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Accurate quantification of tetanus neurotoxin-induced focal spasticity in mice using complex running wheels

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A B S T R A C T

Tetanus neurotoxin (TeNT) enhances activity of motoneurons by blocking spinal inhibitory interneurons. Based on this pathomechanism, we propose that low-dosage intramuscular injections of TeNT could serve as a specific treatment for central paretic muscles. However, in vivo TeNT research is restricted because of the fear of triggering widespread muscle spasms. In addition, no reliable test to measure the in vivo toxicity of low-dosage TeNT is available.

We introduce a novel wheel running-based paradigm with mice to quantify functional effects and thus the toxicity of low-dosage TeNT in vivo. We accustomed three groups of wildtype mice (n = 14) to using a complex running wheel with irregularly spaced crossbars. Each group received an injection with a different low-dosage of TeNT (0.15 ng, 0.1 ng or 0.05 ng TeNT) into both tibialis anterior muscles. The maximum running velocity and accumulative running time of the groups were recorded during the following weeks.

Three days after TeNT injections, the mice exhibited an increase in muscle tone of the injected tibialis anterior muscles but no generalized symptoms. However, we found that normal running in the complex wheel set-up was disturbed such that the maximum running velocity and running time of the mice decreased with the size of the dose. This effect peaked on the fifth and sixth nights after injection and returned to baseline level again within the next two weeks.

With this novel in vivo automated paradigm we can accurately and objectively quantify the duration and degree of TeNT-induced focal increase in muscle tone.

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1. Introduction

Tetanus neurotoxin (TeNT) is a 150 kDa protein that binds to the presynaptic membrane of motoneurons, where it is internalized and retrograde-transported axonally to the spinal cord. There, the toxin is translocated to inhibitory spinal interneurons and blocks their neurotransmitter release by cleaving vesicle-associated membrane protein (VAMP). As a consequence of this, excitability and thus activity of the motoneurons are enhanced, which results in pathologically increased muscle tone (Rossetto et al., 2001).

Dose-dependent TeNT can evoke generalized or simply localized tetanus. Clinically apparent, generalized tetanus is characterized by generalized muscle spasms, rigidity, and autonomic symptoms (Goonetilleke and Harris, 2004). In contrast, intramuscular injections of low-dosage TeNT can cause specifically enhanced muscle activity of the injected muscle for voluntary movements without any sign of widespread muscle hyperexcitability (Fishman et al., 2009; Webster and Laurence, 1963).

This feature sustains the hypothesis that localized injections of TeNT, like injections of botulinum neurotoxin, could be used as a therapeutic agent. As TeNT is a substance that specifically increases muscle activity, we speculate that this toxin could be applied locally in diseases with decreased muscle tone, such as localized flaccid paresis after stroke, multiple sclerosis or spinal cord injury (Fishman et al., 2009; Sanders, 2004; Sasse et al., 2005). Moreover, a recent study showed that a bulldog with obstructive sleep apnea benefited from a local injection of TeNT into an upper airway muscle (Sasse et al., 2005).

A difficulty in the potential therapeutic focal use of TeNT is the safety aspect, due to the dreaded risk of an unintended widespread generalization of tetanus. Up to now, no test is available that can
2. Materials and methods

2.1. Animals

Fourteen male C57/Bl6j 4-week-old mice were acquired from Charles River Laboratories, Sulzfeld, Germany. The mice were housed individually in plastic cages that were equipped with conventional running wheels and offered food and water ad libitum. Lights were off from 6:30 p.m. to 6:30 a.m. which resulted in 12 h light/12 h dark cycles. All experiments were performed under German animal protection laws and were approved by the Government of Lower Saxony.

2.2. Behavioural testing

To assess TeNT effects on muscle tone, we made use of a novel complex running wheel paradigm that was recently used successfully to quantify calf muscle paresis after local botulinum toxin injections (Kutschenko et al., in press). In the course of the experiment, the cages were equipped with two different types of running wheels (conventional and complex running wheels). Both wheel types were constructed by our workshop. One wheel revolution equates to a running distance of 35.5 cm. The wheel axis was connected to a rotation sensor with a resolution of 16/turn. A customized recording device and software (Boenig & Kallenbach oHG, Dortmund, Germany) were used to record running wheel revolutions continuously at a sampling rate of 1/0.48 s. Two running parameters, i.e. maximum velocity \( V_{max} \) and accumulative running time \( T_{ac} \) were then calculated using a custom designed MatLab program (The MathWorks). Each parameter was assessed continuously and the results were logged once daily (9 a.m.).

At the beginning of the experiment, all mice were placed individually into single cages equipped with conventional running wheels. During the first two weeks of the experiment, mice continuously improved their running performance with respect to maximum running velocity \( V_{max} \) and accumulative running time \( T_{ac} \) until a maximum was achieved. Then the conventional wheels were replaced by complex running wheels with irregularly spaced crossbars to make wheel running more difficult (Liebetanz and Merkler, 2006). Following the wheel exchange, the wheel running performance of all mice temporarily decreased; a new plateau was then reached, usually within 10–15 days. At this time point, intramuscular injections of TeNT were administered and wheel running performance was continuously monitored during the following three weeks.

2.3. TeNT injections

Fourteen mice (31.3 ± 2.5 g) were divided randomly into 3 treatment groups of either 4 or 5 mice. TeNT (tetanus toxin, Sigma–Aldrich Logistik GmbH, Schnelldorf, Germany) was reconstituted with 250 µl of sterile purified water and diluted in 20 mL HEPES with 1.25% lactose in accordance to the manufacturer's description. Dilutions of three different low-dosages of TeNT were made (0.05 ng, 0.1 ng and 0.15 ng tetanus toxin per 20 µl). Mice were injected using a microliter syringe (Hamilton 33 G needle) under isoflurane inhalation anesthesia. Each mouse received 10 µl of one of the different dilutions of TeNT into each tibialis anterior muscle (20 µl altogether per mouse). This volume was chosen to assure the dispersion into the whole muscle. Following the injections, the mice were observed daily for any sign of distress.

2.4. Statistics

The effect of TeNT on wheel running performance was quantified by changes on a daily basis in \( V_{max} \) and \( T_{ac} \). Values are expressed as mean ± S.E.M. To analyze the effect of different TeNT dosages with respect to effect size and duration, a two-factorial ANOVA for repeated measurements was performed for each running parameter. Post hoc tests (unpaired t-test) were applied to compare the means of the different dosage groups at different time points when ANOVA revealed significant results. A P level of <0.05 was considered significant in these tests. The statistics were calculated using SPSS 18 software.

3. Results

In the first two nights following the TeNT injections, the accumulative running time \( T_{ac} \) of the mice in all 3 dosage groups decreased regardless of the applied dosage of TeNT (Fig. 1A). Maximum running velocity \( V_{max} \) was not altered (Fig. 1B). Therefore, this initial drop in running performance seems to be an effect of the injection procedure itself, but not a dose-specific effect of the toxin. However, in the third night after the injection, the \( T_{ac} \) and \( V_{max} \) of the mice decreased dose-dependently (Fig. 1). This drop in running performance corresponded to the mice beginning to develop a TeNT-induced focal increase of muscle tone. The dose-dependent decrease was present over the next 18 days. Subsequently, no difference in running performance was apparent between the three dosage groups and the mice again reached their baseline running levels (Fig. 1).

ANOVA for accumulative running time \( T_{ac} \), as well as for maximum running velocity \( V_{max} \), revealed significant main effects of dosage and time course. In addition, an interaction of time course and dosage was exhibited for \( T_{ac} \) as well as for \( V_{max} \) (Fig. 1, Table 1), which indicates that the TeNT dosage governs not only the size of the effect but also its duration. According to the post hoc tests, the \( T_{ac} \) and \( V_{max} \) in mice injected with the highest dosage (0.15 ng TeNT) decreased significantly more these parameters than in either of the lower-dosage groups (0.1 ng and 0.05 ng TeNT). Maximum effect was reached on the fifth and sixth night after the injection. The maximum running velocity and total running time of the middle-dosage group (0.1 ng TeNT) were also significantly more reduced than those of the low-dosage group (0.05 ng TeNT, Fig. 1).
analyses and numerical results are provided in Table 1, which presents the ANOVA results for the maximum velocity ($V_{\text{max}}$) and the accumulative running time ($T_{\text{acc}}$). The results show significant differences in the $V_{\text{max}}$ across different dosages and time courses, indicating a cumulative effect of TeNT on muscle tone.

### Discussion

In the present in vivo study, we demonstrate that focal injections of low-dose TeNT into leg muscles of wildtype mice induce a temporally limited and localized increase in muscle tone. Using an automated wheel running paradigm, we were able to quantify the degree of this spastic muscle tone and to monitor the central action of TeNT over its entire time course. Using this novel test, it was even possible to detect and quantify otherwise clinically only marginally apparent localized increases in muscle tone, as induced by minimal toxin dosages.

Clinical studies have shown that the main cause of disability and of functional motor constrictions of upper motor neuron lesions is not the spasticity of the antagonistic muscles themselves, but the reduction in the muscle strength of the agonists (Fellows et al., 1994; Horstman et al., 2008). For this reason, it seems a promising strategy to treat paresis of the agonistic muscles with localized injections of low-dose TeNT to increase their muscle tone. Animal studies have shown that injections of low-dose TeNT enhance muscle tone also during voluntary movements and that this effect is not prevented by active or passive vaccination against TeNT (Fishman et al., 2009; Webster and Laurence, 1963).

Such a facilitation of central muscle paresis might be an option in neurorehabilitation, for example in the treatment of drop foot in stroke patients. Other potential indications for focal TeNT injections in diseases with decreased muscle tone, such as multiple sclerosis or spinal cord injury, have also been proposed (Fishman et al., 2009; Sanders, 2004). Furthermore, a single experiment with a bulldog points to the therapeutic potential of injecting TeNT into an upper airway muscle in obstructive sleep apnoea (Sasse et al., 2005).

So far, research into the therapeutic potential of TeNT has been restricted by the lack of tests available to reliably measure the toxicity of low-dose TeNT. Especially no in vivo tests exist so far to assess the magnitude as well as time course of functional effects of low-dose TeNT.

The significance of the available visual scale, which was introduced to distinguish 6 degrees of local and generalized tetanus in rabbits (Webster and Laurence, 1963), is limited as it does not allow for continuous monitoring of the time course of focal TeNT effects. A further limitation is that it is highly investigator dependent. In the clinical setting, tetanus vaccines are tested for the absence of toxin by injecting guinea pigs and observing them over the following 3 weeks (Council of Europe, 2008). Alternatively, especially in immunological research of TeNT, mouse lethality curves are calculated as a standard test (Robinson, 1988). However, these animal tests only detect higher dosages of TeNT and furthermore do not assess the time course of the induced effect.
To circumvent animal experiments, several attempts have been made during recent years to develop in vitro tests for assessing the toxicity of TeNT (Behrensford-Nicol et al., 2010). However, as compared to in vivo tests, these approaches only partially cover the stages of the intoxication process, such as ganglioside-binding activity, proteolytic activity, and endopeptidase activity (Behrensford-Nicol et al., 2010). One particular aim of our study was therefore to develop an in vivo test that can quantify the effects of even low-dosage injections of TeNT that induce only mild functional gait disturbances in wildtype mice and therefore represent a better correlate for the prospective therapeutic application of TeNT in neurorehabilitation.

Recent studies have revealed that the analysis of murine wheel running activity, with the use of complex running wheels with irregularly spaced crossbars, is capable of quantifying even latent motor deficiency (Kutschenko et al., in press; Liebetanz and Merker, 2006; Liebetanz et al., 2007). Also in the present study, the use of complex running wheels in combination with injections of low-dosage TeNT into the tibialis anterior muscles of wildtype mice allowed us to objectively, rater-independently, monitor TeNT-induced, slightly and focally increased muscle tone in the injected muscles. After bilateral injections of low-dosage TeNT into the tibialis anterior muscles of mice, their maximum running velocity (V_{max}) and accumulative running time (T_{ac}) decreased in a clear, dose-dependent manner without widespread clinical symptoms. Generalized symptoms due to TeNT injections did not occur in our experiments.

Using this novel paradigm, we were able to significantly reduce the injected TeNT dosages as compared to former in vivo studies and to quantify the magnitude and duration of the induced focal muscle spasms. Our paradigm is capable of detecting spasms of the tibialis anterior muscle that are only slightly evident, but functionally relevant for the continuous adaptation of the stride length on the complex running wheels. Furthermore, our data indicate that V_{max} represents the most meaningful running parameter for detecting functional effects of TeNT injections. V_{max} shows a particularly low intra-individual variation, so that the required animal number is comparatively low.

In addition to the accurate monitoring of TeNT effects, this combination of low-dosage TeNT and wheel running may also be applied as a valuable mouse in vivo model of focal spasticity. So far, spasticity in mice is usually estimated by visually rating whether it is present or not. Alternatively, quantification is achieved measuring the force required to flex the spastic limb against a strain gauge (Baker et al., 2000). However, these hitherto existing approaches do not permit continuous and rater-independent quantification of spasticity in mice. Because of its unique features, this proposed mouse model of focal spasticity particularly offers the opportunity for an automated screening and characterization of potentially anti-spasticity drugs.

In conclusion, our results demonstrate that low-dosage injections of TeNT into the tibialis anterior muscles of wildtype mice induce a temporary focal increase of muscle tone. The degree and duration of the induced effects can be quantified by the analysis of wheel running performance in complex running wheels in a fully automated manner.

This mouse model may serve as an important in vivo tool for future experimental research with respect to the therapeutic potential of TeNT in neurological rehabilitation. The model may furthermore be of particular value in the search for novel anti-spasticity treatments.

Conflict of interest

None.

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