Transynaptic effects of tetanus neurotoxin in the oculomotor system

David González-Forero,1 Sara Morcuende,1 Francisco J. Alvarez,2 Rosa R. de la Cruz1 and Ángel M. Pastor1

1Departamento de Fisiología y Zoología, Universidad de Sevilla, Spain and 2Department of Anatomy and Physiology, Wright State University, OH, USA

Correspondence to: Dr Angel M. Pastor, Departamento de Fisiología y Zoología, Facultad de Biología, Avda. Reina Mercedes, 6, 41012-Sevilla, Spain
E-mail: ampastor@us.es

The question whether general tetanus arises from the independent sum of multiple local tetani or results from the actions of the transynaptic tetanus neurotoxin (TeNT) in higher brain centres remains unresolved. Despite the blood-borne dissemination of TeNT from an infected wound, the access to the central nervous system is probably prevented by the blood–brain barrier. However, several long-term sequelae (e.g. autonomic dysfunction, seizures, myoclonus, and sleep disturbances) present after the subsidence of muscle spasms might be indicative of central actions that occur farther away from lower motoneurons. Subsequently, the obvious entry route is the peripheral neurons followed by the transynaptic passage to the brain. We aimed at describing the pathophysiological correlates of TeNT translocation using the oculomotor system as a comprehensive model of cell connectivity and neuronal firing properties. In this study, we report that injection of TeNT into the medial rectus muscle of one eye resulted in bilateral gaze palsy attributed to firing alterations found in the contralaterally projecting abducens internuclear neurons. Functional alterations in the abducens-to-oculomotor internuclear pathway resembled in part the classically described TeNT disinhibition. We confirmed the transynaptic targeted action of TeNT by analysing vesicle-associated membrane protein2 (VAMP2) immunoreactivity (the SNARE protein cleaved by TeNT). VAMP2 immunoreactivity decreased by 94.4% in the oculomotor nucleus (the first synaptic relay) and by 62.1% presynaptic to abducens neurons (the second synaptic relay). These results are the first demonstration of physiological changes in chains of connected neurons that are best explained by the transsynaptic action of TeNT on premotor neurons as shown with VAMP2 immunoreactivity which serves as an indicator of TeNT activity.

Keywords: deafferentation; neuronal excitability; oculomotor; synaptic plasticity; VAMP2

Abbreviations: TeNT = tetanus neurotoxin; VAMP2 = vesicle-associated membrane protein2; FR = firing rate; SYN = synaptophysin; CR = calretinin


Introduction

Although tetanus should have been a clinical rarity consigned to laboratories long ago, it is estimated that it affects nearly a million people every year. Neither immunization plans nor the facilities to diagnose or treat tetanus are widespread (Thwaites and Farrar, 2003). Following generalized tetanus, recovery is complete after several weeks of intensive care. Nonetheless, the sequelae of tetanus include complaints of bed sores and sleep disturbances that might have a diverse origin. Others, like autonomic dysfunctions, limb contractions, seizures, and myoclonus indicate possible lingering actions of tetanus in the central nervous system (Goonetilleke and Harris, 2004; Wasay et al., 2005). Similarly, tetanus neurotoxin (TeNT) injections in the rat hippocampus reproduce temporal lobe epilepsy (Milward et al., 1999).

There is, therefore, a demand for a fast and reliable diagnosis of tetanus. The current form of clinical diagnosis requires a positive culture of Clostridium tetani obtained from a wound to ascertain the disease (Goonetilleke and Harris, 2004). Moreover, the differential diagnosis for tetanus might be confused by dystonic reactions occasioned...
by strychnine intoxication, meningitis, acute abdominal pain, or psychogenic pseudotetanus among others (Bleck, 1989; Quackenbush, 2003). Tetanus has long been recognized as a strychnine-like syndrome disinhibiting and thus tetanizing, lower motoneurons (Brooks et al., 1957; Kanda and Takano, 1983). It is also known that the action of TeNT relies on its transneuronal passage to reach central synapses (recent reviews by Rossetto et al., 2001; Lalli et al., 2003; Montecucco et al., 2004). TeNT is endocytosed and axonally transported towards the cell bodies along somatic and autonomic motor axons using the routes of transport of neurotrophic factors (Price et al., 1975; Schwab and Thoenen, 1978; Rind et al., 2005). Subsequently, TeNT translocates to presynaptic terminals (Schwab and Thoenen, 1976), where it cleaves synaptobrevin-2/VAMP2 and hence disrupting neurotransmission (Schiavo et al., 2000).

The catalytic and neurotropic properties of TeNT reside in different molecular subunits. Thus, the non-toxic C-fragment of TeNT undergoes transneuronal transport to second and even higher order neuronal centres (Manning et al., 1990; Herreros et al., 2000). This feature has been exploited for neuronal tracing and protein delivery to the CNS (Evinger et al., 1988, 1997). However, the doses needed to demonstrate the transport of the C-fragment exceed by several orders of magnitude the lethal dose of TeNT. Therefore, it is difficult to explore the transsynaptic movement of the toxin along central pathways. Moreover, TeNT produces effects at concentrations below the detection levels of conventional radiolabelling or fluorescent-tagging techniques (Montecucco et al., 2004). Therefore, it has been difficult to ascertain whether clinically relevant doses of TeNT exert significant actions beyond the first intoxicated neuron (i.e. the lower motoneuron) and then spread to higher brain centres.

In this report, we investigated the spread of TeNT through chains of synaptically connected neurons of the oculomotor system. The pontine abducens nucleus is synaptically linked with the contralateral mesencephalic oculomotor nucleus via the abducens internuclear neurons which send their long axons through the medial longitudinal fascicle. Here, we demonstrate the transneuronal and transsynaptic effects of TeNT using physiological and morphological methods. We show that TeNT injected into the medial rectus muscle induces hyperactivity in the firing pattern of abducens internuclear neurons and therefore, demonstrate sequential TeNT effects on interconnected brain centres. We suggest that VAMP2 immunoreactivity can be used as a useful tool to diagnose tetanus.

Material and methods

Seven adult female cats were obtained from the authorized breeders of the University of Cordoba (Spain). All experimental procedures were performed according to the directive of the European Union (86/609/EEC) and the Spanish legislation (BOE 67/8509-12, 1988).

Surgical preparation for chronic recordings

After a vagolytic injection of atropine sulphate (0.5 mg/kg, i.m.), the animal (n = 3) was anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and then stereotaxically implanted with silver bipolar electrodes to stimulate both VI nerves and the medial longitudinal fascicle. Scleral eye coils, 22 mm diameter, made up of teflon-insulated multistranded stainless steel wire, were implanted to record eye movements within a magnetic field (Fuchs and Robinson, 1966). A square window (5 mm side) drilled in the occipital bone was covered with an acrylic chamber to allow neuronal recordings. To restrain the head during recordings, an acrylic holder was attached to the skull via self-tapping screws. Post-operative care was provided, as needed, with antibiotics (streptomycin 20 000 IU/kg, i.m.), corticosteroids (dexamethasone, 5 mg/kg, i.m.) and analgesics (pirazolone, 0.1 g/kg, i.m.).

Extracellular recordings and analysis

Cats were seated in a restraining system with their head immobilized. Extracellular recordings were carried out with glass micropipettes filled with 2 M NaCl (1–3 MΩ resistance). The left abducens nucleus was located with the aid of the antidromic field potential produced by electrical stimulation (50 μs, <0.1 mA) from the VI nerve (Fig. 1A). Motoneurons and internuclear neurons were identified by their antidromic activation from the VI nerve and medial longitudinal fascicle, respectively (Fig. 1A). Extracellular neuronal activity was amplified and filtered at a bandwidth of 10 Hz–10 kHz. The horizontal position of both eyes was recorded using the magnetic field search coil technique (Fuchs and Robinson, 1966). Single unit recordings were obtained when the animal performed spontaneous eye movements or during the vestibulococular reflex induced by sinusoidal rotation (±20 deg at 0.1 Hz) of a servo-controlled table where the set-up rested.

Action potentials were digitalized using a window discriminator and stored in a computer at a time resolution of 10 μs using a CED 1401 A/D card (Cambridge Electronic Design, UK). Eye movements were sampled at 250 Hz. Computer programs were developed to display and select epochs of neuronal and eye movement data to calculate the neuronal sensitivity to eye movement parameters. Thus, firing rate (FR) fitted the simple equation (\(FR = F_0 + k \cdot P + r \cdot V\)) where the slopes \(k\) and \(r\) are the sensitivities to eye position (\(P\)) and velocity (\(V\)), respectively, and the intercept (\(F_0\)) represents the neuronal firing at straight ahead gaze. Alterations in any of these firing parameters before and after injection of TeNT were compared statistically at a level of significance of \(P < 0.05\).

Injection of TeNT

Following several control recording sessions, cats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and injected with TeNT (kindly provided by Dr O. J. Dolly, Imperial College, London). The right medial rectus muscle was isolated under a dissecting microscope and injected with a single dose of 5 ng/kg of TeNT dissolved in 4–5 μl of physiological saline (Fig. 1A). This dose was well under the minimum lethal dose of the toxin batch used (25 ng/kg) and, moreover, effects were locally restricted (González-Forero et al., 2003). In consistence with the clinics of tetanus (Goonetilleke and Harris, 2004), central and motor effects in the oculomotor system of cats persisted longer than 30–40 days, with maximal alterations between 2 and 20 days post-injection. Therefore, data obtained in this interval were grouped and considered as an homogeneous group. Four additional animals were prepared for morphological analyses of VAMP2.
immunoreactivity and synaptic coverage of motoneurons and interneurons. Two were injected with TeNT as above described and two were controls.

**Immunocytochemical analysis of VAMP2 immunoreactivity in the oculomotor and abducens nuclei**

Five days after injections, the four animals (two sham-operated and two treated) were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused transcardially with 1 l of 0.9% saline followed by 2 l of 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The brainstem was removed and 50-μm-thick coronal sections of the oculomotor and the abducens nuclei were obtained with a vibratome. The sections were washed in 0.01 M phosphate buffer, pH 7.1–7.3, with 0.9% saline containing 0.1% Triton X-100 (PBS/TX) and then blocked with normal horse serum (1:10 dilution in PBS/TX) and placed in different mixtures of primary antisera overnight at room temperature. Motoneurons were recognized by immunoreactivity against choline acetyltransferase (ChAT), interneurons with antibodies against calretinin (CR) and synaptic boutons with antibodies against synaptophysin.

Fig. 1 Experimental design: (A) TeNT (5 ng/kg) was injected in the right medial rectus muscle (MR). The abducens nucleus (ABD) contains motoneurons innervating the lateral rectus muscle (LR) and interneurons (grey tracing) projecting to the contralateral oculomotor complex (OCM). ABD neurons were extracellularly recorded (Rec) and identified by their antidromic activation (top traces; open circles) from the VI nerve (VIn St) or medial longitudinal fascicle (MLF St), respectively, and by the collision test (filled triangles) between the orthodromic (filled circles) and antidromic spikes. Besides the antidromic response, MLF stimulation frequently evoked orthodromic spikes (asterisk). The lower two traces demonstrate the tonic-phasic firing rate (FR, in spikes/s) of abducens neurons (an internuclear neuron is shown) during horizontal eye movements of the right eye (RH, in degrees). Other abbreviations: MLF, medial longitudinal fascicle; St, electrical stimulation; VIn, VI cranial nerve; (B and C) histograms showing the distributions of the antidromic activation latencies obtained in 105 abducens motoneurons (B) and 81 internuclear neurons (C) in control (black bars), and 82 motoneurons (B) and 89 internuclear neurons (C) recorded after TeNT (white bars). The incidence of long-latency units increased for internuclear neurons after TeNT treatment (P < 0.05; Kolmogorov–Smirnov test). Bin width: 0.05 ms. The insets illustrate representative examples of antidromic activations (open circle) in a motoneuron (B) and an internuclear neuron (C) after TeNT injection. Note the failure of antidromic discharge after a stimulus applied (down arrow) at short latency after a spontaneous orthodromic spike (filled circle), i.e. the collision test.
(SYN). Triple colour immunofluorescence for VAMP2/ChAT/CR or VAMP2/ChAT/SYN was obtained by mixing the following primary antibodies: VAMP2 (mouse monoclonal antibody IgG; 1:500 dilution; Synaptic systems GmbH, Goettingen, Germany), ChAT (goat polyclonal antibody, pAb; 1:500 dilution; Chemicon, Temecula, CA), and CR (rabbit pAb; 1:2500; Swant, Bellinzona, Switzerland) or SYN (rabbit pAb; 1:500; Chemicon). After extensive washing, ChAT immunostaining was amplified with a biotinylated anti-goat antibody. Following several washes, immunoreactive sites were revealed with donkey anti-rabbit IgG coupled to fluorescein isothiocyanate, donkey anti-mouse IgG coupled or Cy3, and streptavidin coupled Cy5 (Jackson, West Grove, PA).
antibodies and streptavidin were diluted 1:50 in PBS/TX. Finally, sections were washed in PBS, mounted on glass slides and cover-slipped with Vectashield (Vector Labs., Burlingame, CA).

Epifluorescence monochrome images at low (×4) magnifications were documented with a digital colour camera (Spot2 camera; Diagnostic Instruments, Sterling Heights, MI; 24-bits per pixel) prior to confocal imaging at higher magnifications. Camera gains were adjusted automatically by Spot2 software to obtain the best signal-to-noise ratio with ImagePro Plus software (ver. 5.1; Media Cybernetics, Silver Spring, MD). Confocal microscopy (Leica TCS SP2) at higher magnification (×63 oil immersion digitally zoomed at ×2) was used for imaging triple colour immunofluorescent preparations. The number of VAMP2- or SYN-immunoreactive punctae was counted around identified somatic profiles or in the neuropil. The synaptic coverage was defined as the percent ratio between the perimeter occupied by either VAMP2 or SYN terminals and the soma perimeter. The surface density in the neuropil was defined as the number of punctae per 1000 μm².

Results
Axonal conduction velocity of abducens neurons was impaired by TeNT

The left abducens nucleus, contralateral to the injection of TeNT, was located by means of the antidromic field potential produced by the electrical stimulation of the ipsilateral VI cranial nerve (Fig. 1A, VIn St). Unitary identification of motoneurons and internuclear neurons was carried out by both antidromic activation and the collision test from the ipsilateral VI nerve and contralateral medial longitudinal fascicle, respectively (Fig. 1B and C, insets). The antidromic latency of control abducens motoneurons followed a bell-shaped distribution skewed towards long-latency values (Fig. 1B) of mean 0.68 ± 0.12 ms (mean ± SD, n = 105). Although contralateral TeNT application in the medial rectus muscle slightly elevated the proportion of long-latency units (>1 ms) in abducens motoneurons (7.32% versus 1.92%), the differences from control were not significant (P = 0.56; Mann–Whitney rank sum test; n = 82). On the contrary, mean antidromic latency was significantly increased (P < 0.05; Mann–Whitney rank sum test) in internuclear neurons after TeNT treatment (0.81 ± 0.24 ms; n = 89) when compared with control (0.73 ± 0.20 ms; n = 81). Distribution histograms showed a higher incidence of mid-latency and long-latency units after TeNT treatment (Fig. 1C; Kolmogorov–Smirnov test; P < 0.05). These results indicated that medial rectus TeNT-injections led to changes in axonal properties of contralateral internuclear abducens neurons manifested as reduced conduction velocities, whereas abducens motoneuron axonal conduction was not significantly affected. Previously we have shown that TeNT slows the axonal conduction velocity (Gonzalez-Forero et al., 2003).

Qualitative alterations in firing and eye movements after TeNT

In order to study the effects of the TeNT transport along oculomotor pathways, we analysed eye movements and neuronal firing in the abducens nucleus. We reasoned that bilateral motor alterations after a unilateral TeNT injection would unequivocally reflect the action of TeNT on premotor structures. On the contrary, unilateral alterations would discard any significant transneuronal transport of TeNT to the contralateral abducens nucleus. After TeNT injection, the most conspicuous alteration in eye movements was the divergence of gaze of both the injected (right) and the non-treated eyes (left) (Fig. 2B and C). Before treatment, the oculomotor range for the left and right eyes was 77.59 ± 5.68 deg (mean ± SD; n = 3 animals) and 79.09 ± 10.09 deg, with mean centre eye positions in 0.8 ± 3.70 deg and –0.47 ± 2.73 deg, respectively (positive values indicating leftward eye positions). Although the bilateral motor impairment was evident 2 days after treatment, ocular motility and horizontal gaze were maximally affected during the second and third weeks. Thus, the motor range covered by the left and right eyes was reduced to 26.34% (20.44 ± 4.44 deg) and 10.44% (8.26 ± 2.44 deg) and the mean eye position deviated to 9.98 ± 2.29 and –9.11 ± 1.46 deg in the abducting direction, respectively, at 15 days post-injection (Fig. 2D; n = 3; P < 0.05; two-way ANOVA, Duncan’s method). These data show that the unilateral injection of TeNT in the right medial rectus muscle induced bilateral gaze divergence, with a nearly complete impossibility for both eyes to explore the nasal hemifield.

Alterations were also found in the firing of abducens neurons. Control abducens neurons showed a tonic–phasic
firing pattern, with bursts and pauses for on-directed and off-directed saccades, respectively (Fig. 2A). After treatment, firing of internuclear neurons and also abducens motoneurons was affected. Motoneurons were hyperactive (Fig. 2B). Mean FR was significantly increased with respect to control \((P < 0.001; \text{Student's } t\text{-test})\) from 35.03 ± 12.43 \((n = 34)\) to 50.59 ± 23.82 spikes/s \((n = 53)\). Firing activity was also significantly elevated \((P < 0.05, \text{Student's } t\text{-test})\) in internuclear neurons \((77.64 ± 28.28 \text{ spikes/s}, n = 57)\) compared with control \((66.19 ± 19.98 \text{ spikes/s}, n = 39)\) and we noticed an elevated incidence of continuously discharging tonic internuclear neurons (Fig. 2C). Although most internuclear neurons were disinhibited, some \((24.5\%)\) were either mostly silent or tonically discharging at very low rates.

We analysed the time course of changes on the discharge activity of abducens neurons after TeNT application as shown for abducens motoneurons and internuclear neurons (Fig. 2E). Abducens motoneurons showed an increase in mean FR during the second and third weeks after treatment \((P < 0.05; \text{two-way ANOVA, Duncan’s method})\). Actually, the mean FR of abducens motoneurons was correlated with the deviation of the left eye (measured between 2 and 20 days when the effects were more intense). The relationship showed that both parameters were positively correlated \((r = 0.80, P < 0.001; \text{Fig. 2F})\). However, no correlation was found between mean FR of internuclear neurons and mean right eye position \((r = 0.10, P = 0.71; \text{Fig. 2F})\), indicating that signals transferred by the internuclear pathway to the contralateral medial rectus motoneurons and thereafter to the medial rectus muscle were blocked by TeNT. In summary, our data suggest that injection of TeNT in the right medial rectus muscle induces an overall blockade of the afferent neurotransmission in the right oculomotor nucleus and disinhibition in the left abducens nucleus, affecting both motoneurons and internuclear neurons. Although levels and patterns of firing activity were somewhat variable, particularly in internuclear neurons, the tonic discharge and hyperactivity exhibited by most of the units reflects a presynaptic action of TeNT in the abducens nucleus.

Disinhibition of abducens motoneurons
Abducens motoneurons exhibited firing patterns that were qualitatively different from the characteristic tonic-phasic discharge of control motoneurons (Fig. 3A) during spontaneous eye movements. TeNT treatment also affected firing modulation of abducens motoneurons, principally that associated to off-directed eye movements (Fig. 3B). Thus, although bursting activity was preserved during on-directed saccades, pauses or decrements of tonic firing were usually absent or largely attenuated during off-directed saccades. Neuronal sensitivities to eye position during spontaneous eye movements \((k_v)\) and vestibuloocular reflex \((k_v)\) were significantly \((P < 0.001; \text{Student's } t\text{-test})\) reduced in abducens motoneurons from 6.04 ± 2.24 \((n = 30)\) and 6.89 ± 2.23 \((n = 18)\) to 3.43 ± 1.62 \((n = 31)\) and 3.32 ± 2.10 spikes/s/deg \((n = 17)\), respectively. Similarly, mean \(r_v\) decreased by 22.22\% \((0.77 ± 0.31 \text{ versus } 0.99 ± 0.17 \text{ spikes/s/deg/s})\) and mean \(r_v\) by 30.91\% \((0.76 ± 0.38 \text{ versus } 1.10 ± 0.35 \text{ spikes/s/deg/s})\) in the same motoneurons. In parallel with the reduction in neuronal sensitivities, the recruitment eye position threshold was reduced \((-20.64 ± 9.69 \text{ versus } -7.43 ± 6.22 \text{ deg}; P < 0.001, \text{Student’s } t\text{-test})\) and the mean \(F_v\) increased \((63.38 ± 31.60 \text{ versus } 38.13 ± 29.56 \text{ spikes/s}; P < 0.05, \text{Student’s } t\text{-test})\).

The reduced discharge modulation during off-directed eye movements and the elevated FRs probably derive from a TeNT block of inhibitory synapses presynaptic to abducens motoneurons. To analyse differential effects on inhibitory and excitatory synaptic drives, we calculated separately the eye position and velocity sensitivities for each direction of movement (by sorting fixations occurring after on- or off-directed saccades). We found that slopes obtained for on-directed eye movements (excitation) were frequently steeper than those compared with off-directed movements (inhibition) (Fig. 3C). Thus, although experimental \(k_{on}\) \((4.68 ± 3.81 \text{ spikes/s/deg}; n = 27)\) and \(r_{on}\) \((0.87 ± 0.42 \text{ spikes/s/deg/s}; n = 17)\) remained similar to control \(k_v\) and \(r_v\) values, \(k_{off}\) and \(r_{off}\) were dramatically reduced \((1.96 ± 1.71 \text{ spikes/s/deg/deg and } 0.24 ± 0.20 \text{ spikes/s/deg/s}, \text{respectively}; P < 0.05, \text{two-way ANOVA; Duncan’s multiple range tests})\). Finally, greater disparities between \(k_{on}\) and \(k_{off}\) were also exponentially associated to higher firing activities in individual units (Fig. 3D). These are the expected behaviours resulting from increased firing of abducens motoneurons owing to disinhibition.

Firing alterations of internuclear neurons reflect the sequential TeNT action in the oculomotor and abducens nucleus
As indicated above, internuclear neurons recorded after treatment were separated into two classes on the basis of their activity levels and proportion of time they were firing. One class was tonically active units, firing either at high or low rates (Fig. 4A and B, respectively) and the second class was the non-tonic or rarely discharging internuclear neurons (Fig. 4C). Neurons of the first class exhibited either disinhibited firing at high frequencies with a marked loss of modulation during off-directed eye movements (Fig. 4A) or showed depressed and unmodulated firings (Fig. 4B). Non-tonic units were mostly silent, firing only during on-directed eye movements (Fig. 4C). Although tonically discharging units were usually found at all postinjection times, non-tonic internuclear neurons were only transiently found during the initial 11 days.

We found that discharge activity usually was poorly related with eye movements in internuclear neurons. As expected from the higher degree of paralysis of the right eye, the correlations of firing with eye movement parameters were usually higher with the non-injected eye. For the quantitative analysis, only regressions with \(r > 0.65\) were considered. Sensitivities to eye position \((k_v)\) and velocity \((r_v)\) during spontaneous eye
movements were significantly reduced ($P < 0.001$; Student’s $t$-test) in experimental internuclear neurons ($3.46 \pm 2.01$ spikes/s/deg, $n = 42$; and $0.75 \pm 0.46$ spikes/s/deg/s, $n = 21$) in comparison with the control situation ($7.03 \pm 2.56$ spikes/s/deg, $n = 43$; and $1.55 \pm 0.56$ spikes/s/deg/s, $n = 23$). Recruitment threshold diminished in parallel ($-27.37 \pm 23.17$ versus $-10.53 \pm 4.49$ deg), whereas no significant differences ($P = 0.22$) were found in the mean $F_0$ ($74.61 \pm 32.57$ versus $66.72 \pm 25.90$ spikes/s). Finally, eye position and velocity sensitivities during the vestibuloocular reflex ($k_v$ and $r_v$) were also reduced by 22.51 and 43.02%, respectively, reaching significant differences ($P < 0.001$) only in the case of $r_v$. Therefore, these results show a consistent alteration in the modulation of firing of the interneuronal pool after TeNT.

The presence of internuclear neuron subgroups displaying distinct firing alterations could indicate that they result from different actions of TeNT in the oculomotor pathway. A comparison of the rate–position plots obtained from two tonic internuclear neurons (at high and low-rates) and one non-tonic unit is illustrated in Fig. 4D. Non-tonic neurons represented only 27.08% of the total analysed pool ($n = 48$). Eye position sensitivity was significantly higher ($P < 0.001$; Student’s $t$-test) in this group compared with...
tonic units (5.20 ± 2.66 versus 3.03 ± 1.40 spikes/s/deg). In addition, the mean $F_0$ was significantly lower in non-tonic neurons ($P < 0.001$; 34.09 ± 18.79 versus 83.04 ± 31.59 spikes/s). Thus, the main difference between both subgroups seemed to rest on recruitment threshold and mean FR (Fig. 5A). Tonic units had low recruitment thresholds (mean $-34.83 ± 22.32$ deg) and were theoretically recruited over a wide range of eye positions (from $-122.36$ to $10.83$ deg).

On the contrary, non-tonic units were recruited in a much narrower range (from $-11.08$ to $1.61$ deg) located near the primary eye position (mean $-5.26 ± 3.77$ deg). By plotting the mean FR versus the recruitment threshold, both subgroups clustered apart from the control internuclear neurons (Fig. 5A). Lower thresholds and high firing activities corresponded with tonic internuclear neurons, whereas non-tonic units showed reduced discharges and higher thresholds.

The bidirectional changes in threshold and levels of activity in different interneurons could result from different actions of TeNT on efferent and afferent synaptic neurotransmission on the interneuronal group. These two modes of firing are illustrated in Fig. 5B. Non-tonic abducens internuclear neurons might be the result of target disconnection through TeNT actions in the oculomotor nucleus [Fig. 5B (a)] that resembles the ones obtained in these same neurons when target-deprived by chemical lesions (de la Cruz et al., 1994). By contrast, the tonic firing pattern [Fig. 5B (b)] is more likely the result of TeNT presynaptic deafferentation on abducens interneurons that have retrogradely transported the toxin.

**TeNT effects on VAMP2 through the abducens internuclear pathway**

Five days after TeNT treatment we performed an immunocytochemical analysis of VAMP2 in the abducens internuclear pathway. In control cats ($n = 2$), the oculomotor nucleus had less immunoreactivity against VAMP2 as
compared with the overlying periaqueductal grey matter (Fig. 6A). By contrast, the control abducens nucleus had similar VAMP2 immunoreactivity to surrounding regions and could not be delimited from the pontine tegmentum (Fig. 6C). Axon bundles and fibre tracts, as the oculomotor and abducens nerve rootlets and the medial longitudinal fascicle, had no staining for VAMP2. Five days after TeNT treatment \( (n = 2) \), VAMP2 immunoreactivity was decreased in both oculomotor and abducens nuclei (Fig. 6B and D). In the oculomotor nucleus, the ipsilateral side showed an almost complete absence of VAMP2 immunoreactivity (Figs 6B and 7B). To establish the extent of VAMP2 changes at the cellular level, we performed triple immunostainings of SYN (a synaptic vesicle protein insensitive to TeNT and thus used as a presynaptic bouton marker), VAMP2 and ChAT as a cellular marker of motoneurons. In the oculomotor nucleus, VAMP2 decayed more dramatically than SYN in the treated side (Fig. 7A and B), whereas SYN was little altered in the abducens nucleus (data not shown). At high magnification, the neuropil of the control oculomotor nucleus and the motoneuron perimeters contained many boutons immunostained for SYN and VAMP2 (Fig. 7C). Following TeNT, SYN coverage in the treated oculomotor nucleus was significantly reduced by 30% (from a coverage of 81.04% of cell somatic membrane in control to 57.12% in the treated animals; Student’s \( t \)-test; \( P < 0.001 \)). Strikingly, VAMP2 immunostaining, assessed by surface density measurements in the neuropil, disappeared almost completely after TeNT in the treated oculomotor nucleus (94.43% of control; compare Fig. 7D and E).

In the abducens nucleus, we analysed triple immunostainings for VAMP2, CR (a marker of abducens internuclear neurons; de la Cruz \textit{et al.}, 1998) and ChAT (for abducens motoneurons). In control animals, both types of abducens neurons were covered by VAMP2-immunoreactive punctae (Fig. 7F). After TeNT treatment, the abducens nucleus contralateral to the injected side demonstrated a significant decay \( (P < 0.001) \) in VAMP2 immunoreactivity in the neuropil to 37.9% of the control material (Fig. 7G). By contrast, the decay in VAMP2 did not correspond with any statistically significant reduction in the coverage of SYN in abducens neurons. Altogether, these results indicate that TeNT injected in the medial rectus muscle reached the oculomotor nucleus, where it efficiently cleaved VAMP2, and then via the internuclear neurons obtained access to the abducens nucleus, where it also mediated a large reduction in VAMP2 immunoreactivity.

**Discussion**

The results presented here provide the first direct evidence of functional and anatomical alterations in premotor centres induced by peripheral injections of TeNT. Our results indicate that a balance between a severe anatomical deafferentation and/or the loss of trophic support can lead to either disinhibition, thus resembling tetanus in premotor circuits or axotomy-like effects in neurons. First, we propose that the generalized tetanus could be the result of supranuclear effects of premotor structures. Secondly, VAMP2 immunoreactivity could have a potential use in biopsy or post-mortem human tissue in the differential clinical diagnosis of tetanus. Given the
catalytic specificity of TeNT, a mouse bioassay (routinely performed for *Botulinum* neurotoxin, Sesardic *et al.*, 2004) followed by VAMP2 immunolocalization could be considered as a method for fast and direct alternative to the currently low diagnostic success of microbiological cultures.

**Transneuronal actions of TeNT**

We previously reported that the normal firing pattern of abducens neurons dramatically changed towards either a disinhibited or a tonic and depressed discharge following the injection of low or high doses of TeNT in the lateral rectus muscle (González-Forero *et al.*, 2002, 2003). These functional alterations were associated with the blockade of inhibitory inputs only (low dose) or of both inhibitory and excitatory inputs (high dose) and followed by the structural remodelling of synapses on abducens neurons (González-Forero *et al.*, 2004). The present findings demonstrate discharge alterations following the injection of 5 ng/kg of TeNT in the contralateral medial rectus muscle. Thus, both hyperactive and depressed discharging units were found in abducens neurons. The diversity of effects could be explained by a differential access, uptake or accumulation of TeNT into premotor terminals. It is known that TeNT uptake in synaptic terminals and the rate of transport of either the holotoxin or the C fragment depend on neuronal activity (Wellhöner *et al.*, 1973; Miana-Mena *et al.*, 2002).

Our data probably indicate that neurotransmission was completely blocked in the first synaptic relay (oculomotor nucleus) where TeNT possibly achieves the highest concentration, as revealed by the almost complete lack of VAMP2 immunostaining in SYN labelled boutons, but only partially impaired in the abducens nucleus (the second transynaptic relay). We propose that the common effects on motoneurons and internuclear neurons following retrograde transport and transynaptic translocation of TeNT in the abducens nucleus could result from toxicity inside axons of shared common afferents.

**Pathways of passage and actions of TeNT on premotor circuits**

Most of the present knowledge about CNS actions of TeNT derives from studies on spinal motoneurons (Brooks *et al.*,...
This classic work demonstrated that TeNT preferentially blocks inhibitory inputs on spinal motoneurons while leaving unaltered presynaptic inhibition of the monosynaptic reflex and the recurrent inhibition on Ia interneurons (Brooks et al., 1957; Benecke et al., 1977; Takano et al., 1991). The lack of effects in acute studies on premotor structures wrongly led to assume that the main physiological alterations induced by TeNT occur at the level of spinal motoneurons.
TeNT during the disease occurs strictly by disinhibition at the level of motor nuclei. This incomplete clinical description persists and thus generalized tetanus has been clinically explained as the combination of multiple local tetani but not of supramotor structures (Erdmann and Habermann, 1977).


The route of access of TeNT into the CNS is preferentially neural, with no detectable crossing through the blood–brain barrier (Price et al., 1975; Erdmann and Habermann, 1977; Fishman and Carrigan, 1988; Lalli and Schiavo, 2002). Therefore, the central effects described above must be explained by the retrograde ascent and successive synaptic translocations of TeNT after peripheral administration, as shown by morphological tracing studies using high concentrations of toxin labelled with HRP or I\(^{125}\) from the periphery (Erdmann and Habermann, 1977; Dumas et al., 1979; Rind et al., 2005). Our functional results indicate that central transsynaptic effects are possible and thus long-term sequelae like seizures, myoclonus and sleep disturbances (Goonetilleke and Harris, 2004) that occur after recovery of muscle spasms can be owing to central actions of TeNT, either by blocking synapses or inducing instability in circuits as occur in seizures induced by TeNT experimentally (Milward et al., 1999).

**Comparison of effects to target deprivation**

We noticed the presence of high-threshold and non-tonic discharging internuclear neurons with extremely low firing activities as previously reported for target-deprived and axotomized abducens internuclear neurons (de la Cruz et al., 1994, 2000). In contrast, these units were never found in our previous studies after TeNT injection in the lateral rectus muscle (González-Forero et al., 2002, 2003). Therefore, we suggest that the non-tonic discharge pattern observed in some internuclear neurons was probably the consequence of the disconnection between these cells and their motoneuronal target by TeNT. The loss of SYN immunoreactive terminals in the oculomotor nucleus found here after TeNT further reinforces this hypothesis. Physical or functional disconnection of motoneurons from muscle also induces depression of firing activity in spinal and cranial motoneurons, which would be mainly related with the deprivation of trophic support from the muscle and the loss of excitatory afferent inputs resembling the effects of axotomy (Gordon et al., 1980; Pinter et al., 1991; Moreno-López et al., 1997; de la Cruz et al., 2000). It has recently been shown that the transsynaptic transcytosis of some neurotrophins shares common transport pathways and some traffic properties with TeNT (Rind et al., 2005).

Therefore, the TeNT-induced synaptic stripping (as observed by SYN) might result in the loss of trophic support for a proportion of abducens internuclear neurons, at least transiently (11 days). Therefore, it is likely that functional efferent disconnection of internuclear neurons with their motoneuronal target by TeNT also induces some of these ‘axotomy-like or targetless’ changes in a small proportion of internuclear neurons. It can be proposed that a steady state between the processes of TeNT-induced synaptic detachment and the synaptic remodelling at the axonal terminals establishes the level of access to target-derived trophic support, and resulting in the appearance of ‘axotomy’ symptoms.

**Insights into the course of tetanus**

Our data suggest that TeNT activity can spread centrally through interconnected regions during the course of tetanus. However, tracing and functional studies on the spinal motor system failed to report labelling of premotor cell bodies or afferent synaptic blockade on these structures during the development of local tetanus (Brooks et al., 1957; Dimpfel and Habermann, 1973; Schwab and Thoenen, 1976; Benecke et al., 1977). In contrast, in the oculomotor system, strong labelling of premotor neurons with the TeNT C fragment is observed 4 days after its injection at high doses into the eye muscles (Horn and Büttner-Ennever, 1990, 1998). The presence of TeNT effects in the abducens nucleus is further supported by the large depletion of VAMP2 immunoreactivity found here. It is possible that intrinsic differences between oculomotor and spinal motor systems implicate differential transport capabilities and access of TeNT to premotor structures. For instance, differences in the length and basal activity of different peripheral nerves could explain differential rates of uptake and transport (Wellhöner et al., 1973; Miana-Mena et al., 2002).

Functional disruption of supramotor structures could represent a form for spreading motor symptoms of tetanus to several muscle groups, and perhaps it could contribute to the long-term sequelae such as seizures, myoclonus and sleep disturbances (Goonetilleke and Harris, 2004). This can be because of central actions of TeNT, either by blocking synapses or inducing instability in circuits as occur in seizures induced experimentally by TeNT (Milward et al., 1999). In addition, central spreading of TeNT could also contribute to the development of generalized tetanus. The evolution of general tetanus from cephalic tetanus, the so-called descending general tetanus (Mellamby and Green, 1981), has usually the poorer prognosis and the higher lethality with episodic spasms involving both agonist and antagonist muscles (Bleck, 1989; Goonetilleke and Harris, 2004). TeNT spreading through the CNS could result in limb co-contraction and opisthotonus, which resembles decerebration rigidity, and might result from progressive disinhibition of either descending reticular and vestibular systems or the spinal (and cranial) interneurons responsible for the integration of these descending commands (Matsuyama and Jankowska, 2004).
Transynaptic effects of tetanus neurotoxin

Brain (2005), 128, 2175–2188

2187

Acknowledgements

We thank Dr Mark Rich, Department of Neurology, Emory University and Dr Joan Blasi, Dept. Patología y Terapéutica Experimental, Instituto de Investigaciones Biomédicas de Bellvitge for critically reading the manuscript. We also acknowledge Dr George Z. Mentis and Ms Samantha McLaurin for proof reading the manuscript. This work was supported by MCYT (I+D+I)-FEDER BFU2004-01024 and BFU2004-0273-E, Fundación Eugenio Rodriguez Pascual and NSF grant 9984441.

References


