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Botulinum toxin type A reduces pain supersensitivity in experimental diabetic neuropathy:  
Bilateral effect after unilateral injection

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## **Abstract**

We investigated antinociceptive activity of botulinum toxin type A (BTX-A) in a model of diabetic neuropathic pain in rats.

Male Wistar rats were made diabetic by a single intraperitoneal injection of streptozotocin (80 mg/kg). Sensitivity to mechanical and thermal stimuli was measured with the paw-pressure and hot-plate test, respectively. The formalin test was used to measure sensitivity to chemical stimuli. Diabetic animals with pain thresholds lower for at least 25% compared to the non-diabetic group were considered neuropathic and were injected with BTX-A either subcutaneously (3, 5 and 7 U/kg) or intrathecally (1 U/kg). Mechanical and thermal sensitivity was measured at several time-points.

After peripheral application, BTX-A (5 and 7 U/kg) reduced mechanical and thermal hypersensitivity not only on ipsilateral, but on contralateral side, too. The antinociceptive effect started 5 days following BTX-A injection and lasted at least 15 days. Formalin induced hypersensitivity in diabetic animals was abolished as well. When applied intrathecally, BTX-A (1 U/kg) reduced diabetic hyperalgesia within 24 h supporting the assumption of retrograde axonal transport of BTX-A from the peripheral site of injection to central nervous system. The results presented here demonstrate the long-lasting pain reduction after single BTX-A injection in the animals with diabetic neuropathy. The bilateral pain reduction after unilateral toxin application and the effectiveness of lower dose with the faster onset after the intrathecal injection suggest the involvement of the central nervous system in the antinociceptive action of BTX-A in painful diabetic neuropathy.

Key words: botulinum toxin type A, diabetic neuropathy, streptozotocin, rat

## **1. Introduction**

Chronic polyneuropathy is present in 8–26% of diabetic patients and represents a major health problem with substantial impact on the quality of life. It is the most common late diabetic complication and affects both type 1 and type 2 diabetic patients but it is more frequent and severe in the type 1 (Sima et al., 2006). Antidepressants, carbamazepine, gabapentin, opioids and, more recently, duloxetine and pregabalin are used in treatment of painful diabetic neuropathy (Ziegler, 2008). None of these drugs or their combinations provide complete or long-lasting pain relief. Side effects, poor tolerability and ineffectiveness for some percent of diabetic patients are major disadvantages of the current therapeutic options (Ziegler, 2008). Botulinum toxin type A (BTX-A) cleaves SNAP-25 (synaptosomal associated protein of 25 kDa), one of the SNARE proteins essential for neurotransmitter release (Aoki, 2005; Grumelli et al., 2005) and it is nowadays widely used to treat muscular spasms (Truong and Jost, 2006). Additionally, it was observed that BTX-A reduced pain in conditions not associated with muscle hypercontraction, like migraine (Göbel, 2004), trigeminal neuralgia (Allam et al., 2005), chronic focal neuropathies (Ranoux et al., 2008) and low-back pain (Jabbari, 2008). Several experiments on animals demonstrated the antinociceptive effect of BTX-A on inflammatory pain induced by formalin (Cui et al. 2004), carrageenan and capsaicin (Bach-Rojecky and Lacković, 2005a). The pain reduction after single subcutaneous BTX-A injection was demonstrated in experimental models of peripheral neuropathic pain (Bach-Rojecky et al., 2005b; Luvisetto et al., 2006; Park et al., 2006; Favre-Guilmond et al., 2009). The antinociceptive action of BTX-A was independent of the effect on muscle relaxation. Hence, there is a possibility that BTX-A might be a useful long-lasting treatment in painful diabetic neuropathy. Yuan et al. (2009) recently showed that single local injections of BTX-A reduced pain scores on visual analogue scale in 18 diabetic patients within 3 months after the injection.

The most commonly used model of diabetic neuropathy are rodents with type 1 diabetes induced by the pancreatic  $\beta$ -cell toxin streptozotocin (Calcutt, 2004). Diabetic animals display physiologic, neurochemical and behavioural changes suggestive of altered pain perception. Only behavioural methods can directly distinguish painful (hyperalgesia or allodynia) from non-painful sensation. The most common behavioural tests measure changes in sensitivity to mechanical, thermal and chemical noxious stimuli (Calcutt, 2004).

In the present study we investigated antinociceptive activity of BTX-A in a model of diabetic neuropathic pain in rats.

## **2. Materials and methods**

### ***2.1. Animals***

Male Wistar rats (University of Zagreb, School of Medicine) weighing 250-300 g were used. Animals were housed in wire-bottomed cages (4-5 per cage) with free access to food and water. The experiments were carried out according to the Croatian Act on Animal Welfare and the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985). The experiments were approved by the Ethical Committee of the University of Zagreb, School of Medicine (permit No. 07-76/2005-43).

### ***2.2. Drugs***

The following drugs were used: chloral hydrate, streptozotocin and formalin (Sigma, St. Louis, MO, USA); BTX-A (BOTOX<sup>®</sup>, Allergan, Irvine, CA, USA). Each vial of BOTOX<sup>®</sup> contains 100 U (~4.8 ng) of purified Clostridium botulinum type A neurotoxin complex. To obtain respective doses, BTX-A was reconstituted in adequate volume of 0.9% saline.

### ***2.3. Induction of diabetes***

Rats were injected by a single intraperitoneal (i.p.) injection of freshly dissolved streptozotocin in citrate buffer (pH 4.5) at a dose of 80 mg/kg body weight. Control animals

were injected i.p. with the citrate buffer. Tail-vein blood-glucose concentration was determined by colorimetric PAP method 5 days following the induction of diabetes. Animals with the blood glucose concentration above 15 mmol/l were considered diabetic and were included in the study. Hyperglycaemia was re-confirmed before nociceptive measurement (3 weeks after streptozotocin injection).

## **2.4. Nociceptive tests**

### *2.4.1. Mechanical sensitivity measurement*

The sensitivity to mechanical stimuli was measured by the paw-pressure test as described by Randall and Selitto (1957). Mechanical nociceptive thresholds expressed in grams were measured 3 times in 10-min intervals by applying increased pressure to the dorsal surface of the hindpaw until paw-withdrawal or overt struggling were elicited. The measurements were performed bilaterally. Measurements were done by an experimenter who was not aware of the treatment groups.

### *2.4.2. Thermal sensitivity measurement*

Thermal sensitivity was tested using a slight modification of the unilateral hot-plate test originally described for mice (Mendez et al., 2002). The temperature of the hot-plate surface was  $52\pm 0.5$  °C and the cut off time was 20 s in order to prevent paw-tissue damage. Rats were gently restrained and the plantar side of the tested paw was placed on the hot-plate surface. The latency of paw withdrawal from the heated surface was recorded 3 times at 10-min intervals. The measurements were performed bilaterally. Measurements were done by an experimenter who was not aware of the treatment groups.

### *2.4.3. Chemical sensitivity measurement*

Formalin (5%), in a volume 50 µl was injected subcutaneously (s.c.) into the plantar region of the right hindpaw. Immediately after the injection, the number of flinches and shakes of the injected paw was counted in 2-min intervals for 1 h (Kang et al., 2007). In the present study,

the data collected between 0 and 15 min after the formalin injection represented phase 1 and the data collected between 15 and 60 min after the formalin injection represented phase 2. Behavioural studies were performed by an experimenter who was not aware of the treatment groups. Results are presented as the sum of the total number of flinches/shakes of the formalin injected paw during each phase.

### ***2.5. BTX-A injections***

BTX-A was injected by two different routes of administration: 1.) s.c. into the plantar surface of the hindpaw to conscious rat in a volume of 20 µl with a 27 ½ gauge syringe; and 2.) intrathecally at L3-L4 level to anaesthetised rat (chloral hydrate 300 mg/kg, i.p.) in a volume of 10 µl using a Hamilton syringe.

### ***2.6. Experimental protocol***

Diabetic animals were subjected to bilateral measurements of sensitivity to mechanical and thermal stimulation 3 weeks following the induction of diabetes. Only those diabetic animals with nociceptive threshold at least 25% lower than the one found in control non-diabetic group were considered neuropathic (hyperalgesic) and were then subjected to the BTX-A or saline treatment.

*Dose-response experiment:* BTX-A at doses 3, 5 and 7 U/kg was applied peripherally into the hindpaw pad. Sensitivity to thermal and mechanical stimuli was measured bilaterally 5 days following the toxin application. Formalin test was performed only once (10-12 days following the BTX-A peripheral injection).

*The time-course experiment:* BTX-A in a dose of 7 U/kg was applied peripherally and mechanical sensitivity was measured ipsilaterally on day 1, 5, 15 and 28 following the BTX-A peripheral injection.



*Intrathecal application:* BTX-A 1U/kg was applied into the lumbar spinal fluid (central application) and the effects on mechanical and thermal hyperalgesia were tested bilaterally on day 1, 5, 12, 20, 27, 33 and 37.

### **2.7. Statistical analysis**

Results are presented as mean  $\pm$  standard error (S.E.M.). Statistical analysis was performed by an analysis of variance (ANOVA). Intergroup differences were analyzed by the Newman-Keuls post hoc test. A  $P < 0.05$  was considered significant. In the time-course experiment, ANOVA for repeated measurements followed by Tukey's test was employed.

### **3. Results**

Three weeks following a single i.p. streptozotocin injection (80 mg/kg), about 45% of rats with diabetes (blood glucose concentration  $>15$  mmol/l) developed increased sensitivity to mechanical and thermal stimuli compared to the saline-treated non-diabetic control group.

Those animals were included in further experiments as groups with neuropathic pain. In those animals a single unilateral BTX-A (5 and 7 U/kg) injection into the right hindpaw pad significantly decreased mechanical hypersensitivity not only on ipsilateral, but on the contralateral side, as well (Fig.1.).

Fig. 1.

In contrast to the mechanical hyperalgesia which was consistent throughout the whole experiment, changes in the thermal sensitivity were variable. Peripheral injection of BTX-A in a dose of 7 U/kg significantly reduced thermal hypersensitivity on ipsilateral side only. On the contralateral side, sensitivity to thermal stimulation in rats with diabetes seemed increased compared to non-diabetic animals, but this was not significant probably due to the large inter-

group variability. Although BTX-A 7 U/kg appeared to have reversed the thermal latency on the contralateral side, this was not significant either (Fig. 2.).

Fig. 2.

Formalin injection into the ipsilateral hindpaw of diabetic rats induced significant increase in the number of flinches and shakes of the injected paw compared to non-diabetic control only in phase 2 of the test (Fig. 3A and 3B). All three tested doses of BTX-A significantly decreased formalin-induced pain in the second phase of the test (Fig. 3A).

Fig. 3

BTX-A decreased mechanical hypersensitivity 5 days following application into the hindpaw pad and the effect remained significant for 10 more days. When tested 1 day after the injection into the hindpaw, BTX-A was ineffective (Fig. 4.).

Fig. 4.

BTX-A injected intrathecally in the dose of 1 U/kg into the lumbar cerebrospinal fluid decreased bilateral thermal and mechanical hypersensitivity within 24 h after the application. The antinociceptive effect was significant even on day 27 for the thermal (Fig. 5A.) and on day 33 for the mechanical hyperalgesia (Fig. 5B.).

Fig. 5.

#### **4. Discussion**

In the present study we demonstrate the long-lasting reduction of hyperalgesia in experimental diabetic neuropathy after single BTX-A peripheral and central injection. In contrast to all other forms of existing short-lasting therapy, antinociceptive effect of BTX-A in the present experiments lasted up to four weeks. The bilateral pain reduction after unilateral toxin application and the effectiveness of lower dose with the faster onset after the intrathecal injection suggests the involvement of the CNS in the BTX-A antinociceptive action.

The pathology of diabetic neuropathy is characterized by progressive nerve fibre loss and endoneurial microangiopathic changes (Calcutt et al., 2007). Pathogenesis, believed to be the most important, consists of an ischemic process secondary to microangiopathy, glycosylation of structural proteins consequent to chronic hyperglycemia, or injury by reactive oxygen species generated by altered glucose metabolism (Leininger et al., 2006; Romanovsky et al., 2004). However, all recent knowledge is not sufficient to explain the appearance of hyper- and hyposensitivity to pain during the course of diabetic neuropathy.

Streptozotocin is the most common substance used for the induction of diabetes in the rodents. The cytotoxic action of streptozotocin is mediated by reactive oxygen species, liberation of the toxic amounts of NO, alkylation and damage of DNA which result in rapid destruction of pancreatic  $\beta$ -cells (Szkudelski, 2001). Diabetic polyneuropathy develops within weeks of diabetes induction and it is characterized by nerve conduction velocity abnormalities, increased activity of the polyol pathway, and decreased endoneurial blood flow. However, despite these early functional and metabolic abnormalities, animals do not develop structural deficits, such as progressive nerve fibre loss, even after prolonged duration of diabetes, which is the very hallmark of human diabetic polyneuropathy (Calcutt and Backonja, 2007).

Neuropathic pain after single i.p. streptozotocin injection in rodents is mostly characterized by mechanical allodynia and hyperalgesia which progressively develop within 2 weeks.

Inconsistent changes in the thermal nociceptive thresholds have been reported in diabetic animals: thermal hyperalgesia (Courteix et al., 1993), thermal hypoalgesia (Apfel et al., 1994) to unchanged thermal sensitivity (Malcangio and Tomlinson, 1998). It has also been reported that diabetic rats exhibit transient thermal hyperalgesia during the first two weeks of diabetes, which progresses to thermal hypoalgesia within next 2-3 months, similarly to what has been found in diabetic patients (Calcutt, 2004). In our experiments, 3 weeks after the streptozotocin injection less than 50% of animals with diabetes developed mechanical and thermal hyperalgesia. Contrary to the mechanical hyperalgesia, we found that changes in the thermal sensitivity were less pronounced and inconsistent. In the present experiment, unilateral BTX-A (5 and 7 U/kg) injection into the rat hindpaw pad reduced mechanical hyperalgesia on ipsilateral injected side but on the contralateral side as well. The bilateral effect was started to be evident on day 5 after the toxin peripheral injection. In this, as well as in our previous experiments, antinociceptive effect of BTX-A was achieved in doses several times lower than the doses required to produce any measurable effect on motor performance (Cui et al., 2004, Favre-Guimard et al., 2009) or any visible behavioral effect. In our previous experiments we repeatedly questioned the role of the peripheral sensory nerve endings and speculated about the involvement of the CNS in the antinociceptive effect of BTX-A (Bach-Rojecky and Lacković, 2005a; Bach-Rojecky et al., 2008). In our most recent report, unilateral injection of BTX-A bilaterally abolished “mirror pain” induced with repeated acidic saline injection thus indicating involvement of the CNS (Bach-Rojecky and Lacković, 2009). Moreover, the antinociceptive effect of BTX-A was completely prevented by colchicine pre-treatment, additionally suggesting that the central effect is mediated by retrograde axonal transport of BTX-A into the CNS (Bach-Rojecky and Lackovic, *In Press*). At the same time, the bilateral

effect of unilateral BTX-A injection was observed in the paclitaxel induced neuropathy in rats (Favre-Guilhard et al. 2009). The authors assumed that the antinociceptive action of BTX-A is not due to a local effect. Measuring SNAP-25 cleavage at different brain regions Antonucci et al. (2008) recently demonstrated retrograde axonal transport of BTX-A via central neurons and motoneurons and thus offered novel pathways of BTX-A trafficking within neurons. Moreover, after tectal injections of the toxin, cleaved SNAP-25 appeared in cholinergic synapses in the retina. According to the authors, this data indicates retrograde transport to retinal ganglion cells, followed by transecytosis into starburst amacrine cells. In our experiments, BTX-A is most probably transported to the central terminal of the ipsilateral afferent fiber. However, how BTX-A exerts antinociceptive action on a contralateral side remains highly speculative. One of the possibilities might be that it is transynaptically transported to the central endings of the afferent neurons on the contralateral side. However, other possibilities cannot be ruled out, including other places of action with effect on contralateral side. While the significance of axonal transport in the peripheral motoneurons and central neurons is not clear (Antonucci et al. 2008), we suggested the existence of axonal transport of BTX-A most probably within sensory neurons mediating antinociceptive effect of BTX-A.

In order to further investigate involvement of the central nervous system in the antinociceptive effect of BTX-A, we investigated its effect after intrathecal injection.

Intrathecal injection of BTX-A abolished the mechanical and thermal hypersensitivity in diabetic rats in a dose as low as 1 U/kg (10 µl). The effect was bilateral and started within the first 24 h. The observed effectiveness of lower dose and its faster onset after the intrathecal application additionally support the central origin of the BTX-A antinociceptive action.

In our experiments the effect lasted at least 15 days after single peripheral BTX-A injection and up to 33 days when BTX-A was applied intrathecally.

Chemical hypersensitivity was reported to develop in rats with streptozotocin-induced diabetes. The formalin test is used to investigate spinal sensitization in animals and allows investigation of sensory processing beyond peripheral nociceptive pathways (Calcutt and Backonja, 2007). Chemical hyperalgesia during phase 2 of the formalin test in diabetic rats is associated with increased cyclooxygenase-2 expression and prostaglandine E-2 release in the spinal cord (Freshwater et al., 2002; Ramos et al., 2007). Formalin also increased the expression of postsynaptic NMDA and AMPA receptors for glutamate (Li et al., 1999) and enhanced electrophysiologic activity in the dorsal horn neurons (Chen and Pan, 2002). Spinal amplification of pain signals with paradoxical reduction in ongoing signals from the periphery (Calcutt et al., 2000) and decreased spinal release of glutamate (Malmberg et al., 2006) suggest significant role of central sensitization in the formalin induced hypersensitivity in experimental diabetes. Although the investigation of neuropathic pain in diabetes has primarily focused on the peripheral nerves, growing body of evidence suggests spinal cord and higher CNS as generators and amplifiers of pain (Calcutt and Backonja, 2007).

In the present experiment, BTX-A applied peripherally into the rat hindpaw pad 10-12 days before the injection of formalin completely reduced chemical hypersensitivity in diabetic rats for several weeks. Two previously mentioned experiments (Bach-Rojecky and Lacković, 2009, Favre-Guilhard et al., 2009) and the experiments presented here cannot be explained in any other way but assuming the involvement of the CNS. Although the mechanism of the long-lasting antinociceptive effect of BTX-A is not clear, discovery of the axonal transport of BTX-A in motoneurons (Antonucci et al., 2008) and, possibly, in sensory neurons as well (Bach-Rojecky and Lacković, 2009) provide a necessary prerequisite for central action of BTX-A. We believe that the antinociceptive effect of BTX-A might be associated with processes of central sensitization.

## 5. Conclusion

At the same time the results presented here, together with the observation of Yuan et al. (2009) on a small number of diabetic patients, demonstrate the potential of BTX-A as a therapeutic tool to combat neuropathic pain.

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Fig. 1. Dose dependent effect of BTX-A on streptozotocin-induced mechanical hyperalgesia on ipsilateral and contralateral side of the rat hindpaw. Measurements were done 5 days following BTX-A s.c. injection into the hindpaw pad. Results are presented as mean±S.E.M., n=5-7. \*P<0.01, \*\*P<0.001 (Newmann-Keuls post hoc test). Legend: Non-diabetic control stands for the animals without diabetes injected only with citrate buffer. Saline denotes animals with diabetic neuropathy injected with saline into the hindpaw pad; BTX-A 3, 5 and 7 U/kg denote animals with diabetic neuropathy injected with BTX-A 3, 5 or 7 U/kg.

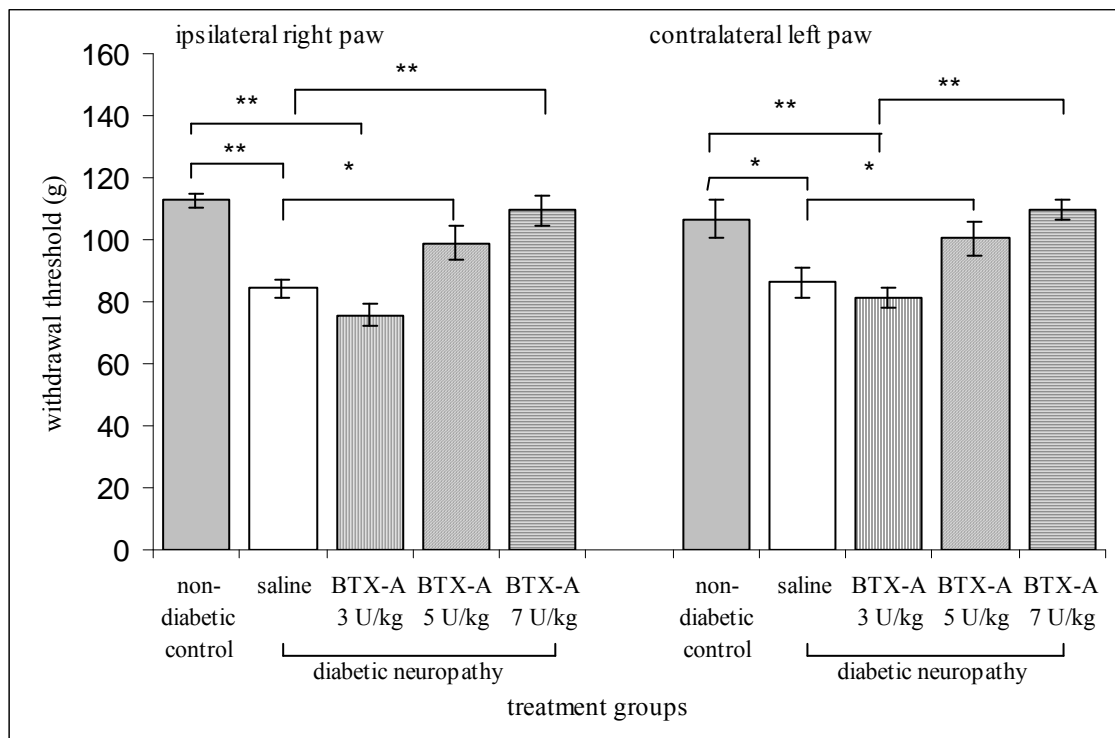


Fig. 2. Dose dependent influence of BTX-A on streptozotocin-induced thermal sensitivity on ipsilateral and contralateral side of the rat hindpaw. Measurements were done 5 days following BTX-A s.c. injection into the hindpaw pad. Results are presented as mean±S.E.M., n=5-7. \*P<0.01; \*\*P<0.001 (Newmann-Keuls post hoc test). Legend: Non-diabetic control stands for the animals without diabetes injected only with citrate buffer. Saline denotes animals with diabetic neuropathy injected with saline into the hindpaw pad; BTX-A 3, 5 and 7 U/kg denote animals with diabetic neuropathy injected with BTX-A 3, 5 or 7 U/kg.

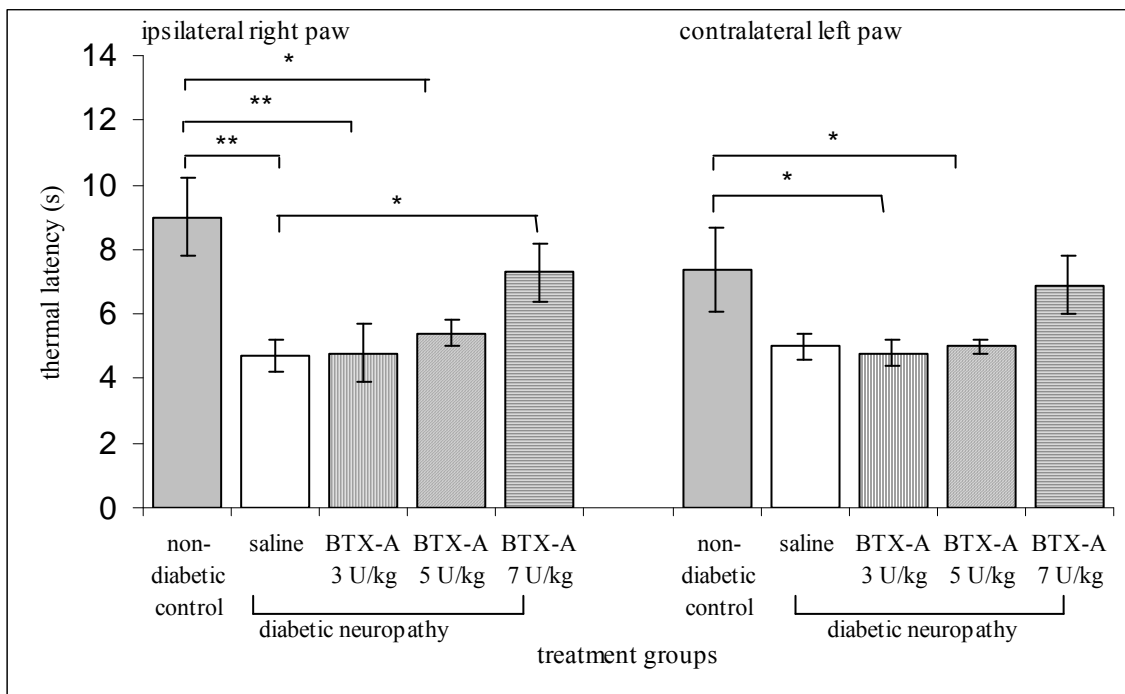
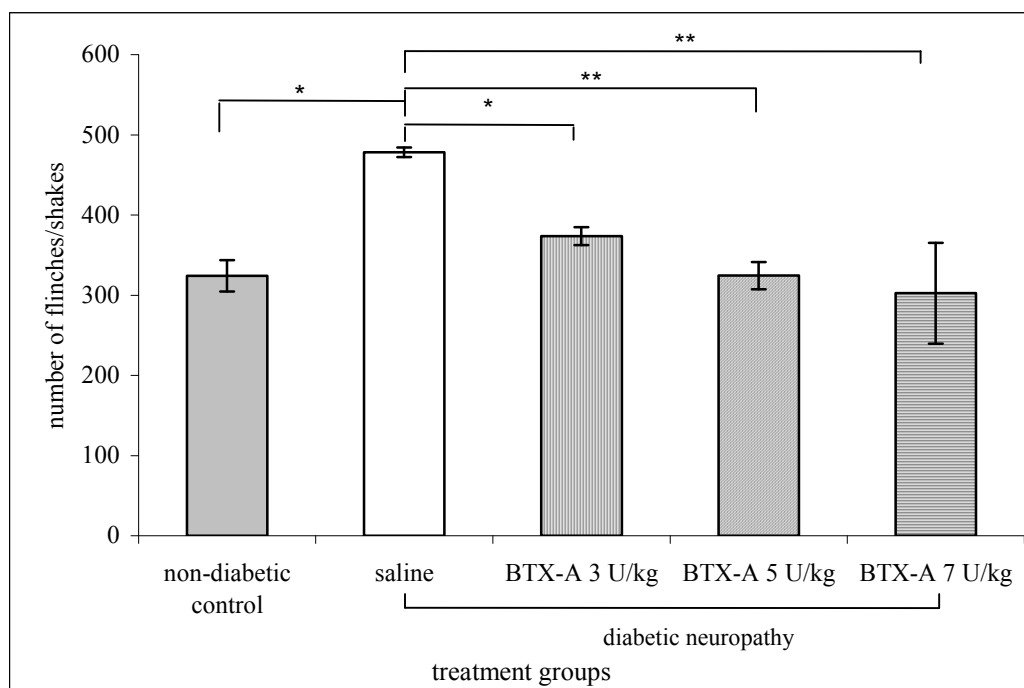


Fig. 3. BTX-A peripheral injection reduces formalin-induced pain in streptozotocin diabetic rats. Measurements were done 10-12 days following BTX-A peripheral application into the hindpaw. Results are presented as mean±S.E.M. for the second phase of the formalin test (3A), n=5-6. \*P<0.05, \*\*P<0.001 (Newman-Keuls post hoc test). Results are presented as mean±S.E.M. for every 4-min interval during 60 min of testing, n=5-6. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001 compared to non-diabetic control; +P<0.05; ++P<0.01 compared to diabetic neuropathy +saline (Newman-Keuls post hoc test).

Legend: Non-diabetic control stands for the animals without diabetes injected only with citrate buffer. Saline denotes animals with diabetic neuropathy injected with saline into the hindpaw pad; BTX-A 3, 5 and 7 U/kg denote animals with diabetic neuropathy injected with BTX-A 3, 5 or 7 U/kg.



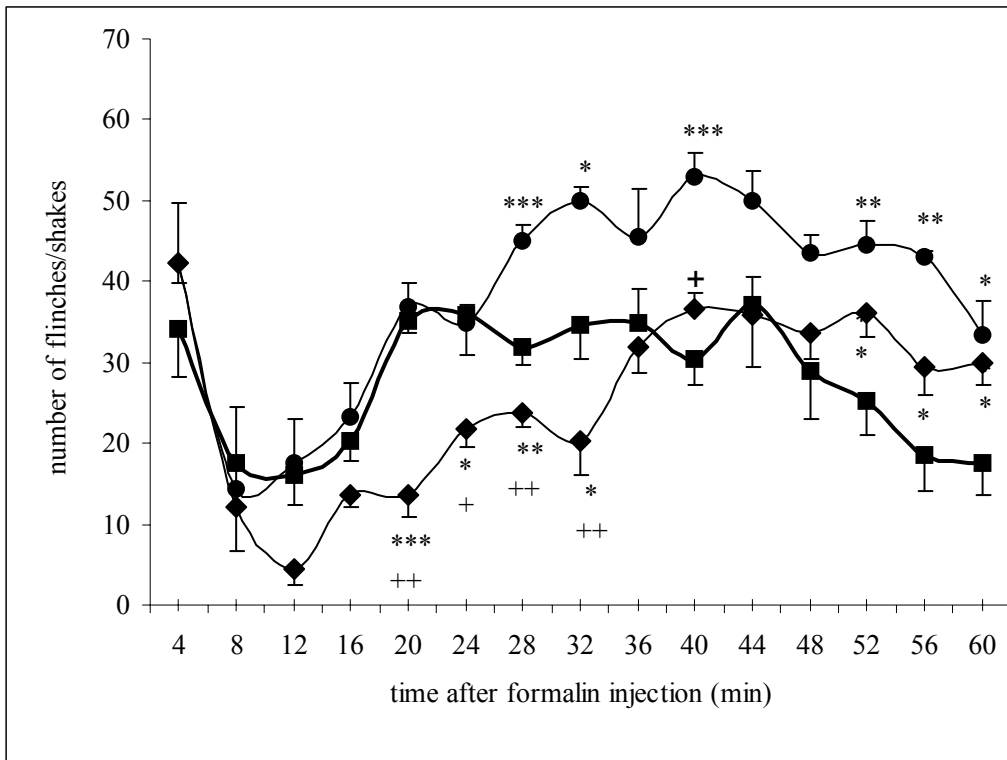


Fig. 4. The time-dependent influence of peripheral BTX-A 7 U/kg injection into the hindpaw on mechanical hyperalgesia in rats with streptozotocin-induced diabetes. Results are presented as mean±S.E.M., n=5-6. \*P<0.001 compared to non-diabetic control; <sup>+</sup>P<0.05, <sup>++</sup>P<0.01 compared to saline-treated rats with diabetic neuropathy (ANOVA for repeated measurements and Tukey's post hoc test).

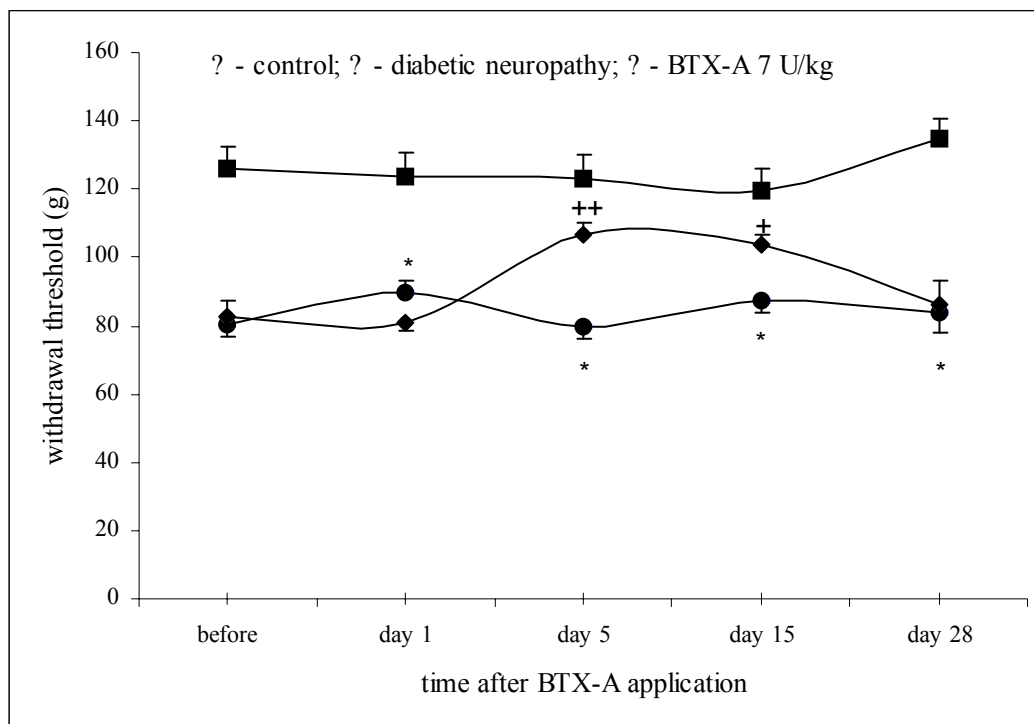


Fig. 5. Time-dependent influence of intrathecal BTX-A 1 U/kg injection on thermal (A) and mechanical (B) hyperalgesia in rats with streptozotocin-induced diabetes. Results are presented as mean±S.E.M., n=5-6. \*P<0.001 compared to non-diabetic control; +P<0.05, ++P<0.01, +++P<0.001 compared to saline-treated rats with diabetic neuropathy (ANOVA for repeated measurements and Tukey's post hoc test).

