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Botulinum toxin B increases intrinsic muscle activity in organotypic spinal cord–skeletal muscle co-cultures

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HIGHLIGHTS

- Botulinum toxin B increases spontaneous muscle activity after 1 day incubation.
- Rocuronium bromide reduces spontaneous muscle activity.
- Application of glutamate receptor antagonists similarly increase muscle activity.

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ABSTRACT

In organotypic spinal cord–skeletal muscle co-cultures, motoneurons are driven by locomotor commands and induce contractions in surrounding muscle fibres. Using these co-cultures, it has been shown that effects of organophosphorus compounds on neuromuscular synapses can be determined *in vitro*. In the present study we aimed to extend this *in vitro* tool for pharmacologic testing of botulinum toxin B. This neurotoxin is widely used for the treatment of dystonia. Besides its effects on the neuromuscular junction, botulinum toxins may also act at centrally located synapses. Incubation with botulinum toxin B (Neurobloc®) induced a significant increase in muscular activity after 24, 48 and 72 h. Application of the NMDA- and AMPA-receptor antagonists AP5 (20 μM) and CNQX (15 μM) induced a similar augmentation of muscle activity after 48 and 72 h, respectively. Administration of the glycine- and GABA(A)-receptor antagonists strychnine (1 μM) and bicuculline (100 μM) did not alter intrinsic muscle activity. In contrast, application of a non-depolarizing muscle relaxant rocuronium bromide reduced the muscle activity in a dose-dependent manner. Our findings suggest that glutamatergic synapses in the spinal cord are more sensitive to botulinum toxin B than synaptic contacts between spinal motoneurons and muscle fibres.

1. Introduction

Botulinum toxin is a food-borne poison produced by the bacterium *Clostridium botulinum*, which induces flaccid paralysis when injected in muscles for the treatment of medical conditions such as torticollis or spasticity (Dressler, 2010). However, an overdose of botulinum toxins may lead to asphyxia by inhibition of respiratory muscles (Souayah et al., 2006; Williams et al., 2014; Witoonpanich et al., 2010). Before use of botulinum toxins for medical purposes, the potency and the high toxicity need to be determined. The commonly used method for potency testing of botulinum toxins is the mouse LD_{50} assay (lethal dose that kills 50% of the tested animals). This LD_{50} assay inflicts severe suffering to the mice and may lead to suffocation within the test period (Adler et al., 2010; Bitz, 2010). Thus, alternatives to this animal testing that reduce the number of animals or replace the detection method with an *in vitro* assay are strongly encouraged by legal authorities (Adler et al., 2010; Council of Europe, 2011). Eight different serotypes of botulinum toxin (A–H) are described so far (Barash and Arnon, 2014; Dover et al., 2014; Humeau et al., 2000; Schiavo et al., 2000). Currently, botulinum toxins A and B are in medical use (Dressler, 2010; Schiavo et al., 2000). Both neurotoxins are thought

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to interrupt acetylcholine release at neuromuscular junctions (Rossetto et al., 2001). However, botulinum toxin A and B display two different molecular mechanisms of action: While botulinum toxin A cleaves the synaptosomal-associated protein of 25 kDa (SNAP-25), botulinum toxin B induces cleavage of the vesicle-associated membrane protein 2 (VAMP-2) (Schiavo et al., 2000). Besides its action on neuromuscular junctions, it was shown that botulinum toxin B acts at central synapses to inhibit neurotransmitter release (Höltje et al., 2013 McMahon et al., 1992; Tighe and Schiavo, 2013).

Lately, an animal-free potency testing assay for botulinum toxin A has been approved by the FDA, Health Canada, and the European Union (Fernández-Salas et al., 2012). By contrast, no alternative method is approved for potency testing of botulinum B so far. Hitherto, we described an *in vitro* assay to determine long-term effects of organophosphorus compounds and botulinum toxin A (Drexler et al., 2011; Eckle et al., 2014). In the present study, we investigated the effect of botulinum toxin B on intrinsic muscle activity in organotypic spinal cord–skeletal muscle co-cultures. This muscle activity is driven by locomotor activity arising from the spinal ventral horn and can be suppressed by organophosphorus compounds or a depolarizing muscle relaxant (Drexler et al., 2013, 2011). These co-cultures are well suitable to investigate long-term effects of drugs on excitatory, inhibitory and neuromuscular synaptic transmission (Streit et al., 1991). In our experiments, organotypic co-cultures were incubated with botulinum toxin B for 3 subsequent days and intrinsic muscle activity was measured at intervals of 24 h. Similar experiments were conducted with a non-depolarizing muscle relaxant to block the neuromuscular junction, with NMDA- and AMPA-receptor antagonists to inhibit the excitatory signaling, and with GABA(A)- and glycine-receptor antagonists to impede the inhibitory neurotransmission in a long-term setting.

2. Material and methods

2.1. Spinal cord–skeletal muscle co-cultures

This study was carried out in strict accordance with the regulations, policies, and principles of the NIH Guide for the Care and Use of Laboratory Animals. Briefly, all procedures were performed in accordance with the German Animal Welfare Regulations (TierSchG), were reviewed by the Institutional Animal Care and Use Committee (Einrichtung für Tierschutz, Tierärztlichen Dienst und Labortierkunde, Eberhard-Karls-University, Tübingen, Germany), and approved by the Government (Regierungspräsidium Tübingen, Tübingen, Germany). All efforts were made to minimize suffering. For tissue harvesting, C57/BL6J mice were decapitated under deep isoflurane anesthesia. Tissue slices derived from the spinal cord and surrounding muscles were prepared as previously described (Drexler et al., 2011). Embryonic spinal columns (day E 13) were cut transversely into 300 μm thick slices using a microslicer (NVSLM1, World Precision Instruments, Sarasota, FL, USA). Slices from the spinal cord and surrounding muscle tissue were glued onto glass coverslips by clotting of chicken plasma and thrombin (Sigma, Taufkirchen, Germany). The coverslips were put into plastic tubes containing nutrient fluid (0.75 mL) and 10 nM neuronal growth factor (from Sigma). A roller

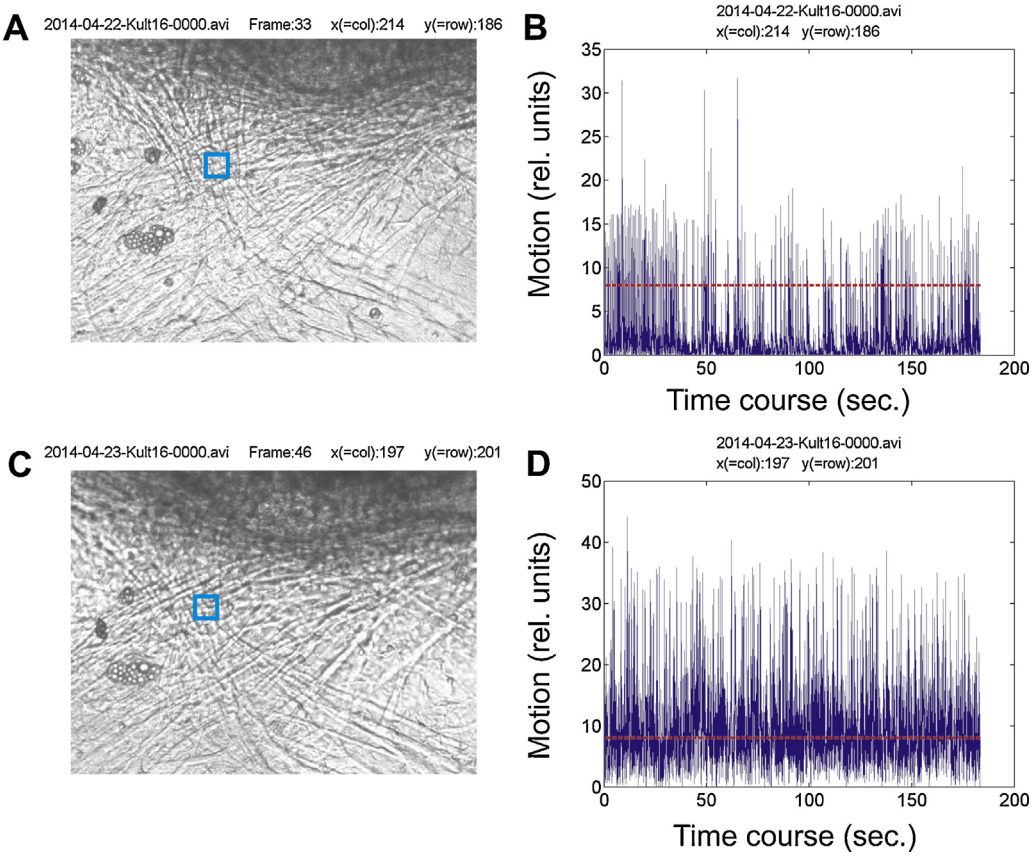

Fig. 1. Detection method of muscle activity. Using videomicroscopy, muscle fibers of an organotypic spinal cord-skeletal muscle co-culture were monitored from DIV 10 to DIV 13. Intrinsic muscle activity was quantified as a change in brightness of pixels in the region of interest (blue frame; A). These changes in brightness were plotted on a time scale. Using a threshold (red dotted line), the frequency of muscle contractions could be detected (B). After 24 h incubation with Neurobloc® (20 U/mL) the same region of interest (blue frame) was re-identified (C) and intrinsic muscle activity was quantified (D) as described above. Note the increase in muscle activity between (B) and (D).

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