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SHORT COMMUNICATION

Regulation of Motoneuronal Calcitonin Gene-related Peptide (CGRP) During Axonal Growth and Neuromuscular Synaptic Plasticity Induced by Botulinum Toxin in Rats

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Abstract

The aim of this study was to examine whether changes in rat motoneuronal calcitonin gene-related peptide (CGRP) can be correlated with axonal growth and plasticity of neuromuscular synapses. Nerve terminal outgrowth was induced by local paralysis with botulinum toxin. Normal adult soleus and tibialis anterior did not show detectable CGRP content at the motor endplates. Following botulinum toxin injection there was a progressive, transient and bimodal increase in CGRP in both motoneuron cell bodies which innervated poisoned muscles and their motor endplates. CGRP content was moderately increased 1 day after paralysis and, after an initial decline, reached a peak 20 days after injection. This was followed by a gradual decrease and a return to normal levels at the 200th day. CGRP changes in intoxicated endplates were less evident in the tibialis anterior than in the soleus muscle. The CGRP content in motoneurons was positively correlated with the degree of intramuscular nerve sprouting found by silver staining. *In situ* hybridization revealed an increase in CGRP mRNA in spinal cord motoneurons 20 days after toxin administration. We conclude that motoneurons regulate their CGRP in situations in which peripheral synapse remodelling and plasticity occur.

Introduction

Calcitonin gene-related peptide (CGRP) is a 37-amino acid peptide (Rosenfeld *et al.*, 1983) present in central neurons and in cell bodies of a large number of cranial and spinal motoneurons (Gibson *et al.*, 1988; Calderó *et al.*, 1992). However, immunohistochemical studies have shown that CGRP immunoreactivity is very weak or absent in most adult motor endplates and that it only abounds in the motor nerve terminals of certain striated muscles (Csillik *et al.*, 1993).

Several studies have suggested that CGRP is an important trophic signal for the development, differentiation and maintenance of muscle cells and neuromuscular connections (New and Mudge, 1986; Fontaine *et al.*, 1987; Esquerda *et al.*, 1989; Miles *et al.*, 1989; Matteoli *et al.*, 1990; Li and Dahlström, 1992), and for the regulation of intramuscular nerve growth (Tsujimoto and Kuno, 1988). Previous investigations have shown that CGRP up-regulation takes place in adult cranial and spinal motoneurons after peripheral nerve injury. A maximum increase in CGRP is followed by a gradual decline in line with the morphological and functional recovery of motor innervation (Streit *et al.*, 1989; Arvidsson *et al.*, 1990; Hass *et al.*, 1990; Calderó *et al.*, 1992).

In this study we attempt to determine whether the motoneuronal CGRP up-regulation that follows peripheral nerve axotomy is associated with the complex cell body reaction that follows injury or if it represents a more specific biological response linked to peripheral nerve growth. For this purpose, we have examined CGRP changes in motoneurons following paralysis with botulinum toxin, a situation in which intramuscular nerve outgrowth and neuromuscular synaptic remodelling occurs (Brown *et al.*, 1981). This study complements recent results reported by Sala *et al.* (1995).

Materials and methods

Animals and tissue preparation

Adult male Sprague–Dawley rats (200–250 g) were anaesthetized with chloral hydrate (450 mg/kg body weight). Rats were locally paralysed with a single injection of 800 μl (20 LD_{50}) of *Clostridium botulinum* type A toxin dissolved in 70 mM phosphate buffer, pH 6.5, containing 0.25% gelatin. Botulinum toxin was applied subcutaneously to the right hind limbs, while the left hind limbs

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received only a single injection of the vehicle solution, which served as a control. Local paralysis, restricted to the leg treated with botulinum toxin, was achieved 24–48 h after injection. Motoneurons innervating the extensor digitorum longus (EDL) muscle were retrogradely traced 2 days before killing the rats (see below). Rats were anaesthetized and killed by intracardiac perfusion of 200 ml physiological saline solution followed by a solution of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Lumbar spinal cords (between levels T12 and L3) and EDL, soleus and tibialis anterior muscles were then dissected and removed.

Retrograde tracing of motoneurons

The location of cell bodies of EDL muscle innervating motoneurons was performed by retrograde labelling using cholera toxin B subunit (CTB; List Biological Laboratories, Campbell, CA). One milligram of lyophilized CTB was reconstituted with 1 ml of distilled water, dialysed against phosphate buffer to remove NaN_3, and then concentrated to 1% with a Centricon-10 microconcentrator (Amicon, Beverly, MA). Three injections (5 µl each) of tracer were applied to selected muscles on both sides, using pulled glass capillary tubes attached to a 10 µl Hamilton syringe. After survival periods of 48–96 h, the rats were re-anaesthetized and perfused.

Immunohistochemistry

Samples were postfixed overnight in paraformaldehyde at 4°C. Samples were frozen following cryoprotection with several changes of 20% (wt/vol) sucrose in 0.1 M phosphate buffer.

Free-floating longitudinal frozen sections (40 µm thick) of lumbar spinal cord were washed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100 and incubated with 3% normal goat serum for 30 min. Sections were then simultaneously incubated with rabbit antiserum against rat CGRP (Peninsula Laboratories, Belmont, CA; diluted 1/500) and goat antiserum against cholera toxin (List Biological Laboratories; diluted 1/4000) for 48 h at 4°C. After several washes, sections were incubated with a mixture of a biotinylated monoclonal antibody against goat immunoglobulins (Sigma; 50 µg/ml) and a fluorescein isothiocyanate (FITC)-labelled monoclonal antibody against rabbit immunoglobulins (Sigma; 1/40). Sections were subsequently washed and incubated with tetramethyl rhodamine isothiocyanate (TRITC)-labelled avidin (Sigma; 10 µg/ml). Secondary incubations were made for 1 h at room temperature.

Cryostat sections (20 µm thick) of dissected muscles were placed on gelatin-coated glass slides and incubated overnight at 4°C with the antiserum against CGRP. After washes, sections were incubated for 1 h at room temperature with FITC-labelled goat anti-rabbit IgG antibody (Sigma; 1/40) mixed with TRITC-conjugated α-bungarotoxin (Molecular Probes; 1 µg/ml) to identify the motor endplates.

In some cases, spinal cord and muscle sections were processed for peroxidase–antiperoxidase immunocytochemical staining.

Silver staining of nerve terminals

EDL muscles from rats treated with botulinum toxin were fixed with 10% formalin for >15 days. Cryostat obtained free-floating sections (40 µm thick) were processed using the Gros–Bielschowsky silver impregnation method in order to visualize nerve terminal arborization. Sections were analysed at 1000×. At least 50 random nerve terminals were drawn for every muscle (using a camera lucida) and their lengths were measured using a digital table analyser (MOP Videoplan, Kontron, Germany). Measurements from botulinum toxin-treated and contralateral muscles were compared. Branching points (number of terminal branches for each endplate) in the same nerve terminals were also counted to see the degree of motor endplate complexity after botulinum toxin paralysis.

In situ hybridization studies

For *in situ* hybridization studies, rats were perfused first with physiological saline solution and then with 4% paraformaldehyde in 0.1 M borate buffer, pH 9.5. The spinal cord segment between levels T12 and L3 was removed, postfixed for 4 h by immersion in the same fixative at 4°C, and left overnight in phosphate buffer containing 30% sucrose at 4°C. Cryostat sections (10 µm thick) were collected on slides coated with Vectabond (Vector Laboratories).

Probes for *in situ* hybridization were made by transcribing linearized SP64 plasmid (Gibson *et al.*, 1988) in which 440 bp of the 3′ non-coding sequences of rat α-CGRP were subcloned. Radiolabelled complementary (antisense) and non-complementary (sense) mRNA probes for *in situ* hybridization were made by transcribing linearized plasmid in the presence of [^{32}P]CTP. Hybridization was carried out following the method of Hamid *et al.* (1987). Briefly, sections were first permeabilized in a solution of 0.1% Triton X-100 in PBS and then with proteinase K 1 mg/ml in Tris/50 mM EDTA pH 8 for 10 min at 37°C. Afterwards, they were postfixed with 4% paraformaldehyde in PBS for 3 min. Before hybridization, the sections were immersed for 10 min in a solution of 0.25% acetic anhydride and 0.1 M triethanolamine. Ten microlitres of labelled probes, diluted at 0.5 ng/µl in hybridization buffer, was applied to each slide and covered with a siliconized coverslip. The sections were incubated for 16–20 h at 42°C, washed, dehydrated, dipped in emulsion (Ilford K-5) and exposed for 5 days at 4°C. Hybridization specificity was checked either by hybridization of complementary probes on sections previously treated with RNase or by incubation of sections with non-complementary probes to α-CGRP mRNA.

Quantification of immunostaining and in situ hybridization radiolabelling

In situ hybridization radiolabelling and CGRP immunostaining were measured using a photomultiplier adapted to a Zeiss Axiovert 10 inverted microscope controlled by Photan software (Zeiss, Oberkochen, Germany). BP 485 excitation and BP 515–565 stop filters were use for immunofluorescence with FITC and BP 546 excitation and LP 590 stop filters for TRITC. No interference was detected between the two fluorochromes. Adjustment and standardization of the photomultiplier was performed using a fluorescent standard (Fl-Standard 474256 from Zeiss). Photometry with transmitted light for quantifying hybridization grain density was standardized by measuring background transmittance in tissue regions devoid of specific labelling. All traced motoneurons innervating EDL muscle were measured for each animal. When *in situ* hybridization was performed, all radiolabelled lumbar motoneurons were measured. Recorded data for each set of samples were analysed using a classification procedure based on a pattern recognition method (Escudero, 1977). In each experiment, CGRP-immunoreactive or CGRP mRNA-radiolabelled motoneurons were classified into three groups (1+, 2+ and 3+) ranging from low to high intensity.

All immunostained muscle sections were examined under an Olympus BH-2 microscope equipped with epifluorescence illumination and also under a confocal laser-scanning Zeiss LSM-310 microscope equipped with 543 nm helium/neon and 488 nm argon ion lasers. CGRP-like immunoreactivity was analysed in at least 150 motor endplates per muscle. Motor endplates were considered CGRP-positive when the intensity of immunoreactivity was clearly visible and CGRP-negative when the immunofluorescence was absent or very weak.

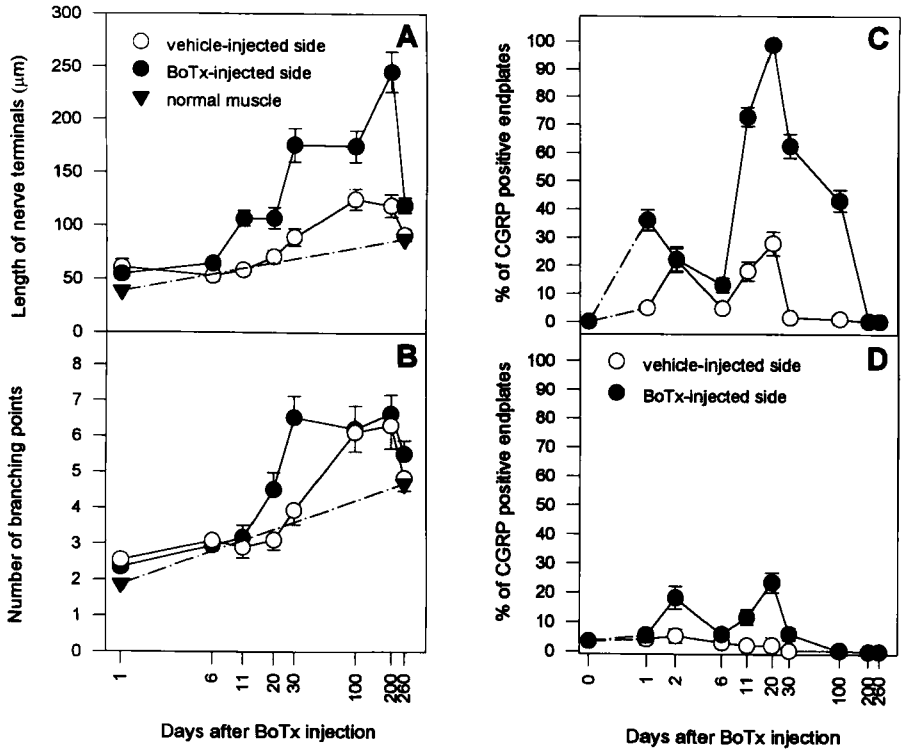

FIG. 1. Time course of changes in length of nerve terminals (A) and number of branching points per endplate (B) from silver-stained right-side muscles (side injected with botulinum toxin, BoTx), left-side muscles (vehicle-injected side) and untreated muscles of the same age (normal muscle). At least 50 endplates were measured on each muscle. Time course of changes in CGRP at the neuromuscular junctions of soleus (C) and tibialis anterior (D) muscles after local injection of botulinum toxin. The proportion of endplates showing positive CGRP immunoreactivity was evaluated in the botulinum toxin-injected (●) and in the contralateral (vehicle-injected, ○) muscles from the same animals. At least 150 endplates were evaluated on each muscle. All points represent mean ±95% confidence intervals from two to four animals. Error bars are sometimes smaller than symbols and consequently not always visible.

Results

After local injection of botulinum toxin, the muscles became paralysed within 24 h and progressively developed nerve terminal sprouting. Outgrowth of motor nerve terminals after paralysis was quantified by measuring the length and number of nerve terminal branching points on each EDL muscle. A notable increase in motor nerve terminal length and complexity was observed (Fig. 1A, B). These plastic changes induced by botulinum toxin had a heterogeneous distribution in the muscles studied, because motor endplates with prominent outgrowth could be seen very near to others exhibiting very short and less complex sprouts. Despite the fact that muscle activity recovered ~30 days after toxin injection, motor nerve terminals continued to grow without any evidence of regression to normal morphology until 200 days following botulinum toxin administration. Contralateral (vehicle-injected) muscles also exhibited some increase in the length and complexity of their motor nerve terminals in a similar way to ipsilateral muscles.

Endplate CGRP-like immunoreactivity in soleus and tibialis anterior muscles was evaluated at the same intervals after botulinum toxin administration (Fig. 1C, D). A rapid increase in the number of positive endplates was observed in soleus muscles 1 day after toxin injection, coinciding with the appearance of the first signs of local paralysis (Fig. 1C). Following this increase, the percentage of CGRP-positive endplates declined progressively until the sixth day. A very considerable increase could be seen 20 days after paralysis, when all endplates studied were intensely positive and presynaptic axon terminals displayed a strong CGRP reaction in most of them. Moreover, using a confocal laser-scanning microscope it was easy to demonstrate that CGRP-like immunoreactivity had a presynaptic distribution clearly dissociated from that of the postsynaptic marker TRITC–α-bungarotoxin (Fig. 2b). CGRP-like immunoreactivity declined progressively as the interval after botulinum toxin treatment increased. At 200 days, immunoreactivity reached low levels, similar to those found in untreated animals. A positive CGRP-like immunoreactive response, with a similar time course but lower intensity, was also detected in the contralateral (vehicle-injected) muscles (Fig. 1C). Although present, changes in the CGRP level in intoxicated endplates from the tibialis anterior muscle were less evident and the recovery of base levels was more rapid than in the soleus muscle (Fig. 1D).

Changes in CGRP-like immunoreactivity in EDL-innervating motoneurons were evaluated after botulinum toxin paralysis. CTB-traced neuron bodies were seen to have cytoplasm filled with fluorescent granules extending to the proximal part of the neuronal processes, but with nuclei remaining unstained (Fig. 3a, b). Measurements of nerve terminal length and branching points in silver-stained sections did not show any significant difference when CTB-injected muscles and non-traced muscles from normal rats were compared (data not shown).

In vehicle-injected samples a considerable proportion of the motoneurons traced with CTB showed positive CGRP-like immunoreactivity. The percentage of CGRP-negative EDL-innervating motoneurons counted in spinal cord control samples was ~25%, and CGRP-like immunoreactivity varied from slight to moderate or intense. Changes in EDL-innervating motoneurons on both sides of the spinal cord (botulinum toxin- and vehicle-injected sides) were evaluated at several time periods ranging from 1 to 200 days after

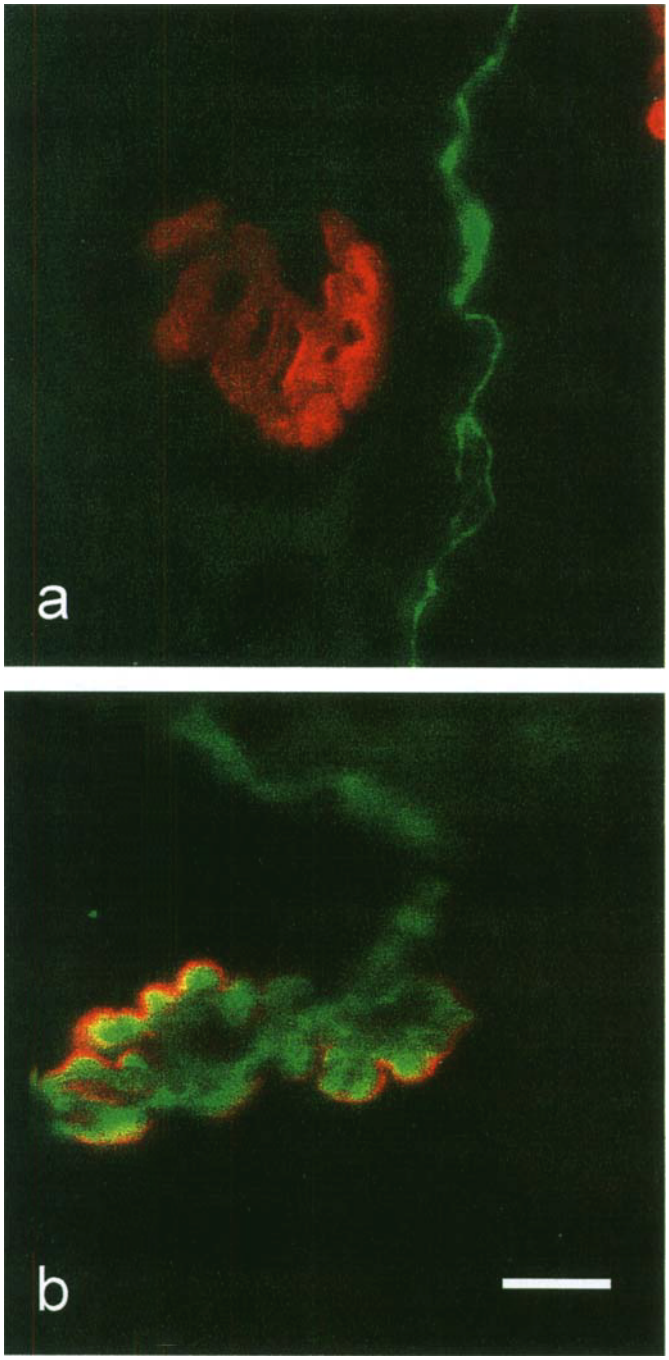

FIG. 2. Confocal laser-scanning micrographs of soleus muscle endplates from a control animal (a) and after 20 days of local injection of botulinum toxin (b), double-labelled for simultaneous demonstration of acetylcholine receptors stained with TRITC–α-bungarotoxin (red) and CGRP by means of immunolabelling with FITC (green). In (a) only red fluorescence is observed at the endplate because of the absence of CGRP immunolabelling; the CGRP-positive (green) nerve fibre running near the endplate is an axon that belongs to a small population of CGRP-positive fibres (sensorial or autonomic) normally present in control muscles which do not innervate skeletal muscle fibres. In (b) red fluorescence surrounds the green buttons connected with the axon terminal, demonstrating the presynaptic location of CGRP labelling. Scale bar (valid for both panels) = 10 μm.

injection (Fig. 4A, B). To simplify the presentation of results, we have plotted changes only for the percentage of 3+ motoneurons in Figure 4A. During the first 2 days after injection, there was an initial peak in the number of motoneurons showing a high intensity of CGRP-like immunoreactivity followed by a gradual decline. However, the main peak in CGRP-like immunoreactivity was produced 20 days after intoxication, coinciding with the maximum increase in CGRP-like immunoreactivity in motor endplates (Fig. 1C). CGRP-like immunoreactivity later dramatically fell to around base levels at 100 days. This type of response could also be observed in EDL-innervating motoneurons on the vehicle-injected side, although to a lesser degree, demonstrating the existence of some type of contralateral response to botulinum toxin intoxication.

The increase in CGRP mRNA expression in lumbar motoneurons

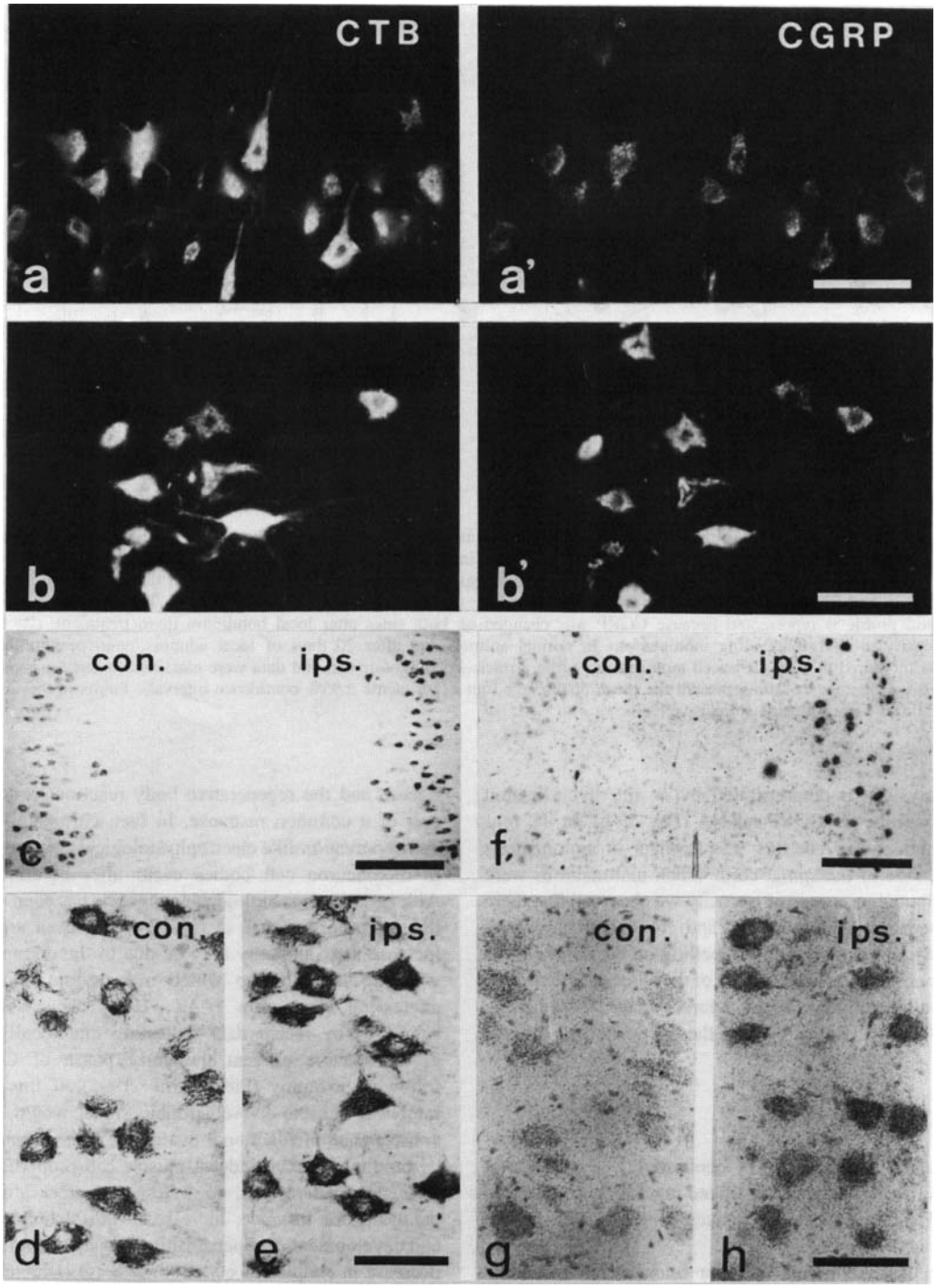

FIG. 3. Local intoxication with botulinum toxin produces up-regulation of CGRP in motoneuron cell bodies. (a, a', b, b') Photomicrographs showing CTB-traced motoneurons double-labelled to demonstrate the presence of CTB with TRITC (a and b) and CGRP-like immunoreactivity with FITC (a' and b'). CTB was injected into EDL muscles and was visualized 72 h after its application. CGRP-like immunoreactivity is shown in the ipsilateral side of the spinal cord 24 h (a') and 20 days (b') after botulinum toxin injection. Note that the fluorescence is much stronger in b' than in a', indicating the increase in CGRP content after local paralysis. (c–e) Photomicrographs showing CGRP-like immunoreactivity obtained using the peroxidase–antiperoxidase procedure. (c) Longitudinal section through the ventral horn of the lumbar spinal cord 30 days after botulinum toxin injection; the CGRP-like activity in motoneurons of the poisoned side (ips.) is markedly increased compared with the vehicle-injected side (con.). (d, e) Higher magnification of both sides seen in (c). (f–h) Autoradiographs following *in situ* hybridization with a specific ^{32}P-riboprobe for rat CGRP mRNA. (f) Longitudinal section through the ventral horn of the lumbar spinal cord 20 days after botulinum toxin injection; note the increase in CGRP mRNA expression in the poisoned side (ips.) compared to the vehicle-injected side (con.). (g, h) Higher magnification of both sides seen in (f) showing that the grain density over motoneurons is higher in g than in h. Scale bars = 100 μm for a–b', d, e, g and h and 500 μm for c and f.

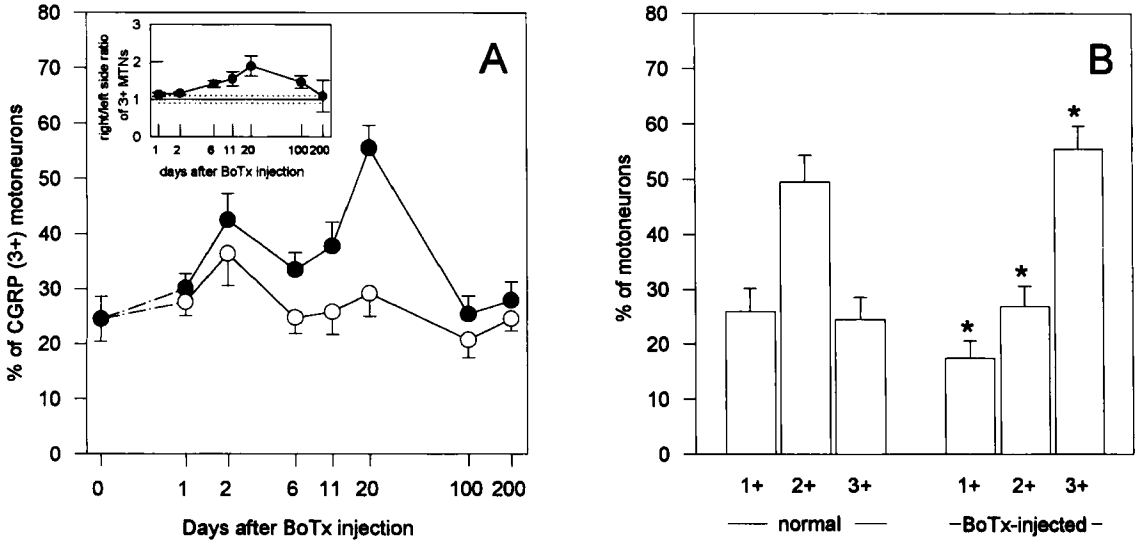

FIG. 4. (A) Time-course changes in CGRP in the motoneuron cell bodies innervating EDL muscle. The proportion of motoneurons showing higher CGRP immunoreactivity (3+ neurons) is represented as a function of time after botulinum toxin injection. ●, motoneurons on the right (botulinum toxin-injected) side of the spinal cord; ○, motoneurons on the left (vehicle-injected) side. Changes in right/left side ratio are shown in the inset graph, in which the steady state obtained from measures in normal animals is represented by a continuous line; the SEM is indicated by dashed lines. The bimodal time course response cannot be seen when the ratio profile is represented because CGRP was changed on both sides after local botulinum toxin treatment. (B) Distribution of CGRP immunolabelling intensity in EDL-innervating motoneurons in normal animals and after 20 days of local administration of botulinum toxin. CGRP-like immunoreactivity was measured in all CTB-traced motoneurons using a microscope photometer, and data were classified in three groups of intensity (1+, 2+ and 3+; see Materials and methods). Data represent the mean of three or four experiments ±95% confidence intervals. Points in the inset graph are the ratio values ±SEM. $*P < 0.001$ versus normal animals, χ^2 test.

20 days after paralysis was demonstrated by *in situ* hybridization using a specific riboprobe for CGRP mRNA (Fig. 3f–h). In the four rats studied, the intensity of labelling was stronger in motoneurons from the paralysed side of the spinal cord. When motoneurons were automatically distributed in groups of intensity we observed that there were more motoneurons exhibiting 2+ and 3+ labelling on the paralysed side, and more exhibiting 1+ labelling on the contralateral side. If the sense riboprobe was used instead of the antisense riboprobe, no over-background labelling was found. Moreover, specific labelling was almost completely eliminated when the sections were treated with RNase.

Discussion

Here we show that the remodelling of adult motor endplates that follows botulinum toxin paralysis is linked to major changes in motoneuronal CGRP and that these changes correlate with plastic changes in motor nerve terminals.

Normal adult soleus and tibialis anterior muscles did not show detectable CGRP-like immunoreactivity at the motor endplates. Following botulinum toxin injection, the motor endplates in both muscles become strongly immunoreactive, although the degree of response is higher in the soleus than in the tibialis anterior muscle. Different fibre type properties and sprouting capacities of these muscles may account for these differences (Brown *et al.*, 1981).

Quantification of CGRP at nerve terminals and motoneuron cell bodies after toxin application gave a bimodal time-course profile for the study period (260 days). Early in muscle paralysis (24–48 h after botulinum toxin injection) a certain increase in CGRP-like immunoreactivity could be detected in both the endplates and motoneuron somas. Since motoneuronal CGRP exhibits similar bimodal behaviour after motor nerve axotomy (Dumoulin *et al.*, 1991; own unpublished observations) it seems that the blockade of acetylcholine release and the regenerative body reaction to nerve transection are part of a common response. In fact, chromatolysis (Watson, 1969) and denervation-like electrophysiological changes (Pinter *et al.*, 1991) in motoneuron cell bodies occur after botulinum toxin paralysis. Although no clear biological hypothesis has been suggested to explain this biphasic response of CGRP, it has been argued that the initial increase after axotomy may be due to the disruption of connections with the target tissue which may physiologically inhibit CGRP expression (Dumoulin *et al.*, 1991). This assumption has been supported by recent data indicating that locally applied fibroblast growth factors prevent the early phase of CGRP up-regulation following axotomy (Piehl *et al.*, 1995). If this were the case, the inhibitory muscle-derived trophic signal would also disappear as a consequence of functional disruption of neuromuscular transmission by botulinum toxin. Indeed, muscle cell properties are changed both after denervation and, to a lesser degree, after botulinum toxin paralysis; for instance, increase in acetylcholine receptor synthesis and development of supersensitivity to acetylcholine (Thesleff, 1960), decrease in endplate acetylcholinesterase (Drachman, 1972), and up-regulation of muscle-derived growth factors such as insulin-like growth factor II [IGF-II (Ishii, 1989)] are present after muscle inactivation by botulinum toxin. Consequently, it would not be surprising if both botulinum toxin and nerve section gave similar responses in the CGRP level by cancelling out muscle-derived signals that might normally down-regulate CGRP levels in motoneurons. Moreover, it should be noted that as botulinum toxin disrupts the calcium-dependent exocytic machinery (Molgó *et al.*, 1990), the release of CGRP might also be blocked by the toxin. In this case, an initial accumulation of CGRP might be expected, within the nerve terminals, further contributing to the first peak.

The most remarkable rise in motoneuron and nerve terminal CGRP levels took place 20 days after botulinum toxin injection. At this time all soleus endplates were strongly immunoreactive, and it is interesting

to note that poisoned nerve terminals were fully grown. In a comparable situation, such as nerve crush, in which nerve growth coexists with CGRP up-regulation, motoneuronal CGRP reaches normal CGRP levels within a time period concurrent with the maximum recovery of motor innervation (Calderó *et al.*, 1992). It has also been seen that during the synaptic remodelling that takes place in postnatal life there is transient up-regulation of endplate CGRP (Matteoli *et al.*, 1990; Li and Dahlström, 1992; own unpublished observations). Summing up, it seems that CGRP increases when motor nerve terminals are either immature or unstable. However, as reported here, within a period of 20–200 days, the level of CGRP slowly drops as nerve terminal sprouts continue growing. This indicates that in the long term a clear dissociation occurs between CGRP level and the stability of nerve–muscle contacts after botulinum toxin treatment. Since the maximum CGRP-like immunoreactivity attained after intoxication drops over a time period which coincides with the functional recovery of muscle paralysis, it is possible that restoration of the neurotransmitter release mechanisms leads to depletion of CGRP retained inside nerve terminals. This CGRP could be used to promote the differentiation of new postsynaptic structures at the extrajunctional muscle fibre areas.

In agreement with Sala *et al.* (1995), local paralysis by botulinum toxin also determines, to some degree, a contralateral CGRP response in motoneuron somas and endplates. These changes coincide with the moderate outgrowth that we have detected in contralateral motor nerve terminals following botulinum toxin injection. Since botulinum toxin has denervation-like effects in spinal cord motoneurons, our results are in harmony with reports which have demonstrated that unilateral axotomy can induce chromatolytic-like changes in the contralateral motoneurons (Lieberman, 1971) and promotes contralateral motor nerve terminal sprouting (Rotshenker and Tal, 1985). Moreover, Caroni and Becker (1992) have recently shown contralateral changes in motoneuron GAP-43 and tubulin-α1 following either local paralysis with botulinum toxin or subcutaneous injections of IGF-I. The reason for these contralateral reactions after axotomy or local paralysis is not clear at present; however, it seems that retrograde signals transmitted through commissural interneurons which connect the ipsi- and contralateral sides of the spinal cord could hold the key to this hidden mechanism (Rotshenker and Tal, 1985).

The coexistence of motoneuronal CGRP up-regulation with exacerbated nerve terminal growth after botulinum toxin intoxication may be difficult to reconcile with the 'antisprouting activity' of externally applied CGRP demonstrated by Tsujimoto and Kuno (1988) in tetrodotoxin-paralysed muscles. We have not been able to prevent botulinum toxin-induced nerve terminal sprouting by subcutaneous injections of CGRP (J. E. Esquerda, J. Molgó, O. Tarabal, J. X. Comella and J. Calderó, in preparation). Since botulinum toxin and tetrodotoxin have different actions on neuromuscular transmission (i.e. on presynaptic calcium currents; Katz and Miledi, 1967; Gundersen *et al.*, 1982) and presumably on nerve–muscle interactions, the exact role of exogenous CGRP in controlling nerve terminal growth requires further examination (for discussion of this point see Calderó *et al.*, 1992).

We conclude that motoneuronal CGRP changes are closely correlated with axon growth and neuromuscular synapse plasticity. The nature of the muscle-derived signals or neurotrophic factors involved in the regulation of motoneuronal CGRP expression and function remain to be determined.

Acknowledgements

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Abbreviations

CGRP	calcitonin gene-related peptide
CTB	cholera toxin B subunit
EDL	extensor digitorum longus
EDTA	ethylenediamine tetraacetic acid
FITC	fluorescein isothiocyanate
PBS	phosphate-buffered saline
TRITC	tetramethyl rhodamine isothiocyanate

References

Arvidsson, U., Johnson, H., Piehl, H., Culheim, S., Hökfelt, T., Risling, M., Terenius, L. and Ulfhake, B. (1990) Peripheral nerve section induces increased levels of calcitonin gene-related peptide (CGRP)-like immunoreactivity in axotomized motoneurons. *Exp. Brain Res.*, **79**, 212–216.

Brown, M. C., Holland, R. L. and Hopkins, W. G. (1981) Motor nerve sprouting. *Annu. Rev. Neurosci.*, **4**, 17–42.

Calderó, J., Casanovas, A., Sorribas, A. and Esquerda, J. E. (1992) Calcitonin gene-related peptide in rat spinal cord motoneurons: subcellular distribution and changes induced by axotomy. *Neuroscience*, **48**, 449–461.

Caroni, P. and Becker, M. (1992) The downregulation of growth-associated proteins in motoneurons at the onset of synapse elimination is controlled by muscle activity and IGF-1. *J. Neurosci.*, **12**, 3849–3861.

Csillik, B., Tajti, L., Kovacs, T., Kukla, E., Rakic, P. and Knyiharscsillik, E. (1993) Distribution of calcitonin gene-related peptide in vertebrate neuromuscular junction—relationship to the acetylcholine receptor. *J. Histochem. Cytochem.*, **41**, 1547–1555.

Drachman, D. B. (1972) Neurotrophic regulation of muscle cholinesterase: effects of botulinum toxin and denervation. *J. Physiol. (Lond.)*, **226**, 619–627.

Dumoulin, F. L., Raivich, G., Streit, W. J. and Kreutzberg, G. W. (1991) Differential regulation of calcitonin gene-related peptide (CGRP) in regenerating rat facial nucleus and dorsal root ganglion. *Eur. J. Neurosci.*, **3**, 338–342.

Escudero, L. F. (1977) *Reconocimiento de Patrones*. Ed. Paraninfo, Madrid.

Esquerda, J. E., Ciutat, D. and Comella, J. X. (1989) Absence of histochemical immunoreactivity to calcitonin gene-related peptide (CGRP) in spinal cord motoneurons from (+)tubocuranine-treated chick embryos. *Neurosci. Lett.*, **105**, 1–6.

Fontaine, B., Klarsfeld, A. and Changeux, J.-P. (1987) Calcitonin gene-related peptide and muscle activity regulate acetylcholine receptor α-subunit mRNA levels by distinct intracellular pathways. *J. Cell Biol.*, **105**, 1337–1342.

Gibson, S. J., Polak, J. M., Giaid, A., Hamid, Q. A., Kar, S., Jones, P. M. and Denny, P. (1988) Calcitonin gene-related peptide messenger RNA is expressed in sensory neurons of the dorsal root ganglia and also in spinal motoneurones in man and rat. *Neurosci. Lett.*, **91**, 283–288.

Gundersen, C. B., Katz, B. and Miledi, R. (1982) The antagonism between botulinum toxin and calcium in motor nerve terminals. *Proc. R. Soc. Lond. Ser. B*, **216**, 369–376.

Hamid, Q., Wharton, J., Terenghi, G., Hassall, C. J. S., Aimi, J., Taylor, K. M., Nakazato, H., Dixon, J. E., Burnstod, G. and Polak, J. M. (1987) Localization of atrial natriuretic peptide mRNA and immunoreactivity in rat and human atrial appendages. *Proc. Natl Acad. Sci. USA*, **84**, 760–6764.

Hass, C. A., Streit, W. J. and Kreutzberg, G. W. (1990) Rat facial motoneurons express increased levels of calcitonin gene-related peptide mRNA in response to axotomy. *J. Neurosci. Res.*, **27**, 270–275.

Ishii, D. N. (1989) Relationship of insulin-like growth factor II gene expression in muscle to synaptogenesis. *Proc. Natl Acad. Sci. USA*, **86**, 2898–2902.

Katz, B. and Miledi, R. (1967) Tetrodotoxin and neuromuscular transmission. *Proc. R. Soc. Lond. Ser. B*, **167**, 8–22.

Li, J. Y. and Dahlström, A. (1992) Development of calcitonin gene-related peptide, chromogranin A, and synaptic vesicle markers in rat motor endplates, studied using immunofluorescence and confocal laser scanning. *Muscle Nerve*, **15**, 984–992.

Lieberman, A. R. (1971) The axon reaction. A review of the principal features of perikaryal responses to axon injury. *Int. Rev. Neurobiol.*, **14**, 49–124.

Matteoli, M., Balbin, S., Sala, C., Chini, B., Cimino, M., Vitadello, M. and Fumagalli, G. (1990) Developmentally regulated expression of calcitonin gene-related peptide at mammalian neuromuscular junction. *J. Mol. Neurosci.*, **2**, 175–184.

Miles, K., Greengard, P. and Huganir, R. L. (1989) Calcitonin gene-related peptide regulates phosphorylation of the nicotinic acetylcholine receptor in rat myotubes. *Neuron*, **2**, 1517–1524.

Molgó, J., Comella, J. X., Angaut-Petit, D., Pécot-Dechavassine, M., Tabti, N., Faille, L., Mallart, A. and Thesleff, S. (1990) Presynaptic actions of botulinal neurotoxins at vertebrate neuromuscular junctions. *J. Physiol. (Paris)*, **84**, 152–166.

New, H. V and Mudge, A. W. (1986) Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature*, **323**, 809–811.

Piehl, F., Ru-Rong, J., Culheim, S., Hökfelt, T., Lindholm, D. and Hughes, R. A. (1995) Fibroblast growth factors regulate calcitonin gene-related peptide mRNA expression in rat motoneurons after lesion and culture. *Eur. J. Neurosci.*, **7**, 1739–1750.

Pinter, M. J., Vanden Noven, S., Muccio, D. and Wallace, N. (1991) Axotomy-like changes in cat motoneuron electrical properties elicited by botulinum toxin depend on the complete elimination of neuromuscular transmission. *J. Neurosci.*, **11**, 657–666.

Rosenfeld, M. G., Mermod, J. J., Amara, S. G., Swanson, L. W., Sawchenko, P. E., Rivier, J., Vale, W. W. and Evans, R. M. (1983) Production of novel neuropeptide encoded by the calcitonin gene via tissue-specific RNA processing. *Nature*, **304**, 129–135.

Rotshenker, S. and Tal, M. (1985) The transneuronal induction of sprouting and synapse formation in intact mouse muscles. *J. Physiol. (Lond.)*, **434**, 257–270.

Sala, C., Andreose, J. S., Fumagalli, G. and Lomo, T. (1995) Calcitonin gene-related peptide: possible role in formation and maintenance of neuromuscular junctions. *J. Neurosci.*, **15**, 520–528.

Streit, W. J., Dumoulin, F. L., Raivich, G. and Kreutzberg, G. W. (1989) Calcitonin gene-related peptide increases in rat facial motoneurons after peripheral nerve transection. *Neurosci. Lett.*, **101**, 143–148.

Thesleff, S. (1960) Supersensitivity of skeletal muscle produced by botulinum toxin. *J. Physiol. (Lond.)*, **151**, 598–607.

Tsujimoto, T. and Kuno, M. (1988) Calcitonin gene-related peptide prevents disuse-induced sprouting of rat motor nerve terminals. *J. Neurosci.*, **8**, 3951–3957.

Watson, W. E. (1969) The response of motor neurones to intramuscular injection of botulinum toxin. *J. Physiol. (Lond.)*, **202**, 611–630.