Calcitonin Gene-Related Peptide: Possible Role in Formation and Maintenance of Neuromuscular Junctions

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The expression and content of calcitonin gene-related peptide (CGRP) and secretogranin II (SgII) in adult rat motor neurons were examined by *in situ* hybridization, Northern blot analysis, and immunocytochemistry. Normal motor nerve terminals did not contain detectable CGRP or SgII. Ten to 15 days after a peripheral nerve crush about 80% of the motor nerve terminals reinnervating the soleus (SOL) muscle contained detectable CGRP but no SgII. Thereafter, the percentage of CGRP-positive terminals declined towards zero. In the spinal cord, CGRP expression was higher than normal 1 d after a sciatic nerve crush and increased during the next few days. No increase in SgII expression was observed.

Nerve blocks by tetrodotoxin (TTX) and botulinum toxin (BoTX) increased CGRP content and expression in motor neurons but had no effect on SgII. After 10 d of BoTX treatment and 33 d of TTX treatment (the longest time points studied), more than 90% of the motor nerve terminals stained for CGRP. The density of large dense core vesicles (LDCVs) was also higher than normal in such terminals. Some increase in CGRP content and expression occurred in the non-treated side.

In a group of rats, the peroneal nerve was stimulated electrically with brief, intermittent pulse trains at 100 Hz. The stimulation was applied below a TTX block that had started 7 or 19 d earlier. One minute of such stimulation was sufficient to remove CGRP from most of the terminals.

These results show (1) that CGRP is upregulated in motor neurons and accumulate in motor nerve terminals during reinnervation and muscle paralysis by BoTX and TTX, (2) that no detectable changes in expression or content of SgII occur in the same conditions, and (3) that nerve stimulation causes CGRP to disappear rapidly from the motor nerve terminals, indicating that CGRP is released by nerve impulse activity. An hypothesis for how CGRP may contribute to the formation and maintenance of neuromuscular junctions is presented.

[Key words: calcitonin gene-related peptide, secretogranin II, neuromuscular junction, botulinum toxin, tetrodotoxin, transmitter release, synaptogenesis]

Calcitonin gene-related peptide (CGRP) is a neuropeptide generated by tissue-specific alternative splicing of the calcitonin gene (Rosenfeld et al., 1983). CGRP has a variety of effects on neural and non-neural tissue, including muscle, and may act as an anterograde neurotrophic factor at the neuromuscular junction (Fontaine et al., 1986; Changeux et al., 1992). In agreement with this proposal, CGRP is present in motor neurons (Rosenfeld et al., 1983; New and Mudge, 1986), transported to motor nerve terminals (Kashihara et al., 1989) where it is stored in large dense core vesicles (LDCV) (Matteoli et al., 1988). CGRP binding sites in muscle have been demonstrated (Jennings and Mudge, 1989; Roa and Changeux, 1991), and in cultured myotubes or adult muscle fibers CGRP has been shown to induce AChR expression (Fontaine et al., 1986), prolong AChR channel open time (Lu et al., 1993), and modulate AChR-channel properties (Eusebi et al., 1988; Mülle et al., 1988).

Postnatally, motor nerve terminals initially have high levels of CGRP and secretogranin II (SgII). Subsequently, as the neuromuscular junctions mature, CGRP and SgII contents decline to very low levels, often undetectable by immunohistochemical techniques (Böj et al., 1989; Matteoli et al., 1990; Li and Dahlström, 1992; Andreose et al., 1994). Sg II, a tyrosine sulphated protein present in large dense-core vesicles (LDCVs) (Huttner et al., 1991), is thought to play a role in the packaging of peptides in LDCV (Gerdes et al., 1989) and has been recently shown to be the precursor of the neuropeptide secretoneurin (Kirchmair et al., 1993). The accumulation of CGRP and SgII in developing motor nerve terminals, which suggests that CGRP and SgII may play a role in the formation of neuromuscular junctions, led to the first question asked in this work; do CGRP and SgII also accumulate in motor nerve terminals during reinnervation of adult muscle after a nerve crush?

CGRP, but not SgII, did accumulate in reinnervating nerve terminals and was also upregulated, as reported by others, after axotomy (Streit et al., 1989; Arvidsson et al., 1990; Noguchi et al., 1990; Piehl et al., 1991). We, therefore, next asked whether muscle paralysis by tetrodotoxin (TTX) or botulinum toxin (BoTX), which elicits axonal sprouting (Brown, 1984) and formation of new neuromuscular contacts (Angaut-Petit et al., 1990), would also increase CGRP expression and content in motor neurons. TTX and BoTX, like nerve crush, did upregulate CGRP expression but not SgII expression. In addition, CGRP accumulated in the motor nerve terminals. This raised the third

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Figure 1. CGRP, but not SgII, immunoreactivity appears in reinnervating SOL motor nerve terminals. *A, C, E, and G* show endplates labeled for both AChRs and neurofilament with FITC-coupled markers. Neurofilament labeling always extended outside the area of AChR labeling (arrowheads in *A* and *C*), making it easy to distinguish between denervated and reinnervated junctions. *B, D, F, and H* show the same endplates labeled with monospecific anti-CGRP (*B, D, and F*) or SgII (*H*) polyclonal rabbit antibodies followed by biotinylated secondary antibodies and Texas red-Streptavidin. The SOL endplates are either normal (*A* and *B*) or reinnervated 10 d after a SOL nerve crush (*C–H*). Magnifications: $\times 700$ for *A, B, E, and F*; $\times 900$ for *C* and *D*; $\times 400$ for *G* and *H*.

question asked in this work; can the CGRP that accumulates below a TTX block be released by stimulating the nerve below the block? It is still an open question whether nerve impulse activity can release CGRP from motor axons in rat skeletal muscles (Sakaguchi et al., 1991).

Materials and Methods

Animals, surgery, BoTX and TTX treatments, and nerve stimulation. Adult (250–300 gm b.wt.) and 1-week-old Wistar rats (Charles River, Italy and Møllegaards Ltd, Denmark) were used. Surgical operations were performed under Equithesin anesthesia (42.5 mg chloral hydrate and 9.7 mg pentobarbital in 1 ml solution, 0.4 ml/100 g weight, i.p.). Denervation was done by crushing either the SOL (for immunostaining in the muscle) or the sciatic nerve (for Northern blot and *in situ* hybridization in the spinal cord). Paralysis of leg muscles was obtained (1) by injecting botulinum toxin type A (BoTX) subcutaneously in one leg (4 ng/100 g) or (2) by continuously infusing tetrodotoxin (TTX, 1 mg/100 ml saline, 25 ml/hr) into a 8 mm long cuff around the sciatic from an external pump as previously described (Fumagalli et al., 1990). The presence of nerve block and muscle paralysis was checked daily by inspection of the animal's motor behavior and absence of motor reflexes and, at the time of the acute experiment, by stimulating the nerves and observing no contraction. When the TTX blocked nerve was stimulated below the block, vigorous muscle contraction was always observed. In the stimulation experiments, electrodes were implanted alongside the fibular nerve and trains of 60 pulses (bipolar, 0.2 msec long) at 100 Hz were delivered to the fibular nerve every 5 sec for periods ranging from 1 min to 4 hr. At the end of the stimulation period, muscles were removed, fixed, and processed for immunohistochemistry. The experiments involving local application of paralytic toxins have been inspected and permitted by the Norwegian Experimental Board and Ethical Committee for Animal Experiments and were overseen by the

responsible veterinarian for the animal house. During the experiments, the animals did not appear to suffer pain.

Tissue preparation. For immunohistochemical analysis, anesthetized animals were perfused with 4% formaldehyde in 0.1 M phosphate buffer. Muscles were dissected, infiltrated with 12–18% sucrose, and frozen in liquid nitrogen. For *in situ* hybridization and Northern blot experiments, freshly dissected lumbar spinal cords were quickly frozen in liquid nitrogen. For electron microscopy studies, EDL muscles were fixed in 2% glutaraldehyde in 0.1 M Cacodylate buffer, pH 7.3. Endplate-rich fragments were then postfixed in 1% OsO₄, dehydrated, and embedded in Epon 812. Thin sections were cut with a Reichert FC4 Ultracut microtome, stained with uranyl acetate and lead citrate, and observed in a Philips CM10 electron microscope. For morphometric analysis, pictures were taken and printed at the same magnification; the data were obtained using the NIH Image 1.37 program on a Macintosh IIfx computer equipped with a drawing board.

Immunohistochemistry. A solution containing phosphate-buffered saline (PBS), 1% bovine serum albumin, and 0.3% Triton X-100 was used to dilute immunoreagents and to rinse sections. The rabbit anti-CGRP antiserum (gift from Dr. J. M. Polak) has been previously characterized (Gibson et al., 1984). The rabbit anti-SgII (gift from Dr. P. Rosa) has been previously characterized (Rosa et al., 1985). Donkey biotinylated anti-rabbit IgGs (Amersham) followed by Texas Red Streptavidin (Amersham), was used as second step immunoreagent. Endplates were identified by FITC- α -bungarotoxin (Sigma) and a monoclonal antibody directed against the 150 kDa subunit of neurofilaments (Vitadello et al., 1986), followed by FITC-goat anti-mouse IgGs (Amersham), was used to visualize the nerve endings. Presynaptic axons and postsynaptic AChRs, both labeled by fluorescein, had clearly different staining patterns (e.g., Fig. 1) and it was, therefore, easy to make sure whether endplates were reinnervated or not.

Probes. A rat CGRP specific, 1.4 kilobases long cDNA (from a psp64 vector given by S. G. Amara) was inserted in the opposite sense orientation with respect to the SP6 promoter. A rat SgII specific, 600 bases

Figure 2. Transient appearance of CGRP in SOL motor nerve terminals during reinnervation. Filled circles show the percentage of reinnervated endplates at different times after a SOL nerve crush. Columns show percentage of terminals with immunohistochemically detectable CGRP in SOL muscles on the side of the nerve crush (open columns) and contralaterally (stippled columns). Values are mean $\pm$ SE.

long cDNA (from a pGEM 4Z vector given by H. Gerdes and W. Huttner) was inserted in the opposite sense orientation with respect to the SP6 promoter and in sense orientation with respect to the T7 promoter. For *in situ* hybridization high specific activity, 35 S-UTP-labeled, single-strand RNA antisense probes were synthesized from the linearized templates (Melton et al., 1984). For Northern blot analysis, the CGRP, SgII, and S18 (ribosomal RNA S18 subunit) specific fragments were excised from the vectors and used to synthesize random primer probes in presence of 32 P-dCTP.

In situ hybridization. *In situ* hybridization histochemistry was carried out according to Chesselet et al. (1987). In each experiment, spinal cords of treated and control animals were collected on the same slide. Sections were fixed with 4% paraformaldehyde in PBS for 5 min and, after rinsing in PBS and $2\times$ SSC (0.15 M NaCl in 0.015 M Na citrate), were acetylated by immersion in 0.1 M triethanolamine containing 0.025% acetic anhydride. After further rinsing in PBS and $2\times$ SSC, the sections were incubated at room temperature for 30 min in 0.1 M Tris-glycine pH 7.0, rinsed in $2\times$ SSC, dehydrated in 70%, 80%, and 95% ethanol, and air dried. Fifty milliliters of the hybridization mixture were applied to each slide, which was then covered with a cover slip and stored overnight at 50°C in a humid chamber. The hybridization mixture contained 50% formamide, $2\times$ SSC, 1 mg/ml sheared salmon sperm DNA, 1 mg/ml yeast tRNA, 10 Dextran sulphate, $1\times$ Denhardt's solution (0.002% Ficoll, 0.02% polyvinylpyrrolidone, 10 mg/ml bovine serum albumin), 10 mM dithiothreitol, and 35 S-RNA probe (50,000 cpm/ml). After hybridization, the sections were washed in 50% formamide in $2\times$ SSC at 50°C, incubated with 100 mg/ml RNase A (Sigma) in SSC at 37°C for 30 min, rinsed in $2\times$ SSC, and then stored overnight under agitation in a solution containing 0.05% Triton X-100 in $2\times$ SSC. The sections were finally dehydrated with ethanol, air dried, and dipped in a K5 nuclear track emulsion (Ilford) diluted 1:1 with distilled water. After 3 weeks of exposure at 4°C, the slides were developed in Kodak D19 at 20°C for 5 min, fixed in Kodak fix, and air dried. Controls consisted in treatment of the sections with 100 mg/ml RNase A in SSC at 37°C for 30 min following paraformaldehyde fixation or hybridization with sense probes.

For autoradiographic grain density analysis, sections were first stained with methylene blue and then observed in a Zeiss microscope. The bright-field images were digitally stored using Image 1.37; the slides were then washed with a fluid soap to remove the stain and observed under dark-field optics. For each individual neuron, the area of interest was drawn on the bright-field image and the corresponding grain density value was measured on the dark-field image. The density values were subtracted for the background measured in motor neuron-free areas.

Slides coated with gelatin containing known amount of radioactivity were used as grain density standard.

Northern blot. Total RNA was extracted from tissue by standard procedures (Sambrook et al., 1989). RNA was denatured at 55°C in formaldehyde and run in a 2.2 M formaldehyde 1.4% agarose electrophoretic gel as described in (Sambrook et al., 1989). The RNA was then transferred to Hybond N⁺ membranes (Amersham) and prehybridized for at least 2 hr at 42°C in 50% formamide, 4% dextran sulphate, 1 M NaCl, 2% powdered milk, 200 mg/ml denatured fish sperm. The probe was added to a final concentration of 10^6 cpm/ml and incubated for at least 12 hr. The membranes were washed at room temperature in $2\times$ SSC, 1% SDS, then in $0.1\times$ SSC, 1% SDS at 50°C, and finally exposed to Kodak XAR films with an intensifying screen at -80°C . In general, the same blot was used to hybridize with the CGRP and SgII probes. A ribosomal S18 probe was used as reference for the amount of loaded RNA.

Results

Nerve crush

The SOL nerve was crushed just outside the SOL muscle, and the content of CGRP and SgII in the reinnervating motor nerve terminals examined by immunohistochemical techniques. In innervated control muscles, the neuromuscular junctions stained for both AChRs and neurofilaments (Fig. 1*A*) but not for CGRP (Fig. 1*B*) or SgII. After the nerve crush, neurofilament staining disappeared from all the endplates for 4 d and then reappeared at an increasing number of endplates (Fig. 1*C,E,G*). Sixteen days after the nerve crush, essentially all the endplates contained neurofilament-positive terminals and were, therefore, reinnervated (Fig. 2). Many reinnervated terminals contained detectable amounts of CGRP (Fig. 1*D,F*). The percentage of terminals containing CGRP increased to 80% around day 16 and then declined (Fig. 2). CGRP staining also appeared in the contralateral leg, but the intensity of the staining was weaker and the percentage of positive endplates smaller than in the operated leg (Fig. 2). No reinnervating terminals contained detectable amounts of SgII at any time (example in Fig. 1*H*). In contrast, both SgII and CGRP accumulate in rat motor nerve terminals during normal development, as shown in a separate work in which the same antibodies and immunohistochemical techniques were used (Andreose et al., 1994).

The sciatic nerve was crushed in the thigh and CGRP and SgII expression in the spinal cord examined by *in situ* hybridization (Fig. 3*A–E*) and Northern blots (Fig. 3*F*). Both techniques showed that CGRP mRNA content had increased 1 d after the crush and continued to rise for at least another 4 d. *In situ* hybridization showed, in addition, that CGRP mRNA content increased also contralaterally to the treated side but less than on the treated side (Fig. 3*E*), confirming earlier results (Piehl et al., 1991). In contrast, SgII mRNA did not increase (Fig. 3). Densitometric analysis of the Northern blots combined with normalization to the S18 value in each lane to correct for differences in amount of RNA, resulted in the following ratios between the spinal cords from experimental and control (non-treated) animals 1, 3, 5, and 10 d after the nerve crush: 3.21 ± 0.39 , 4.88 ± 0.58 , 4.95 ± 0.57 , and 4.32 ± 0.53 , respectively, for CGRP, and 0.8 ± 0.07 , 1.04 ± 0.02 , 1.15 ± 0.04 , 1.04 ± 0.15 , respectively, for SgII (mean $\pm$ SD, $n = 3$ for each time point).

Nerve block by botulinum toxin or tetrodotoxin

Application of both BoTX and TTX markedly increased the immunoreactivity for CGRP in the motor nerve terminals of the paralysed muscles. Five days after injection of BoTX, almost

Figure 3. Sciatic nerve crush upregulates CGRP, but not SgII, expression in spinal motor neurons. *In situ* hybridization on anterior horns of the left (*A* and *C*) and right (*B* and *D*) side of lumbar spinal cords 5 d after a crush to the right sciatic nerve. *A* and *B* are from the same spinal cord cross section after reaction with a CGRP specific probe. *C* and *D* are from an adjacent cross section after reaction with a SgII specific probe. *E* shows autoradiographic grain density over lumbar motor neurons on the right (nerve crush) and left (contralateral) sides, as indicated. The grain clusters coincided with large neuronal cell bodies as seen with bright-field illumination (not shown). *F* shows Northern blots of CGRP and SgII mRNA at different times after the nerve crush. The same blot was used to hybridize with the CGRP, SgII, and S18 probes. *A–D*: $\times 500$.

all the SOL endplates ($94\% \pm 5$, mean $\pm$ SE; $n = 5$) were intensely stained by CGRP antibodies (example in Fig. 4*A,B*). CGRP-positive terminals appeared more slowly in the EDL during TTX treatment, but after 30 d of paralysis essentially all the terminals were CGRP positive (Fig. 5*A,B*; Fig. 6*A*).

BoTx and TTX also affected the motor nerve terminals in the nontreated leg, but to a lesser degree. The time course for this contralateral effect during TTX treatment is shown in Fig. 6*A*. During BoTx treatment, $38 \pm 6\%$ and $52 \pm 5\%$ of the terminals in the contralateral SOL were CGRP positive after 5 and 10 d, respectively.

Accumulation of CGRP immunoreactivity occurred together with a significant increase in the density of the LDCVs (Table 1). Also, in the 1-week-old rats, the density of LDCV was relatively high (Table 1). Essentially, all the LDCVs were found at a distance from the presynaptic plasma membrane, away from the clusters of small synaptic vesicles.

Both BoTx and TTX increased the content of CGRP mRNA in the lumbar spinal cord (Fig. 4*E*; Fig. 5*E,F*). Densitometric analysis of Northern blots 3 and 5 d after the BoTx injection (Fig. 4*E*) showed that the ratio between the CGRP content in spinal cords from BoTx-treated and nontreated animals in-

creased to 1.82 ± 0.03 and 2.17 ± 0.04 (mean $\pm$ SD, $n = 3$ for each time point, $p < 0.01$). In three rats the spinal cords were examined by *in situ* hybridization for CGRP mRNA after the sciatic nerve on one side had been blocked by TTX for 33 d. The density of autoradiographic grains over profiles of motor neuron cell bodies was measured in cross sections of the spinal cord between lumbar segments L2 and L5. The density was 3.1 ± 0.5 times higher on the treated than on the untreated (contralateral) side ($p < 0.01$), indicating that CGRP mRNA expression had been upregulated by the TTX block (Fig. 5*E,F*).

Table 1. Density of large dense-core vesicles

Control adult	(8)	0.89 ± 0.05
1 week old	(5)	$1.87 \pm 0.11^*$
BoTx (7 d)	(4)	$2.21 \pm 0.31^*$
TTX (33 d)	(6)	$1.57 \pm 0.10^*$

Data are number of large dense-core vesicles (LDCV) per mm^2 in nerve terminals of neuromuscular junctions in EDL muscles (mean $\pm$ SE). Numbers within parentheses show number of animals. From 18 to 29 endplates were analyzed in each animal.

* Statistically different from adult, $p < 0.01$ in *t* Student's test.

Figure 4. BoTx increases CGRP, but not SgII, immunoreactivity and mRNA content in spinal motor neurons. Five days after BoTx injection, neuromuscular junctions labeled for AChR and neurofilament (*A* and *C*) are positive for CGRP (*B*), but not SgII (*D*). *E* shows Northern blots of CGRP and SgII mRNAs from spinal cords of normal animals (day 0) and of animals treated with BoTx for 3 and 5 d. The same blot was used to hybridize with the CGRP, SgII, and S18 probes. *A* and *B*: $\times 820$; *C* and *D*: $\times 450$.

Neither BoTx nor TTX had any detectable effect on SgII content or expression (Fig. 4*C,D,E*; Fig. 5*C,D,G,H*).

Stimulation of the nerve below the TTX block

CGRP may accumulate in the terminals of TTX-blocked nerves because TTX blocks invasion of action potentials into nerve terminals and, hence, impulse-dependent neuropeptide release. In that case, it should be possible to empty the terminals of CGRP by stimulating the nerve below the TTX block. Accordingly, in two rats with a local sciatic block of 19 d duration, the more distally placed common peroneal nerve was stimulated electrically for 4 or 5 h with brief, intermittent, pulse trains at 100 Hz. These relatively long-lasting periods of stimulation were chosen for the first experiments to avoid missing a possible effect. Later, a time course study of 1 to 30 min was performed after the sciatic nerve had been blocked for 7 d. As shown in Fig. 6*B* and *C*, all the stimulations caused a striking reduction of the percentage of CGRP-positive motor nerve terminals, indicating that 1 min of intermittent, high frequency nerve impulse activity is sufficient to remove CGRP from the majority of the terminals. Regardless of the duration of the stimulation, however, detectable CGRP remained in approximately 5–10% of the terminals.

Stimulation of the peroneal nerve activated the EDL but not the SOL muscle. Accordingly, the stimulation emptied most of the EDL terminals of CGRP but had no effect on the SOL terminals in the same leg. In the SOL, the percentage of CGRP-positive terminals varied between 32% and 46% ($n = 8$) in the 7 d blocked legs and between 65% and 76% ($n = 2$) in the 19 d blocked legs.

The results show that the disappearance of CGRP from the EDL terminals is due to impulse activity in the nerve and not to any unspecific effect of the stimulation.

Discussion

The results obtained provide answers to the questions raised in the introductory remarks. First, during reinnervation, CGRP accumulated in motor nerve terminals and then disappeared as the junctions matured. CGRP mRNA expression in the motor neurons also increased. SgII did not accumulate, nor did its expression increase. Second, during muscle paralysis by BoTx and TTX, as during reinnervation, CGRP accumulated in the motor nerve terminals and CGRP expression increased, whereas SgII did not accumulate nor did its expression increase. Third, electrical stimulation of a TTX blocked nerve below the block emptied the motor nerve terminals of CGRP. The implications of these findings are discussed below.

Factors causing CGRP upregulation

Axotomy and nerve blocks by BoTx or TTX upregulate CGRP expression, stimulate axonal growth, and alter many other properties of motor neurons in similar ways (Watson, 1969; Brown and Irons, 1977; Sumner, 1977; Czéh et al., 1978; Pinter et al., 1991; Brown, 1984). These findings suggest that CGRP upregulation is only one aspect of a much wider response of motor neurons to injury and muscle paralysis.

Nerve injury and muscle paralysis alter the production of

Figure 5. Local nerve block by TTX upregulates CGRP expression in motor neurons and causes accumulation of CGRP in motor nerve terminals. Thirty-three days after TTX application to the sciatic nerve, neuromuscular junctions in EDL, labeled for AChR and neurofilaments (*A* and *C*), are positive for CGRP (*B*) but not SgII (*D*). In addition, CGRP mRNA content in lumbar motor neurons is increased on the treated side (*F*) relative to the contralateral side (*E*) of the same spinal cord section. SgII mRNA content is not increased (*G* and *H*). *A* and *B*: $\times 500$; *C* and *D*: $\times 770$; *EE-H*: $\times 500$.

neurotrophic growth factors in muscle and non-neural cells along the nerve. These factors are retrogradely transported to the cell bodies of motor neurons in the spinal cord, where they promote survival, support regeneration, and have other effects. For example, damaged nerves and denervated muscles increase their expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) (Meyer et al., 1992; Funakoshi et al., 1993; Henderson et al., 1993), while axons central to a lesion increase their retrograde transport of BDNF, NT-3, and ciliary neurotrophic factor (CNTF) (Curtis et al., 1993; Lindsay et al., 1994). Muscle extracts, which contain such factors, become more potent after denervation or muscle inactivation (Henderson et al., 1983, 1986), in which case they also upregulate CGRP expression (Popper et al., 1992). This potency decreases again if the denervated muscles are stimulated directly (Hill and Bennett, 1983). Motor nerve terminals on muscles paralyzed by BoTx sprout, and this sprouting is prevented by direct electrical stimulation of the muscle (Brown et al., 1980). The growth factors produced in the BoTx-treated muscle may act locally on the terminals but may also reach the motor neurons in the spinal cord because the mechanism for retrograde transport appears intact during BoTx treatment (Kristensson and Olsson, 1978). Similarly, a local nerve block by TTX alters the electrical properties of motor neurons in the spinal cord, and these changes are prevented by direct stimulation of the nerve below the block, which activates the distally located muscle but not the centrally located motor neurons (Czéh et al., 1978). Together, these findings indicate that the observed upregulation of CGRP is caused, at least in part, by growth factors, which are produced in higher than normal amounts in paralyzed muscles and damaged nerves. This conclusion does not rule out the possibility that interrupted flow of trophic factors from the periphery may contribute to

some of the responses of the motor neurons, particularly in the first stages after axotomy (Wu et al., 1993).

SgII expression is not regulated

Of the two secretory proteins examined in this study, CGRP and SgII, only CGRP was affected by the different treatments. SgII is expressed in many neurons and endocrine cells, where it is a marker for the regulated pathway of secretion (Huttner et al., 1991). At developing rat motor endplates, both CGRP and SgII are present postnatally but then disappear as the junctions mature (Matteoli et al., 1990; Andreose et al., 1994). The postnatal decline in CGRP and SgII content is accompanied by a decrease in LDCV density (Table 1). Since SgII has been suggested to be a constitutive component of LDCV (Huttner et al., 1991), it was surprising that TTX and BoTx treatments increased the density of LDCV without affecting the expression or content of SgII. The present findings show that SgII and CGRP can be independently regulated. In addition, they suggest that SgII in motor neurons has a function that is unrelated to reinnervation and LDCV biogenesis.

Accumulation and release of CGRP at the endplate

The neuropeptide CGRP is stored in LDCVs (Matteoli, et al., 1988). Accordingly, we found an increase both in the density of LDCVs and in CGRP immunoreactivity in the terminals of motor axons blocked by BoTx and TTX. Release of neuropeptides from LDCV requires relatively high impulse frequencies (Lundberg and Hökfelt, 1983), presumably because greater Ca influx is required to reach the higher average cytosolic Ca^{2+} levels necessary for neuropeptide release (Verhage et al., 1991). Accordingly, we found that high-frequency stimulation of the nerve below the TTX block emptied the majority of motor nerve terminals in less than 1 min. Such impulse-dependent release

Figure 6. Nerve block by TTX causes CGRP to accumulate in motor nerve terminals (*A*) and nerve stimulation below the block causes the accumulated CGRP to disappear (*B* and *C*). *A* shows percentage of CGRP-positive motor nerve terminals in EDL muscles at different times after local application of TTX to the sciatic nerve; *filled circles* represent blocked side, *open circles* contralateral side. *B* and *C* show percentage of CGRP positive terminals in EDL muscles after a TTX block of the sciatic nerve lasting 19 (*B*) and 7 (*C*) d. Before removing the muscles, the peroneal nerve was stimulated intermittently at 100 Hz below the block for 4 to 5 hr (*B*) or 1 to 30 min (*C*).

of CGRP from motor nerve terminals has not been convincingly demonstrated (Sakaguchi et al., 1991), possibly because the stimulation frequencies used have been too low. The stimulation frequency used in the present experiments occurs normally in EDL (Hennig and Lomo, 1985). Whether lower frequency stimulation, corresponding to the long 20 Hz trains prevailing in the normal SOL, can also release CGRP and whether SOL and EDL motor nerve terminals differ in their response to different impulse frequencies, was not examined in this work.

The rapid disappearance of CGRP during stimulation of the nerve below the TTX block indicates that CGRP accumulated in the motor nerve terminals because action potentials were prevented from invading them. BoTx also causes accumulation of CGRP in the terminals, but in this case, BoTx blocks exocytosis of ACh (Thesleff, 1989) apparently by cleaving SNAP-25, a protein component of the putative vesicle docking and fusion apparatus in nerve terminals (Schiavo et al., 1993). Since action potentials invade BoTx poisoned terminals (Dreyer et al., 1983; Angaut-Petit et al., 1990), the observed accumulation of CGRP indirectly shows that BoTx also blocks fusion of

LDCVs with the terminal membrane and, hence, the release of neuropeptides.

Axotomy, TTX, and BoTx treatments in one leg increased CGRP content in the motor nerve terminals of the opposite leg. Previously, it has been shown that axotomy in one leg induces motor nerve terminal sprouting (Rotshenker and McMahan, 1976) or enhanced transmitter release from motor nerve terminals (Herrera and Grinnell, 1981) in the other leg. The mechanisms behind these contralateral effects of axotomy remain unclear, but appear to be due to generation of intraspinal signals that pass transneuronally from the treated to the nontreated side of the spinal cord (Rotshenker and Tal, 1985). A similar mechanism may be suggested for the contralateral effects of TTX and BoTx since these toxins have axotomy-like effects in the spinal cord. In addition, systemic effects of the toxins should be considered.

Conclusions and hypothesis

To speculate fruitfully about the role of CGRP in the formation and maintenance of neuromuscular junctions, it is helpful to consider (1) that CGRP expression is transiently high during the formation of neuromuscular junctions in ontogenesis (Matteoli et al., 1990; Li and Dahlström, 1992) and reinnervation (this work), (2) that nerve impulses appear capable of releasing CGRP from motor nerve terminals (this work), (3) that muscle inactivity upregulates CGRP in motor neurons (this work), and that this upregulation may be caused by increased release and retrograde transport of trophic factors from the periphery, as suggested by the ability of extracts of inactive muscles to upregulate CGRP expression (Popper et al., 1992), (4) that CGRP may downregulate the production of trophic factors in muscle, as suggested by the inhibiting effect of CGRP on motor nerve terminal sprouting (Tsujimoto and Kuno, 1988), and (5) that CGRP from the nerve may enhance postsynaptic transmission efficiency (Lu et al., 1993), while trophic factors from the muscle, such as BDNF and NT-3, may enhance presynaptic transmitter release (Lohof et al., 1993).

These considerations suggest a novel role for CGRP in neuromuscular junction formation, which, in the absence of more direct evidence, we think of as an interesting and fruitful working hypothesis. At early stages of synaptogenesis, when the expression of neuronal CGRP and muscular trophic factors is high, enhanced release of these factors may rapidly strengthen the developing junctions and, therefore, increase the amount of nerve-evoked muscle activity. As the junctions mature, this activity may then downregulate the expression of trophic factors in the muscle, which, in turn, may cause downregulation of CGRP expression in the motor neurons. Finally, the expressions of CGRP (and presumably other neuronal factors) in motor neurons and trophic factors in muscle reach the low levels typical of mature junctions where they balance each other in maintaining the mature properties of the junctions. Any treatment, like TTX or BoTx, which destroys this balance by paralyzing the muscle, upregulates the expression of trophic factors in the muscle, which, in turn, upregulates the expression of CGRP (and other proteins) in the motor neurons. Together, these effects may then contribute to setting the stage for repair and reformation of junctions.

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