EJP
European Journal of Pain

ORIGINAL ARTICLE

Role of central versus peripheral opioid system in antinociceptive and anti-inflammatory effect of botulinum toxin type A in trigeminal region

V. Drinovac Vlah[1], B. Filipović[2,3], L. Bach-Rojecky[1], Z. Lacković[2]

1 Department of Pharmacology, University of Zagreb Faculty of Pharmacy and Biochemistry, Croatia
2 Laboratory of Molecular Neuropharmacology, Department of Pharmacology, University of Zagreb School of Medicine, Croatia
3 Department of Otorhinolaryngology & Head and Neck Surgery, University Hospital Sveti Duh, Zagreb, Croatia

Correspondence
Zdravko Lacković
E-mails:
lackoviczdravko@gmail.com; lac@mef.hr

Funding sources
This work has been fully supported by Croatian Science Foundation under the project IP-2014-09-4503.

Conflicts of interest
None declared

Accepted for publication
12 October 2017

doi:10.1002/ejp.1146

Abstract

Background: Although botulinum toxin type A (BT-A) is approved for chronic migraine treatment, its site and mechanism of action are still elusive. Recently our group discovered that suppression of CGRP release from dural nerve endings might account for antimigraine action of pericranially injected BT-A. We demonstrated that central antinociceptive effect of BT-A in sciatic region involves endogenous opioid system as well. Here we investigated possible interaction of BT-A with endogenous opioid system within the trigeminal region.
Methods: In orofacial formalin test we investigated the influence of centrally acting opioid antagonist naltrexone (2 mg/kg, s.c.) versus peripherally acting methylnaltrexone (2 mg/kg, s.c.) on BT-A's (5 U/kg, s.c. into whisker pad) or morphine's (6 mg/kg, s.c.) antinociceptive effect and the effect on dural neurogenic inflammation (DNI). DNI was assessed by Evans blue-plasma protein extravasation.
Results: Naltrexone abolished the effect of BT-A on pain and dural plasma protein extravasation, whereas peripherally acting methylnaltrexone did not change either BT-A's effect on pain or its effect on dural extravasation. Naltrexone abolished the antinociceptive and anti-inflammatory effects of morphine, as well. However, methylnaltrexone decreased the antinociceptive effect of morphine only partially in the second phase of the test and had no significant effect on morphine-mediated reduction in DNI.
Conclusions: Morphine acts on pain in trigeminal region both peripherally and centrally, whereas the effect on dural plasma protein extravasation seems to be only centrally mediated. However, the interaction of BT-A with endogenous opioid system, with consequent inhibition of nociceptive transmission as well as the DNI, occurs primarily centrally.
Significance: Botulinum toxin type A (BT-A)'s axonal transport and potential transcytosis suggest that its antinociceptive effect might involve diverse neurotransmitters at different sites of trigeminal system. Here we discovered that the reduction in pain and accompanying DNI involves the interaction of BT-A with central endogenous opioid system (probably at the level of trigeminal nucleus caudalis).

1. Introduction

Pain models in the head and neck area have recently been employed to study the sensitization of trigeminal dural afferents, which are implicated in pathophysiology of migraine and other types of headache (Filipović et al., 2012; Lacković et al., 2016). Botulinum toxin type A (BT-A), registered as a preventive drug in chronic migraine (Dodick et al., 2010), was demonstrated to reduce pain and associated dural neurogenic inflammation (DNI) in preclinical models of pain in the trigeminal region (Filipović et al., 2012; Lacković et al., 2016). However, up to now its site and mechanism of antinociceptive action are still elusive (Matak and Lacković, 2014). Our group recently discovered the presence of BT-A's enzymatic activity, e.g. cleaved synaptosomal-associated protein of 25 kDa (clSNAP-25), in calcitonin gene-related polypeptide (CGRP) positive dural nerve fibres following peripheral (extracranial) injection of BT-A into rats' whisker pad. Inhibition of CGRP release from trigeminal nerve fibres innervating dura, mediated by SNAP-25 cleavage, might explain BT-A's mechanism of pain reduction in migraine and other headaches (Lacković et al., 2016).

There are currently two hypotheses which might explain how extracranially applied BT-A could be transported to meningeal nerve endings:

(1) through axons of intracranial nociceptors, which reach extracranial tissue by crossing through cranial sutures (Burstein et al., 2014; Zhang et al., 2016) or
(2) through nociceptive primary afferents to trigeminal ganglion (TG) from where trans-synaptic transport to the neurons projecting to dura mater occurs and/or trigeminal nucleus caudalis (TNC) from where trans-synaptic transport to the terminal of the converging meningeal afferent could occur (Ramachandran and Yaksh, 2014; Ramachandran et al., 2015; Lacković et al., 2016).

Opioid system is an important factor in neuromodulation of pain at different levels throughout the central and peripheral nervous system (Holden et al., 2005; Stein, 2013). Opioid analgesics effectively reduce migraine pain. However, due to misuse and safety issues they are usually employed as the last treatment option (Finocchi and Viani, 2013). Recently we have provided evidence of the involvement of endogenous opioid system in BT-A's antinociceptive action. We demonstrated that antinociceptive effect of BT-A can be dose-dependently blocked by opioid antagonists, applied either systemically or intrathecally, in several pathophysiologically different types of experimental pain: inflammatory pain induced by formalin injection into the hind paw-pad, neuropathic pain induced by partial sciatic nerve transection (Drinovac et al., 2013) and bilateral pain induced by intramuscular carrageenan (Drinovac Vlah et al., 2016). On the basis of these results, we propose that under BT-A treatment spinal inhibitory neurotransmitter systems are indirectly activated, thus the central antinociceptive effect might be more complex than simple inhibition of neurotransmitter release from primary afferent terminals.

Possible interactions between endogenous opioids and BT-A on pain in trigeminal region were not investigated before. In comparison to sciatic region, pain in trigeminal region is accompanied by dural neurogenic inflammation (DNI), which involves vasodilatation, plasma protein extravasation and inflammatory cells' infiltration (Williamson and Hargreaves, 2001; Filipović et al., 2012; Lacković et al., 2016). Therefore, the aim of the present research was to examine whether opioid antagonist naltrexone blocks the antinociceptive effect of BT-A and its effect on DNI in the orofacial formalin test. Furthermore, we investigated the effects of peripherally acting opioid antagonist methylnaltrexone to examine where does the interaction with opioid system take place. And lastly, to check the rationality of our approach we investigated the effects of naltrexone and methylnaltrexone on opioid agonist morphine, which served as a control in this study.

2. Methods

2.1 Animals

Animal care and experimental procedures were in accordance with the 2010/63/EU Directive on the protection of animals for scientific purposes and the recommendations of International Association for the Study of Pain (Zimmerman, 1983). Experiments were approved by Croatian Ministry of Agriculture Veterinary and Food Safety Directorate (permit no. EP 03-2/2015). ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (Kilkenny et al., 2010) were followed in the preparation of the present manuscript. Experiments were conducted on 3-month-old male Wistar rats (Department of Pharmacology, University of Zagreb School of Medicine, Croatia), weighing 300–400 g, housed with three rats per cage, kept on 12-h light/dark cycle with free access to food and water. Animals were randomly

allocated to experimental treatments. The experimenter conducting the behavioural testing was unaware of the treatments given to the animals. Behavioural testing was performed in a quiet laboratory at daylight between 8:30 am and 1:30 pm. Prior to orofacial formalin injection rats accommodated to the experimental environment for 10 min. Each experimental group contained six animals, except morphine treated groups which contained five animals per group.

2.2 Substances

In this study we used: Evans blue (Merck KGaA, Darmstadt, Germany), formalin (Kemika, Zagreb, Croatia), ketamine (Richter Pharma AG, Wels, Austria) and xlyazine (Alfasan International BV, Woerder, Netherlands), isoflurane (Abbott Laboratories Ltd., Queenborough, UK), naltrexone hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), methylnaltrexone bromide (Relistor®, Wyeth Pharmaceuticals Inc., Philadelphia, PA, USA), BT-A (Botox®, Allergan Inc., Irvine, CA, USA).

Vial of Botox® contains 100 units (4.8 ng) of purified *Clostridium botulinum* neurotoxin type A complex. Vial of Relistor® contains 12 mg of methylnaltrexone bromide in 0.6 mL of water. To obtain the needed doses, all substances were dissolved or diluted in physiological saline (0.9% NaCl).

2.3 Pharmacological treatments

Botulinum toxin type A (5 U/kg) was injected subcutaneously (s.c.) into the left whisker pad in a volume of 20 μL. Dose is chosen based on previous experiments performed in our laboratory (Matak et al., 2011). Morphine, non-selective opioid agonist used as a positive control to BT-A treatment, was s.c. applied in a dose of 6 mg/kg (Sevostianova et al., 2005).

Naltrexone, nonselective opioid antagonist and methylnaltrexone, opioid antagonist that does not cross the blood–brain barrier (Yuan and Israel, 2006), were injected s.c. in a dose of 2 mg/kg (Brown and Goldberg, 1985; Foss et al., 1996; Correa et al., 2010). Morphine, naltrexone and methylnaltrexone were s.c. injected in a volume of 250 μL into a loose skin over the neck. All injections were applied to conscious, gently restrained rats using a 27 1/2-gauge needle. To examine methylnaltrexone in our experimental conditions, and especially to assure that the chosen dose of methylnaltrexone (2 mg/kg) is sufficient to block peripheral opioid receptors, we tested the effects of different doses of methylnaltrexone (0.15, 2 and 4 mg/kg) on morphine's analgesia in a preliminary study. Methylnaltrexone in clinically used therapeutic doses (12 mg per 62–114 kg; 0.15–0.18 mg/kg) does not appear to interfere with the central analgesic effects of opioid pain medications (Webster et al., 2015). This is in line with our results since the dose of 0.15 mg/kg had no significant effect on morphine's analgesia in both phases of the orofacial formalin test. In addition, the dose of 4 mg/kg had similar effects as the chosen dose (2 mg/kg). A preliminary study was performed on three animals per group and the results are shown in Fig. 1.

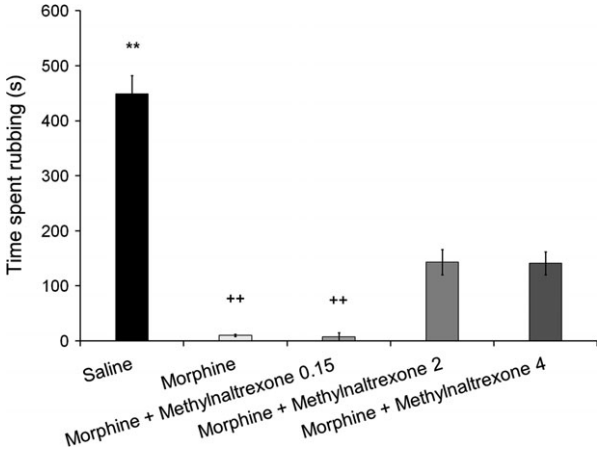

Figure 1 Methylnaltrexone dose–response: the effects of 0.15, 2 and 4 mg/kg methylnaltrexone on morphine's analgesia in the second phase of the orofacial formalin test. Formalin injection and measurement of nociception was preformed 40 min following morphine systemic s.c. treatment and its co-administration with different doses of methylnaltrexone. Mean ± SEM, $N = 3$, $^{**}p < 0.01$ compared to morphine, morphine + methylnaltrexone 0.15, morphine + methylnaltrexone 2, morphine + methylnaltrexone 4; $^{++}p < 0.01$ compared to morphine + methylnaltrexone 2, morphine + methylnaltrexone 4 (Tukey *post hoc*).

2.4 Formalin-induced facial pain: Behavioural testing

Conscious, gently restrained rats were s.c. injected with saline-diluted 2.5% formalin solution (50 μL) into the left whisker pad and placed in transparent cages for 45 min observation period. Facial grooming and rubbing were manually measured using stopwatch (Carl Roth Int., Karlsruhe, Germany), in 3 min periods (number of seconds). Phase I (0–12 min) nocifensive behaviour corresponds to acute nociceptive pain caused by direct stimulation of nerve endings with formalin, whereas phase II (12–45 min)

represents inflammatory pain characterized by the release of inflammatory mediators and sensitization (Raboisson and Dallel, 2004).

According to our previous study protocols, BT-A was applied 5 days before nociceptive testing, whereas morphine, naltrexone and methylnaltrexone were injected 40 min prior to the facial formalin test (Matak et al., 2011; Drinovac et al., 2013). Control animals received saline in appropriate volumes.

2.5 Assessment of dural neurogenic plasma extravasation

Anaesthetized animals (isoflurane inhalational anaesthesia: 5% induction, 2.5% maintenance) were injected i.v. (tail vein) with 1 mL of Evans blue solution (40 mg/kg). After behavioural tests (approx. 1 h after induction of pain) animals were deeply anesthetized with ketamine (70 mg/kg) and xylazine (7 mg/kg), and transcardially perfused with 500 mL of saline solution. Supratentorial dura (ipsilateral and contralateral to formalin treatment) was dissected and weighed. Collected tissue was incubated in 2 mL of formamide at 37 °C for 48 h. After extraction of Evans blue dye in formamide, absorbance of Evans blue formamide extracts was measured spectrophotometrically (Iskra, Ljubljana, Slovenia) at 620 nm. Amount of extravasated dye in tissue was calculated using standard concentration curve (Filipović et al., 2012.).

2.6 Data analysis

Results, presented as mean ± SEM, were analysed by one-way ANOVA followed by the Tukey *post hoc* test for between-group differences. $p < 0.05$ was considered significant.

3. Results

3.1 Opioid antagonist naltrexone abolishes BT-A's effects on pain and dural plasma protein extravasation in orofacial formalin test

Peripheral s.c. BT-A pre-treatment (5 U/kg) significantly reduced formalin-induced pain (time spent rubbing of formalin-injected area) during the second phase of the test, which is in line with our previous findings (Matak et al., 2011). Time spent rubbing the formalin-injected area was reduced from 355 ± 42 s to 170 ± 28 s with BT-A treatment ($p < 0.01$, Fig. 2A). BT-A reduced dural plasma protein extravasation caused by formalin injection (baseline: 5.67 ± 0.86 ng/mg tissue, saline: 21.57 ± 2.28 ng/mg tissue, BT-A: 11.58 ± 1.48 ng/mg tissue; $p < 0.01$, Fig. 2B), which was previously demonstrated in our laboratory as well (Filipović et al., 2012).

Naltrexone (2 mg/kg, s.c.), applied 40 min prior to facial formalin injection, abolished the antinociceptive effect of BT-A (332 ± 20 s; $p < 0.01$, Fig. 2A) as well as its effect on dural plasma protein extravasation (19.68 ± 2.13 ng/mg tissue; $p < 0.05$, Fig. 2B). Tested dose of naltrexone alone did not influence formalin-induced pain (354 ± 39 s) and dural extravasation (20.16 ± 2.21 ng/mg tissue).

It was previously demonstrated that BT-A has no effect on formalin-induced acute pain (Cui et al., 2004; Matak et al., 2011). Accordingly, no differences in pain scores were observed during the first phase between the groups (saline: 57 ± 6 s; BT-A: 61 ± 10 s; BT-A + naltrexone: 56 ± 10 s; naltrexone: 53 ± 8 s; Fig. 2A).

3.2 Peripheral opioid antagonist does not change the effects of BT-A on pain and dural plasma protein extravasation

In contrast to naltrexone, neither BT-A's effect on pain nor its effect on DNI were changed by peripherally acting methylnaltrexone (2 mg/kg) s.c. injection (Fig. 3). In this experiment, the time spent rubbing in the second phase was reduced from 495 ± 39 s to 310 ± 26 s ($p < 0.01$) with BT-A treatment and 326 ± 48 s ($p < 0.05$) with BT-A + methylnaltrexone treatment (Fig. 3A). There were no differences between groups in the first phase of the test (saline: 95 ± 10 s; BT-A: 93 ± 16 s; BT-A + methylnaltrexone: 87 ± 14 s). The amount of extravasated dye was as follows: baseline 11 ± 1.85 ng/mg tissue, saline 39.6 ± 3.65 ng/mg tissue, BT-A 17.23 ± 3.82 ng/mg tissue, BT-A + methylnaltrexone 22.13 ± 5.32 ng/mg tissue (Fig. 3B).

The used dose of methylnaltrexone as a single treatment did not influence formalin-induced pain (first phase: 93 ± 15 s; second phase: 493 ± 27 s) and dural plasma protein extravasation (40.41 ± 3 ng/mg tissue).

3.3 Effects of morphine and its combination with naltrexone versus methylnaltrexone on pain and dural plasma protein extravasation in orofacial formalin test

Morphine applied s.c. in a dose of 6 mg/kg almost completely reduced pain in orofacial formalin test (Fig. 4A): in the first phase from 95 ± 10 s to 0 s ($p < 0.01$), whereas in the second phase from

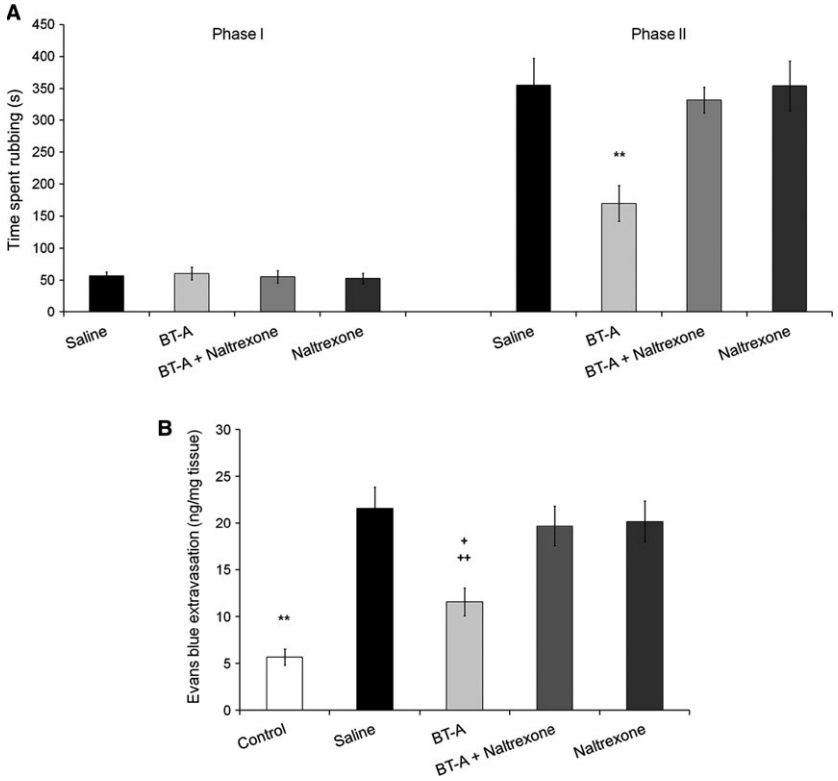

Figure 2 The effects of BT-A and naltrexone on formalin induced pain and dural plasma protein extravasation. Formalin injection and nociceptive measurement were preformed 5 days after BT-A (5 U/kg) peripheral s.c. injection into whisker pad and 40 min after naltrexone (2 mg/kg) systemic s.c. injection. Samples of *dura mater* for extravasation analysis were taken 1 h following formalin injection. Control = non-treated animals; all other groups = treated with 2.5% formalin. (A) Naltrexone abolishes antinociceptive effect of BT-A in the second phase of orofacial formalin test. Mean ± SEM, $N = 6$, $^{**}p < 0.01$ compared to saline, BT-A + naltrexone and naltrexone (Tukey *post hoc*); (B) BT-A decreases formalin-induced DNI, which is abolished by naltrexone. Mean ± SEM, $N = 6$, $^{**}p < 0.01$ compared to saline, BT-A + naltrexone and naltrexone; $^{+}p < 0.05$ compared to BT-A + naltrexone and naltrexone; $^{++}p < 0.01$ compared to control (Tukey *post hoc*).

495 ± 39 s to 10 ± 10 s ($p < 0.01$). This antinociceptive effect was prevented by centrally acting naltrexone (2 mg/kg) co-treatment (first phase: 86 ± 20 s; second phase: 454 ± 20 s). In comparison to naltrexone, methylnaltrexone (2 mg/kg) had no significant effect on acute pain (7.2 ± 5 s) and decreased the antinociceptive effect of morphine only partially in the second phase (166 ± 23 s; $p < 0.01$). Morphine reduced plasma protein extravasation in dura ($p < 0.01$), which was prevented by naltrexone ($p < 0.01$), but methylnaltrexone had no significant effect on it (baseline: 11 ± 1.85 ng/mg tissue, saline 39.6 ± 3.65 ng/mg tissue; morphine: 15 ± 2.7 ng/mg tissue; morphine + naltrexone: 37.47 ± 1.52 ng/mg tissue; morphine + methylnaltrexone: 17.97 ± 2.94 ng/mg tissue; Fig. 4B). Naltrexone and methylnaltrexone had no effect on pain and dural protein plasma extravasation alone, as demonstrated on Figs. 2 and 3.

4. Discussion and conclusions

Botulinum toxin type A is clinically used as a second line prophylactic therapy in a chronic migraine (Jackson et al., 2012; Weatherall, 2015). Its application might be beneficial in other types of headaches (e.g. tension-type headache, medication overuse headache, trigeminal neuralgia, etc.) as well (Sandrini et al., 2011; Jackson et al., 2012; Shackleton et al., 2016). However, mechanism of its antinociceptive action and potential sites of action in trigeminal innervation area have not yet been defined. Preclinical research suggests that peripherally applied BT-A undergoes microtubule-dependent retrograde axonal transport within trigeminal TRPV1 (transient receptor potential vanilloid type 1) positive neurons to reach TNC (Matak et al., 2014). Furthermore, to explain the presence of clSNAP-25 in nerve endings innervating dura (Matak et al., 2011; Filipović et al.,

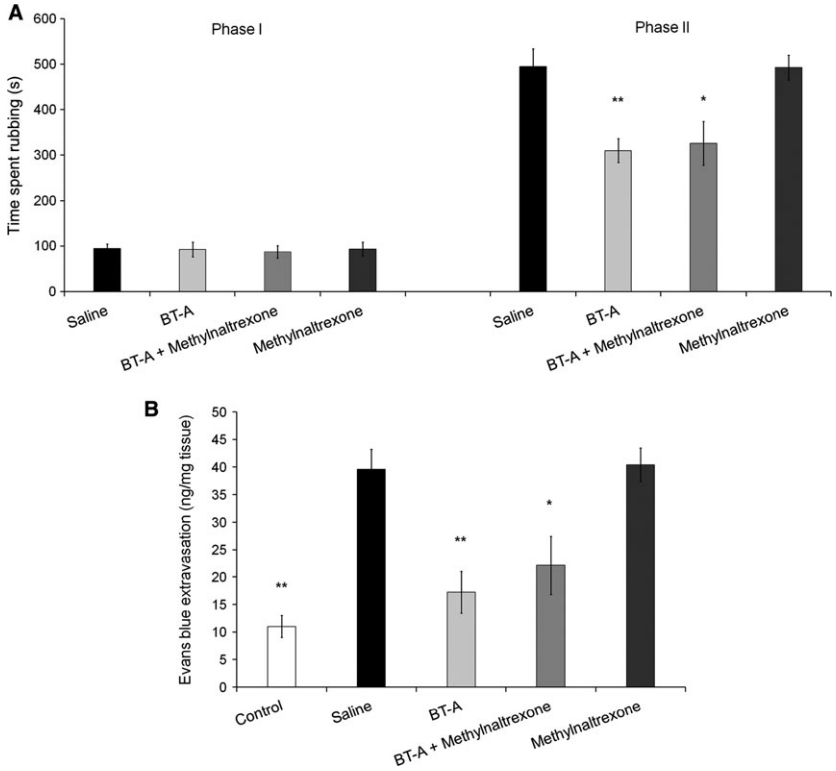

Figure 3 Methylnaltrexone does not interfere with BT-A's effects on nociception and dural plasma protein extravasation in orofacial formalin test. Formalin injection and measurement of nociception were preformed 5 days following BT-A (5 U/kg) peripheral s.c. into whisker pad and 40 min following methylnaltrexone (2 mg/kg) systemic s.c. treatment. Samples of *dura mater* for extravasation analysis were taken 1 h following formalin injection. (A) Methylnaltrexone has no influence on antinociceptive action of BT-A in the second phase of orofacial formalin test. Mean ± SEM, $N = 6$, $*p < 0.05$ compared to saline and methylnaltrexone; $**p < 0.01$ compared to saline and methylnaltrexone (Tukey *post hoc*); (B) Methylnaltrexone does not change the effect of BT-A on dural plasma protein extravasation. Mean ± SEM, $N = 6$, $*p < 0.05$ compared to saline and methylnaltrexone; $**p < 0.01$ compared to saline and methylnaltrexone (Tukey *post hoc*).

2012), trans-synaptic transport of BT-A within TG (Kitamura et al., 2009; Ramachandran et al., 2015), or possibly TNC (Ramachandran and Yaksh, 2014; Lacković et al., 2016) to neurons projecting to *dura mater* was proposed to occur as well. Such possibilities were recently observed in cell culture of rat's hippocampal neurons in a form of non-acidified organelles (Bomba-Warczak et al., 2016) or autophagosomes (Wang et al., 2015). Accordingly, there are several sites potentially involved in antinociceptive and anti-inflammatory effect of BT-A in the trigeminal region: peripheral nerve endings, TG, TNC and nerve endings innervating dura mater.

Recently our group discovered the presence of clSNAP-25 within CGRP positive dural nerve fibres along with the reduction in CGRP content in dura mater in rats with CFA-induced inflammation of temporomandibular joint pre-treated with BT-A (Lacković et al., 2016). In addition, two recent studies on cranial pain models, using electrophysiological techniques suggested that extracranially applied BT-A inhibits surface expression of high-threshold mechanosensitive ion channels (Burstein et al., 2014), and TRPV1 and TRPA1 channels in dural nerve endings of meningeal nociceptors (Zhang et al., 2016). Although the reduction in CGRP release from dural nerve fibres, along with the reduction in channel expression on meningeal nerves seem as an acceptable explanation for BT-A's mechanism of action on pain and possibly DNI, here we propose an additional mechanism which includes opioid system contributing to BT-A's antinociceptive effect.

The role of the endogenous opioid system in neuromodulation and control of pain is well known. It was suggested that spinal opioid system is involved in antinociceptive effects of BT-A (Drinovac et al., 2013; Drinovac Vlah et al., 2016). This assumption was based on the result that intrathecal opioid antagonists, naltrexone or naloxonazine, dose-dependently inhibit the antinociceptive effect of

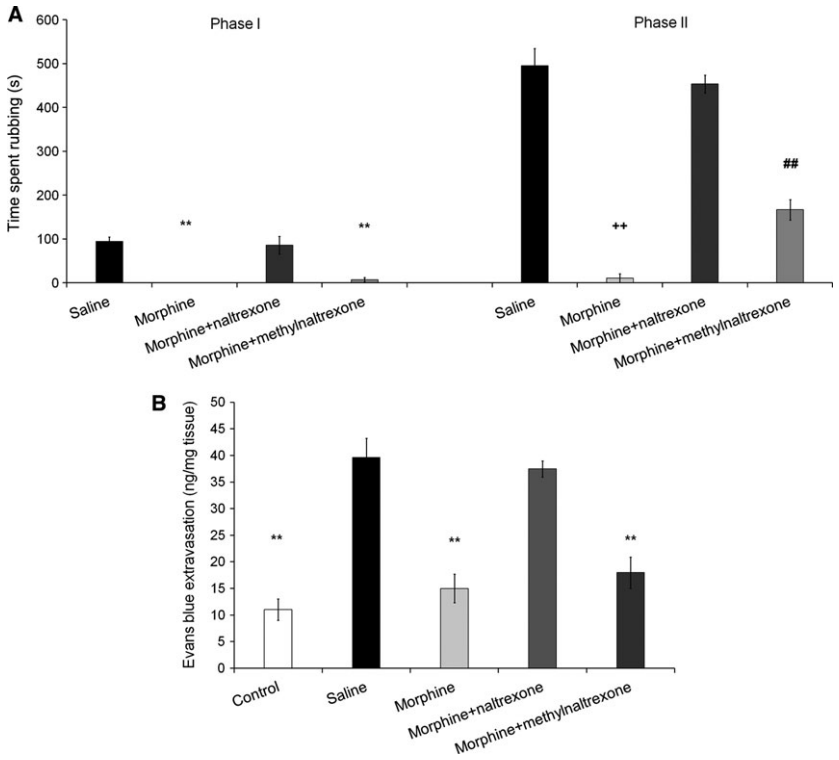

Figure 4 Comparison of morphine and its combination with naltrexone versus methylnaltrexone: effects on formalin-induced pain and dural plasma protein extravasation. Formalin injection and measurement of nociception were preformed 40 min following morphine systemic s.c. treatment and/or its co-administration with s.c. naltrexone (2 mg/kg) or methylnaltrexone (2 mg/kg). Samples of dura mater for extravasation analysis were taken 1 h following formalin injection. (A) Naltrexone completely prevented morphine's antinociceptive effect in both phases of the test, whereas methylnaltrexone only in the second phase and partially. Mean ± SEM, $N = 5$. First phase: $^{**}p < 0.01$ compared to saline and morphine + naltrexone (Tukey *post hoc*). Second phase: $^{++}p < 0.01$ compared to saline, morphine + naltrexone, morphine + methylnaltrexone; $^{\#\#}p < 0.01$ compared to saline, morphine and morphine + methylnaltrexone (Tukey *post hoc*); (B) Methylnaltrexone does not change the effect of morphine on dural plasma protein extravasation, whereas naltrexone prevents it. Mean ± SEM, $N = 5$, $^{**}p < 0.01$ compared to saline and morphine + naltrexone (Tukey *post hoc*).

peripherally applied BT-A (Drinovac et al., 2013; Drinovac Vlah et al., 2016). In parallel, those antagonists prevent BT-A-mediated reduction in c-Fos expression, a marker of neuronal activation, in dorsal horn (Drinovac et al., 2013). In comparison to pain originating from the sciatic area, pain in a trigeminal region is accompanied with neurogenic inflammation of meninges, which can be diminished by peripheral injection of BT-A in the trigeminal region (Filipović et al., 2012; Lacković et al., 2016).

In this study, non-selective centrally acting antagonist of opioid receptors naltrexone (2 mg/kg, s.c.) abolished the antinociceptive effect of peripherally applied BT-A in the second phase of orofacial formalin test (Fig. 2A, $p < 0.01$). In parallel, naltrexone abolished BT-A's effect on dural plasma protein extravasation (Fig. 2B, $p < 0.01$). These results, similarly as previous results in sciatic region (Drinovac et al., 2013), might suggest that BT-A indirectly increases the release of endogenous opioid peptides and/or the number of opioid receptors to reduce pain and accompanying DNI in trigeminal region as well.

The presence of opioid receptors and endogenous opioid peptides was never investigated directly in dural tissue. However, the distribution of enkephalin-, endorphin- and dynorphin-containing neurons in various brain areas are well studied in animals and *post mortem* in humans (Froehlich, 1997). The occurrence of the mRNA and/or protein expression of opioid receptors was reported in trigeminal ganglia (Xie et al., 1999) and TNC (Mansour et al., 1995). It was demonstrated as well that μ-opioid receptors are co-localized with substance P (SP) and CGRP in lamina I and II of the TNC (Li et al., 1998). Opioid receptors are located at the cerebral perivascular nerves and cerebrovascular endothelium, where endogenous opioid peptides cause

vasoconstriction or vasodilatation and consequently regulate the cerebral blood flow in certain states, such as hypoxia (Shankar and Armstead, 1995; Benyó and Wahl, 1996). Accordingly, to assess whether the interference of BT-A with opioid system occurs peripherally at the level of dural nerve endings and TG or is centrally mediated at TNC we used methylnaltrexone, which is unable to cross the blood–brain barrier due to reduced lipid solubility (Chandrasekaran et al., 2010). In contrast to centrally acting naltrexone, methylnaltrexone (2 mg/kg s.c.) had no effect on BT-A's antinociceptive and anti-inflammatory effect in dura matter (Fig. 3). We assume that methylnaltrexone occupies peripheral receptors situated: (1) at the cell membranes of the inflamed whisker pad tissue; (2) in trigeminal ganglion; (3) at the trigeminal nerve endings innervating dura; whereas the receptors located in the CNS, including TNC are not affected. In line with these results, we propose that the interaction of BT-A with endogenous opioid system occurs centrally and not peripherally (which would also include meninges).

Since the effect of opioid agonists on dural plasma protein extravasation was not investigated before in the model of pain in trigeminal region, we employed morphine in a full analgesic dose (6 mg/kg s.c.) as a positive control to BT-A treatment. It was previously shown that opioids block vasodilatation evoked by electrical stimulation of trigeminal ganglion in rat, but not that evoked by i.v. CGRP (Williamson et al., 2001). Accordingly, their clinical efficacy in migraine does not involve direct vasoconstriction (in contrast to triptans); it is assumed to be a consequence of activation of opioid receptors located on trigeminal sensory fibre terminals innervating dural blood vessels and/or presynaptic terminals of central trigeminal projections (Williamson et al., 2001).

In this study, morphine (6 mg/kg, s.c.) diminished pain and dural plasma protein extravasation induced by facial formalin injection, as expected (Fig. 4). While naltrexone (2 mg/kg, s.c.) blocked the antinociceptive effect of morphine (6 mg/kg, s.c.) in the second phase of the orofacial formalin test, peripherally acting methylnaltrexone (2 mg/kg, s.c.) reduced it only partially. On the other hand, naltrexone blocked the antinociceptive effect of morphine in the first phase, whereas methylnaltrexone had no significant effect (Fig. 4). The dose of methylnaltrexone used in this study was 2 mg/kg, which was chosen to parallel naltrexone dose, and which was used in animal studies described in the literature (Brown and Goldberg, 1985; Foss et al., 1996). In our preliminary experiment to determine the methylnaltrexone dose, a higher dose of 4 mg/kg had similar effects as 2 mg/kg (Fig. 1). Therefore, it could be assumed that 2 mg/kg was sufficient to block peripheral opioid receptors and insufficient to cross the blood–brain barrier (Brown and Goldberg, 1985). This assumption is additionally supported by our results showing that methylnaltrexone (2 mg/kg) partially inhibited of morphine's analgesic effect only in the second phase of formalin test (inflammatory pain) because of its known action in inflamed tissue or TG (Stein, 2013; Sullivan et al., 2016).

We anticipated that methylnaltrexone would reduce at least to some extent the effect of morphine on dural plasma protein extravasation as well. However, in our experimental conditions methylnaltrexone did not change morphine-mediated reduction in dural plasma extravasation, in contrast to naltrexone which completely prevented this effect. Accordingly, we assume that opioid agonists primarily, if not exclusively, inhibit the nociceptive neurotransmission within the TNC which might indirectly reduce the extent of dural plasma protein extravasation possibly by inhibition of CGRP and SP release from trigeminal afferents innervating dura mater.

In conclusion, using naltrexone and peripheral opioid antagonist methylnaltrexone, we found that morphine acts on pain in the trigeminal region both peripherally and centrally, whereas the effect on dural plasma protein extravasation seems to be only centrally mediated. However, the interaction of BT-A with endogenous opioid system, which inhibits nociceptive transmission as well as dural neurogenic inflammation, occurs primarily centrally. This observation suggests that BT-A's antinociceptive effect, in addition to CGRP, involves diverse neurotransmitters at different sites of the trigeminal system.

Acknowledgements

We thank our technician Božica Hržan for excellent technical assistance.

Author contributions

All authors contributed to conception and design of the study, analysis and interpretation of data. V.D.V. and B.F. preformed the acquisition of data. V.D.V. drafted the manuscript and all other authors revised it critically. All authors have approved the final version of the manuscript. All authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

References

Benyó, Z., Wahl, M. (1996). Opiate receptor-mediated mechanisms in the regulation of cerebral blood flow. *Cerebrovasc Brain Metab Rev* 8, 326–357.

Bomba-Warczak, E., Vevea, J.D., Brittain, J.M., Figueroa-Bernier, A., Tepp, W.H. et al. (2016). Interneuronal transfer and distal action of tetanus toxin and botulinum neurotoxins A and D in central neurons. *Cell Rep* 16, 1974–1987.

Brown, D.R., Goldberg, L.I. (1985). The use of quaternary narcotic antagonists in opiate research. *Neuropharmacology* 24, 181–191.

Burstein, R., Zhang, X., Levy, D., Aoki, K.R., Brin, M.F. (2014). Selective inhibition of meningeal nociceptors by botulinum neurotoxin type A: Therapeutic implications for migraine and other pains. *Cephalalgia* 34, 853–869.

Chandrasekaran, A., Tong, Z., Li, H., Erve, J.C., DeMaio, W. et al. (2010). Metabolism of intravenous methylnaltrexone in mice, rats, dogs, and humans. *Drug Metab Dispos* 38, 606–616.

Correa, J.D., Paiva-Lima, P., Rezende, R.M., Dos Reis, W.G., Ferreira-Alves, D.L., Bakhle, Y.S., Francischi, J.N. (2010). Peripheral mu-, kappa- and delta-opioid receptors mediate the hypoalgesic effect of celecoxib in a rat model of thermal hyperalgesia. *Life Sci* 86, 951–956.

Cui, M., Khanijou, S., Rubino, J., Aoki, K.R. (2004). Subcutaneous administration of botulinum toxin A reduces formalin-induced pain. *Pain* 107, 125–133.

Dodick, D.W., Turkel, C.C., DeGryse, R.E., Aurora, S.K., Silberstein, S.D. et al. (2010). OnabotulinumtoxinA for treatment of chronic migraine: Pooled results from the double-blind, randomized, placebo-controlled phases of the PREEMPT clinical program. *Headache* 50, 921–936.

Drinovac Vlah, V., Bach-Rojecky, L., Lacković, Z. (2016). Antinociceptive action of botulinum toxin type A in carrageenan-induced mirror pain. *J Neural Transm (Vienna)* 123, 1403–1413.

Drinovac, V., Bach-Rojecky, L., Matak, I., Lacković, Z. (2013). Involvement of μ-opioid receptors in antinociceptive action of botulinum toxin type A. *Neuropharmacology* 70, 331–337.

Filipović, B., Matak, I., Bach-Rojecky, L., Lacković, Z. (2012). Central action of peripherally applied botulinum toxin type A on pain and dural protein extravasation in rat model of trigeminal neuropathy. *PLoS ONE* 7, e29803.

Finocchi, C., Viani, E. (2013). Opioids can be useful in the treatment of headache. *Neurol Sci* 34(Suppl 1), S119–S124.

Foss, J.F., Orelind, E., Goldberg, L.I. (1996). Effects of methylnaltrexone on morphine-induced cough suppression in guinea pigs. *Life Sci* 59, PL235–PL238.

Froehlich, J.C. (1997). Opioid peptides. *Alcohol Health Res World* 21, 132–136.

Holden, J.E., Jeong, Y., Forrest, J.M. (2005). The endogenous opioid system and clinical pain management. *AACN Clin Issues* 16, 291–301.

Jackson, J.L., Kuriyama, A., Hayashino, Y. (2012). Botulinum toxin A for prophylactic treatment of migraine and tension headaches in adults: A meta-analysis. *JAMA* 307, 1736–1745.

Kilkenny, C., Browne, W., Cuthill, I.C., Emerson, M., Altman, D.G. (2010). Animal research: Reporting in vivo experiments: The ARRIVE guidelines. *Br J Pharmacol* 160, 1577–1579.

Kitamura, Y., Matsuka, Y., Spigelman, I., Ishihara, Y., Yamamoto, Y. et al. (2009). Botulinum toxin type a (150 kDa) decreases exaggerated neurotransmitter release from trigeminal ganglion neurons and relieves neuropathy behaviors induced by infraorbital nerve constriction. *Neuroscience* 159, 1422–1429.

Lacković, Z., Filipović, B., Matak, I., Helyes, Z. (2016). Activity of botulinum toxin type A in cranial dura: Implications for treatment of migraine and other headaches. *Br J Pharmacol* 173, 279–291.

Li, J.L., Ding, Y.Q., Li, Y.Q., Li, J.S., Nomura, S., Kaneko, T., Mizuno, N. (1998). Immunocytochemical localization of mu-opioid receptor in primary afferent neurons containing substance P or calcitonin gene-related peptide. A light and electron microscope study in the rat. *Brain Res* 794, 347–352.

Mansour, A., Fox, C.A., Burke, S., Akil, H., Watson, S.J. (1995). Immunohistochemical localization of the cloned mu opioid receptor in the rat CNS. *J Chem Neuroanat* 8, 283–305.

Matak, I., Lacković, Z. (2014). Botulinum toxin A, brain and pain. *Prog Neurobiol* 119–120, 39–59.

Matak, I., Bach-Rojecky, L., Filipović, B., Lacković, Z. (2011). Behavioral and immunohistochemical evidence for central antinociceptive activity of botulinum toxin A. *Neuroscience* 186, 201–207.

Matak, I., Rossetto, O., Lacković, Z. (2014). Botulinum toxin type A selectivity for certain types of pain is associated with capsaicin-sensitive neurons. *Pain* 155, 1516–1526.

Raboisson, P., Dallel, R. (2004). The orofacial formalin test. *Neurosci Biobehav Rev* 28, 219–226.

Ramachandran, R., Yaksh, T.L. (2014). Therapeutic use of botulinum toxin in migraine: Mechanisms of action. *Br J Pharmacol* 171, 4177–4192.

Ramachandran, R., Lam, C., Yaksh, T.L. (2015). Botulinum toxin in migraine: Role of transport in trigemino-somatic and trigemino-vascular afferents. *Neurobiol Dis* 79, 111–122.

Sandrini, G., Perrotta, A., Tassorelli, C., Torelli, P., Brighina, F., Sances, G., Nappi, G. (2011). Botulinum toxin type-A in the prophylactic treatment of medication-overuse headache: A multicenter, double-blind, randomized, placebo-controlled, parallel group study. *J Headache Pain* 12, 427–433.

Sevostianova, N., Danysz, W., Bespalov, A.Y. (2005). Analgesic effects of morphine and loperamide in the rat formalin test: Interactions with NMDA receptor antagonists. *Eur J Pharmacol* 525, 83–90.

Shackleton, T., Ram, S., Black, M., Ryder, J., Clark, G.T., Enciso, R. (2016). The efficacy of botulinum toxin for the treatment of trigeminal and postherpetic neuralgia: A systematic review with meta-analyses. *Oral Surg Oral Med Oral Pathol Oral Radiol* 122, 61–71.

Shankar, V., Armstead, W.M. (1995). Opioids contribute to hypoxia-induced pial artery dilation through activation of ATP-sensitive K+ channels. *Am J Physiol* 269, H997–H1002.

Stein, C. (2013). Targeting pain and inflammation by peripherally acting opioids. *Front Pharmacol* 4, 123.

Sullivan, L.C., Chavera, T.S., Jamshidi, R.J., Berg, K.A., Clarke, W.P. (2016). Constitutive desensitization of opioid receptors in peripheral sensory neurons. *J Pharmacol Exp Ther* 359, 411–419.

Wang, T., Martin, S., Papadopulos, A., Harper, C.B., Mavlyutov, T.A. (2015). Control of autophagosome axonal retrograde flux by presynaptic activity unveiled using botulinum neurotoxin type A. *J Neurosci* 35, 6179–6194.

Weatherall, M.W. (2015). The diagnosis and treatment of chronic migraine. *Ther Adv Chronic Dis* 6, 115–123.

Webster, L.R., Brenner, D.M., Barrett, A.C., Paterson, C., Bortey, E., Forbes, W.P. (2015). Analysis of opioid-mediated analgesia in Phase III studies of methylnaltrexone for opioid-induced constipation in patients with chronic noncancer pain. *Pain Res* 8, 771–780.

Williamson, D.J., Hargreaves, R.J. (2001). Neurogenic inflammation in the context of migraine. *Microsc Res Tech* 53, 167–178.

Williamson, D.J., Shepheard, S.L., Cook, D.A., Hargreaves, R.J., Hill, R.G., Cumberbatch, M.J. (2001). Role of opioid receptors in neurogenic dural vasodilation and sensitization of trigeminal neurones in anaesthetized rats. *Br J Pharmacol* 133, 807–814.

Xie, G.X., Meuser, T., Pietruck, C., Sharma, M., Palmer, P.P. (1999). Presence of opioid receptor-like (ORL1) receptor mRNA splice variants in peripheral sensory and sympathetic neuronal ganglia. *Life Sci* 64, 2029–2037.

Yuan, C.S., Israel, R.J. (2006). Methylnaltrexone, a novel peripheral opioid receptor antagonist for treatment of opioid side effects. *Expert Opin Investig Drugs* 15, 541–552.

Zhang, X., Strassman, A.M., Novack, V., Brin, M.F., Burstein, R. (2016). Extracranial injections of botulinum neurotoxin type A inhibit intracranial meningeal nociceptors' responses to stimulation of TRPV1 and TRPA1 channels: Are we getting closer to solving this puzzle? *Cephalalgia* 36, 875–886.

Zimmerman, M. (1983). Ethical guidelines for investigations of experimental pain in conscious animals. *Pain* 16, 109–110.