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

Lidija Bach-Rojecky [a], Melita Šalković-Petrišić [b], Zdravko Lacković [b,*]

[a] Department of Pharmacology, University of Zagreb School of Pharmacy and Biochemistry, Zagreb, Croatia
[b] Laboratory of Molecular Neuropharmacology, Department of Pharmacology and Croatian Institute for Brain Research, University of Zagreb School of Medicine, Zagreb, Croatia

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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.

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 and Kamiya, 2006). Antidepressants, carbamazepine, gabapentin, opioids and, more recently, duloxetine and pregabalin are used in the 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, 2005). The pain reduction after single subcutaneous BTX-A injection was demonstrated in experimental models of peripheral neuropathic pain (Bach-Rojecky et al., 2005; Luvisetto et al., 2007; Park et al., 2006; Favre-Guilmard 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 injection 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 β-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

* Corresponding author. Department of Pharmacology, University of Zagreb School of Medicine, Šalata 11, 10 000 Zagreb, Croatia. Tel./fax: +385 1 45 66 843.
E-mail address: lac@mef.hr (Z. Lacković).

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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®, Allergan, Irvine, CA, USA). Each vial of BOTOX® 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 threshold expressed in grams was 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 (Menendez et al., 2002). The temperature of the hot-plate surface was 52 ± 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 for the second phase and additionally for every 4-min interval during 60-min testing.

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 27½ 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.

2.6.1. 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).

2.6.2. The time-course experiment

BTX-A in a dose of 7 U/kg was applied peripherally and mechanical sensitivity was measured ipsilaterally on days 1, 5, 15 and 28 following the BTX-A peripheral injection.

2.6.3. Intrathecal application

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

2.7. Statistical analysis

Results are presented as mean ± standard error (S.E.M.). Statistical analysis was performed by an analysis of variance (ANOVA). Inter-group 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 citrate buffer-treated non-diabetic control group. These animals were included in further experiments as groups with neuropathic pain. In these 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.).

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

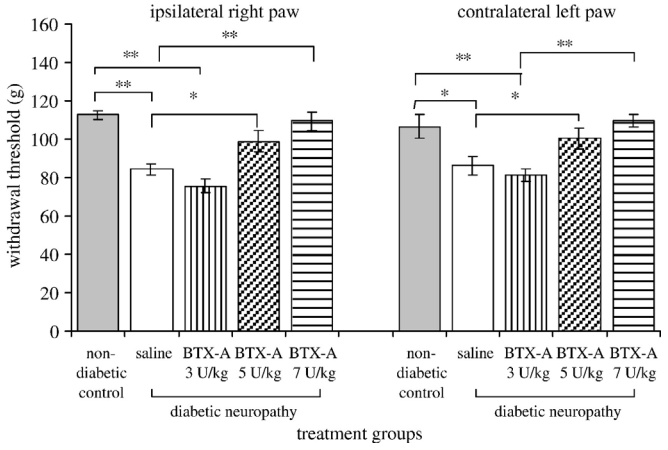

Fig. 1. Dose dependent effect of BTX-A on streptozotocin-induced mechanical hyperalgesia on ipsilateral and contralateral side of the rat hindpaw. Measurements were done 5 days following BTX-A s.c. injection into the hindpaw pad. Results are presented as mean ± S.E.M., $n = 5–7$. $^{*}P<0.01$ and $^{**}P<0.001$ (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.

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 intra-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.).

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,B). All three tested doses of BTX-A significantly decreased formalin-induced pain in the second phase of the test (Fig. 3A).

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.).

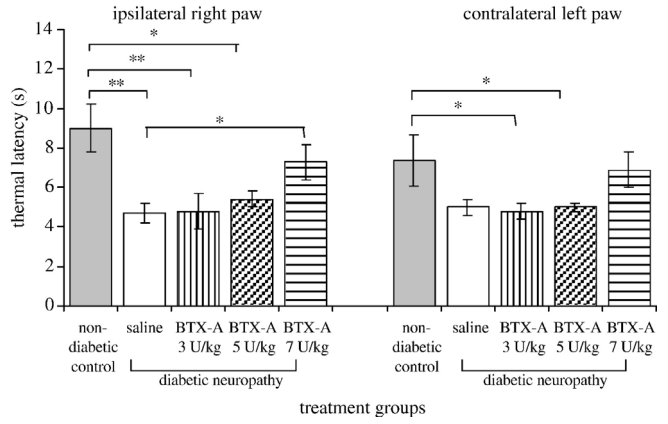

Fig. 2. Dose dependent influence of BTX-A on streptozotocin-induced thermal sensitivity on ipsilateral and contralateral side of the rat hindpaw. Measurements were done 5 days following BTX-A s.c. injection into the hindpaw pad. Results are presented as mean ± S.E.M., $n = 5–7$. $^{*}P<0.01$ and $^{**}P<0.001$ (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.

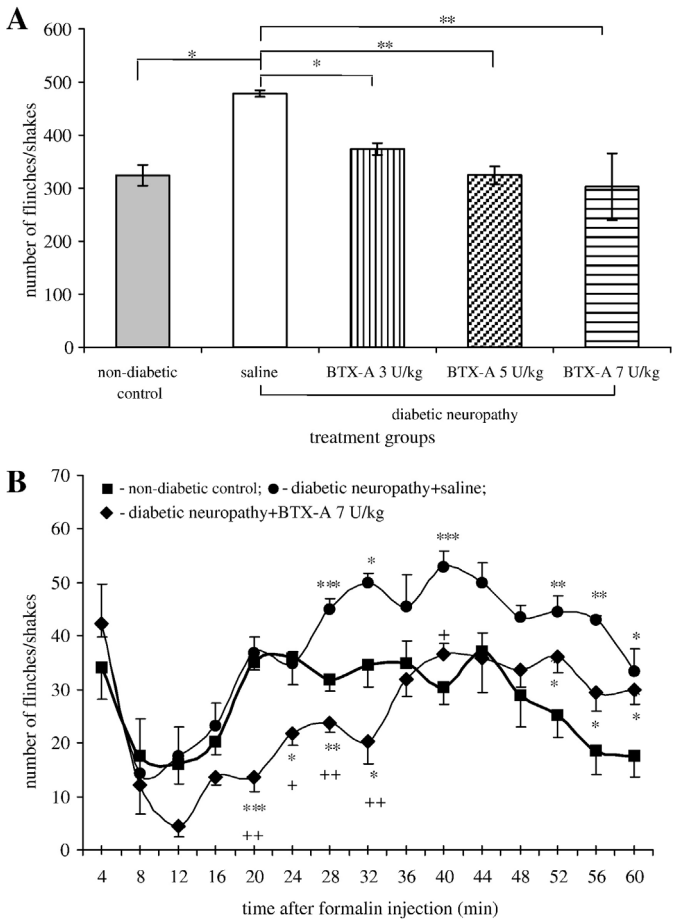

Fig. 3. BTX-A peripheral injection reduces formalin-induced pain in streptozotocin diabetic rats. Measurements were done 10–12 days following BTX-A peripheral application into the hindpaw. Results are presented as mean ± S.E.M. for the second phase of the formalin test (A), $n = 5–6$. $^{*}P<0.05$ and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

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).

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 4 weeks. 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 CNS in the BTX-A antinociceptive action.

The pathology of diabetic neuropathy is characterized by progressive nerve fibre loss and endoneurial microangiopathic changes (Calcutt and Backonja, 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

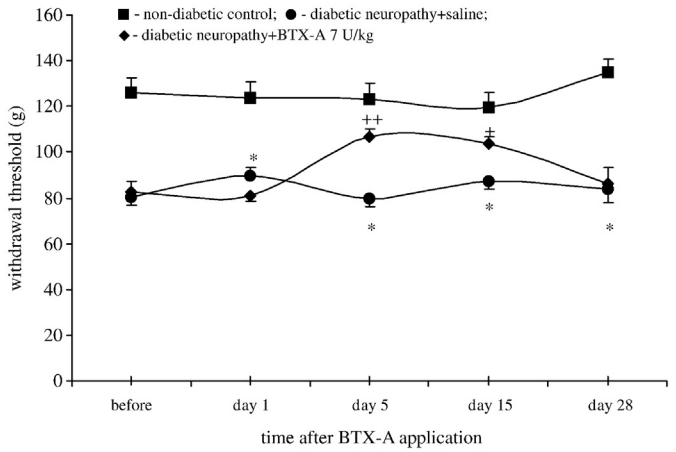

Fig. 4. The time-dependent influence of peripheral BTX-A 7 U/kg injection into the hindpaw on mechanical hyperalgesia in rats with streptozotocin-induced diabetes. Results are presented as mean ± S.E.M., $n = 5–6$. $^{*}P<0.001$ compared to non-diabetic control; $^{+}P<0.05$ and $^{++}P<0.01$ compared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

metabolism (Leinninger 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.

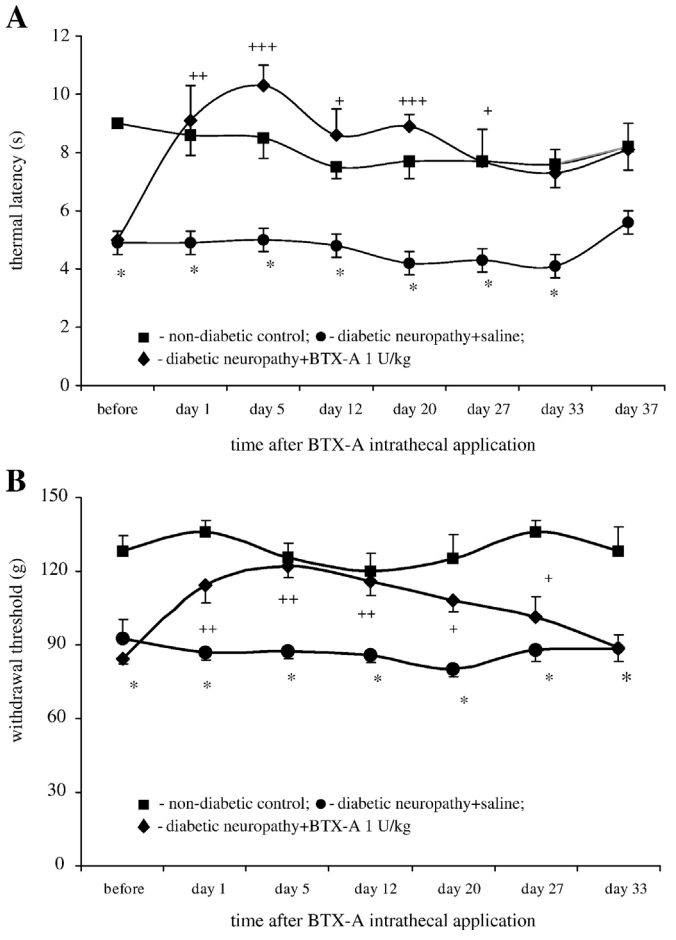

Fig. 5. Time-dependent influence of intrathecal BTX-A 1 U/kg injection on thermal (A) and mechanical (B) hyperalgesia in rats with streptozotocin-induced diabetes. Results are presented as mean ± S.E.M., $n = 5–6$. $^{*}P<0.001$ compared to non-diabetic control; $^{+}P<0.05$, $^{++}P<0.01$, and $^{+++}P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

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 β-cells (Szkudelski, 2001). Diabetic polyneuropathy develops within 2–3 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 2 weeks of diabetes, which progresses to thermal hypoalgesia within the 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-Guilmard et al., 2009) or any visible behavioural 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, 2005; 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 Lacković, 2009). At the same time, the bilateral effect of unilateral BTX-A injection was observed in the paclitaxel induced neuropathy in rats (Favre-Guilmard 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 transcytosis into starbust amacrine cells. In our experiments, BTX-A is most probably transported to the central terminal of the ipsilateral afferent fibre. 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 the 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 the 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 prostaglandin 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, the 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-Guilmard 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, the 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) provides 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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