE (1993) Experimental rationale for the therapeutic use of neurotrophins in amyotrophic lateral sclerosis. Exp Neurol 124:64–72.

Semba K, Egger MD (1986) The facial "motor" nerve of the rat: control of vibrissal movement and examination of motor and sensory components. J Comp Neurol 247:144–158.

Sendtner M, Kreutzberg GW, Thoenen H (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature 345:440–441.

Sendtner M, Stöckli KA, Thoenen H (1992) Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. J Cell Biol 118:139–148.

Siesjo B (1981) Cell damage in the brain: a speculative synthesis. J Cereb Blood Flow Metab 1:155–185.

Simon RP, Griffiths T, Evans MC, Swane JH, Meldrum BS (1984) Calcium overload in selectively vulnerable neurons of the hippocampus during and after ischemia: an electron microscopic study in the rat. J Cereb Blood Flow Metab 4:350–361.

Son YJ, Thompson WJ (1995a) Schwann cell processes guide regeneration of peripheral axons. Neuron 14:125–132.

Son YJ, Thompson WJ (1995b) Nerve sprouting in muscle is induced and guided by processes extended by Schwann cells. Neuron 14:133–141.

Sparrow JR, Kiernan JA (1979) Uptake and retrograde transport of proteins by regenerating axons. Acta Neuropathol (Berl) 47:39–47.

Sumner AJ (1990) Aberrant reinnervation. Muscle Nerve 13:801–803.

Sunderland S (1946) Course and rate of regeneration of motor fibers following lesions of the radial nerve. Arch Neurol Psychiatry 56:133–157.

Takimoto I, Fujibayashi K (1988) Effect of flunarizine on experimentally induced facial nerve injury. Acta Otolaryngol (Stockh) 446:152–156.

Thomander L (1984) Reorganization of the facial motor nucleus after peripheral nerve regeneration. Acta Otolaryngol (Stockh) 97:619–626.

Thomas PK (1988) Clinical aspects of PNS regeneration. Adv Neurol 47:9–29.

Uematsu D, Greenberg JH, Hickey WF, Reivich M (1989) Nimodipine attenuates both increase in cytosolic free calcium and histologic damage following focal cerebral ischemia and reperfusion in cats. Stroke 20:1531–1537.

Van den Kerckhoff W, Drewes LR (1985) Transfer of the Ca-antagonists nifedipine and nimodipine across the blood-brain barrier and their regional distribution in vivo. J Cereb Blood Flow Metab 5:459–460.

Van der Zee CEEM, Brakkee JH, Gispen WH (1991) Putative neurotrophic factors and functional recovery from peripheral nerve damage in the rat. Br J Pharmacol 103:1041–1046.

Van der Zee CEEM, Schuurman T, Gerritsen van der Hoop R, Traber J, Gispen WH (1990) Beneficial effect of nimodipine on peripheral nerve function in aged rats. Neurobiol Aging 11:451–456.

Van der Zee CEEM, Schuurman T, Traber J, Gispen WH (1987) Oral administration of nimodipine accelerates functional recovery following peripheral nerve damage in the rat. Neurosci Lett 83:143–148.

Vaughan ED, Richardson D (1993) Facial nerve reconstruction following ablative parotid surgery. Br J Oral Maxillofac Surg 31:274–280.

Wadworth AN, McTavish D (1992) Nimodipine. A review of its pharmacological properties and therapeutic efficacy in cerebral disorders. Drugs Aging 2:262–286.

Wasserschaff M (1990) Coordination of reinnervated muscle and reorganization of spinal cord motoneurons after nerve transection in mice. Brain Res 515:241–246.

Watson CRR, Sakai S, Armstrong W (1982) Organization of the facial nucleus in the rat. Brain Behav Evol 20:19–28.

Yagi N, Nakatani H (1986) Crocodile tears and thread test of lacrimation. Ann Otol Rhinol Laryngol 95:13–16.

Yawo H, Kuno M (1985) Calcium dependence of membrane sealing at the cut end of the cockroach giant axon. J Neurosci 5:1626–1632.

Yu WHA, Yu MC (1983) Acceleration of the regeneration of the crushed hypoglossal nerve by testosterone. Exp Neurol 80:349–360.