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Source / Izvornik: **Neuroscience Letters**, 2016, 617, 213 - 217

Journal article, Accepted version

Rad u časopisu, Završna verzija rukopisa prihvaćena za objavljivanje (postprint)

<https://doi.org/10.1016/j.neulet.2016.02.020>

Permanent link / Trajna poveznica: <https://um.nsk.hr/um:nbn:hr:105:820906>

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## Središnja medicinska knjižnica

**Ibragić S., Matak I., Dračić A., Smajlović A., Muminović M., Proft F., Sofić E., Lacković Z., Riederer P. (2016) *Effects of botulinum toxin type A facial injection on monoamines and their metabolites in sensory, limbic and motor brain regions in rats. Neuroscience letters, 617. pp. 213-7. ISSN 0304-3940***

<http://www.elsevier.com/locate/issn/03043940>

<http://www.sciencedirect.com/science/journal/03043940>

<http://dx.doi.org/10.1016/j.neulet.2016.02.020>

<http://medlib.mef.hr/2749>

**EFFECTS OF BOTULINUM TOXIN TYPE A FACIAL INJECTION ON MONOAMINES AND THEIR METABOLITES IN SENSORY, LIMBIC AND MOTOR BRAIN REGIONS IN RATS**

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## **Abstract**

Despite its toxicity, botulinum neurotoxin type A (BTX-A) is a valuable therapeutic agent for several motor, autonomic and pain disorders. Numerous studies have described its peripheral as well as central effects. Using reversed-phase High Performance Liquid Chromatography with Electrochemical Detection (HPLC-ED) and gradient elution, we quantified the concentrations of dopamine (DA), noradrenaline (NA), serotonin (5-HT) and their metabolites in 10 brain regions, ipsilateral and contralateral from the site of unilateral BTX-A administration (5 U/kg) into the rat whisker pad. In regions associated with nociception and pain processing we also examined possible BTX-A effects in combination with formalin-induced inflammatory orofacial pain. The dominant BTX-A effects on the monoamines and their metabolites were insignificant. The only significant increase caused by BTX-A alone was that of NA in striatum and serotonin in hypothalamus. While antinociceptive effects of BTX-A are most probably not related to central monoamine concentrations, the localized increased NA and 5-HT concentrations might play a role in reported BTX-A efficacy for the treatment of depression.

**Keywords:** Botulinum neurotoxin type A, monoamines, pain, depression, High Performance Liquid Chromatography with Electrochemical Detection

## **Highlights**

- Effects of Botulinum toxin A (BTX-A) on central monoamines and their metabolites were analyzed.
- Antinociceptive effects of BTX-A are most probably not related to central monoamine concentrations.
- Monoamine hypothesis: low levels of serotonin (5-HT), noradrenaline (NA) and/or dopamine (DA) linked to depression.
- Observed increased NA and 5-HT concentrations might play a role in BTX-A efficacy for treatment of depression.
- Changes in neurotransmission by BTX may be able to influence depression, sleep and pain.

## 1. INTRODUCTION

Botulinum neurotoxins (BTX), produced by anaerobic bacteria of the genus *Clostridium* (*Clostridium botulinum*, *Clostridium barati*, *Clostridium argentinense*), are the most potent toxins known [40]. Despite the toxicity, Botulinum neurotoxin type A (BTX-A) local application in small doses is proven to be effective for numerous clinical conditions such as dystonias, spasms, strabismus, bruxism, hyperhidrosis, overactive bladder, gastroparesis, migraine and musculoskeletal pain [11]. Structurally, BTX consist of a heavy and a light chain linked by a disulphide bond and non-covalent interactions. The carboxy-terminus of the heavy chain binds with extraordinary affinity and specificity to nerve terminals [6]. Although differing in clinical manifestation, the fundamental mode of action – inhibition of neurotransmission – is common to all *Clostridium* neurotoxins [5,33,44]. *In vitro* and *in vivo* animal studies show that BTX alters acetylcholine, glutamate, noradrenaline, serotonin, dopamine, and glycine transmission, and may change the electrophysiologic properties, differentiation, and survival of central neurons [10]. Inhibition of neurotransmitter release by BTX is caused by the specific cleavage of a group of proteins integral to the exocytotic process, the SNARE proteins (soluble NSF-attachment protein receptors). The light chain plays a major role in that step, acting as a Zn<sup>2+</sup>-dependent endopeptidase. Cleavage of one or more of the SNARE proteins leads to a block in the release of vesicular contents to the extracellular environment [44]. Along with the well known peripheral effects, preclinical and clinical studies have provided evidence for central effects of BTX-A mediated by toxin axonal transport to CNS [2, 3, 6, 26, 27]. The aim of this study was to investigate possible changes of concentrations of neurotransmitters and their metabolites in sensory, limbic and motor brain regions after facial administration of BTX-A in rats.

## 2. MATERIALS AND METHODS

### 2.1. Animals

Male Wistar rats (University of Zagreb School of Medicine, Croatia), weighing 300-400 g, kept on 12 h/12h light and dark cycle, were used in all experiments. The experiments were conducted according to the European Communities Council Directive (86/609/EEC), the Croatian Law on Animal Protection (Zakon o zaštiti životinja NN 135/06), and recommendations of the International Association for the Study of Pain by Zmmerrmann

[51]. All efforts were made to reduce the number of animals used and to reduce their suffering. Brain regions were analyzed from either two or four differently treated groups of rats, each group containing a total of 10 rats. Animal procedures were approved by the institutional review board of the University of Zagreb School of Medicine (permit No. 07-76/2005-43).

## **2.2. BTX-A injections**

Conscious, restrained rats were injected unilaterally with 20  $\mu$ l of saline-diluted BTX-A therapeutic preparation (Botox®, INN: Clostridium botulinum type A neurotoxin complex, Allergan Inc., Irvine, CA, USA) using a 27 1/2-gauge needle into the whisker pad. 1 unit (1 U) of BTX-A preparation contains 48 pg of 900 kDaBoNT/A complex. 5 U/kg dose was chosen based on previous experiments [28].

## **2.3. Behavioral testing**

The effect of BTX-A was assessed in a model of formalin-induced orofacial pain 7 days post peripheral toxin injection. Prior to behavioral measurement, the rats were allowed to accommodate to testing cage environment. The rats were then briefly restrained and injected with 50  $\mu$ l of saline-diluted 2.5% formalin (0.92 % formaldehyde, Formalin, Kemika, Zagreb, Croatia) into the whisker pad ipsilateral to BTX-A pretreatment and returned to cages for observation period of 45 min. The number of seconds of formalin-induced ipsilateral facial rubbing/grooming was measured in 3 min periods during phases I and II of formalin-induced pain [36]. Phase I (0-12 min) behavior represents the immediate pain response characterized by direct chemical stimulation of peripheral nerve endings with formalin. Phase II (12-45 min) behavior is characterized by delayed hyperalgesic response maintained by ongoing afferent input and central sensitization.

## **Brain region tissue preparation**

One hour after formalin injection, the animals were deeply anesthetised (chloralhydrate, Sigma, St Louis, USA; 300 mg/kg i.p.), the brain tissue was quickly excised and immersed into the liquid nitrogen. The brain tissue was then kept on -80°C. Prior to homogenization, the frozen brainstem was placed to cryostat-cooled environment (-25°C) for dissection of multiple ipsilateral and contralateral regions without thawing. The brain was carefully sectioned into thick coronal sections (~3 mm) by using a microtome knife blade, and the outlines of different regions were identified visually using the rat stereotaxic atlas by Paxinos

and Watson, and appropriate landmarks for each region (central canal, obex, aqueduct, ventricles, etc.). The nuclei were excised manually using pre-cooled scalpel and forceps. Dissected tissue was then kept on -80 °C until homogenization.

## 2.5. HPLC analysis

### 2.5.1. Chemicals

Noradrenaline (NA), 3-methoxy-4-hydroxyphenylglycol (MHPG), dopamine (DA), homovanillic acid (HVA), 5-hydroxytryptamine (serotonin, 5-HT), 5-hydroxyindoleacetic acid (5-HIAA), triethylamine (TEA), ethylene-diamine-tetra-acetic acid (EDTA), 1-octanesulfonic acid (OSA), 3,4-dihydroxyphenylacetic acid (DOPAC), *trisodium salt* of calcium diethylenetriaminepentaacetate (DTPA) and methanol Chromasolv<sup>®</sup> were purchased from Sigma-Aldrich, Steinheim, Germany; sodium dihydrogen phosphate monohydrate (NaH<sub>2</sub>PO<sub>4</sub> • H<sub>2</sub>O) and phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) from Merck, Darmstadt, Germany; sodium hydroxide from AppliChem, Darmstadt, Germany. Ultrapure water was produced in-house using a Barnstead NANOpure<sup>®</sup> Diamond<sup>™</sup> system (Thermo Fisher Scientific, Essex, UK). All chemicals were of puriss p.a. grade.

### 2.5.2. Preparation of samples

First, 15 - 25 mg of tissue of the region of interest was weighed and transferred into 5 ml tubes [scale: „Feinwaage HL52" (Mettler-Toledo GmbH, Gießen, Germany); tubes: Polypropylen-Zentrifugenröhrchen 5 ml, 75 x 12 mm, REF 55.526 (Sarstedt AG & Co., Nümbrecht, Germany)]. The tissue was kept frozen on dry ice as far as possible to prevent *post mortem* changes. The tissue was sonicated [sonicator: „Ultraschallhomogenisator Branson Digital Sonifier" (G. Heinemann Ultraschall- und Labortechnik, Schwäbisch-Gmünd, Germany)] in 1 : 10 to 1:50 dilution within an aqueous solution of H<sub>3</sub>PO<sub>4</sub> (150 mM) and DTPA (500 µM) on ice under argon atmosphere. The inert gas argon was applied to oust oxygen from the tube during sonication to prevent analytes' degradation by oxidation. An amplitude of 15 % was used and sonication was performed until the tissue was visibly homogenised. Homogenates were centrifuged (20 min; 4-8 °C; 40700 g). Supernatants were transferred into 1.5 ml-reaction tubes and stored at -80 °C until analysis. Prior to analysis, thawed homogenates were filtered via microcentrifugal filters (membrane of regenerated cellulose; pore size 0.2 µm; Amchro GmbH, Hattersheim, Germany; 6 min, room temperature, 21400

g). For analysis the filtrates without further treatment were injected into the HPLC-system via an autosampler in appropriate volume.

### 2.5.3. Preparation of standard solutions

A solution containing 500 µg/ml of each analyte was prepared and diluted to contain 500 ng/ml of each component. Finally, to plot the standard curve three different analytes' concentrations were used: 20 ng/ml, 40 ng/ml, 80 ng/ml.

### 2.5.4. Instrumentation and Chromatographic Conditions

The concentrations of NA, MHPG, DA, DOPAC, HVA, 5-HT and HIAA were determined using HPLC-ED. The HPLC system was an Agilent Technologies 1100 chromatograph coupled to an electrochemical detector (model 1640, Bio-Rad, Munich, Germany) equipped with a glassy carbon working electrode and an Ag/AgCl reference electrode. On the day of analysis, samples were allowed to thaw, placed in the autosampler and kept at + 15 ° C before injection. Varying injection volumes were used, however the standard injection volume per sample was 50 µl. Calculation of the analytes' concentrations in samples was performed relating the injection volume of the sample to that of the external standard. The separation column was EC 250/4.6 NUCLEOSIL<sup>®</sup> 100-5 C18 (Macherey-Nagel, Düren, Germany) and it was thermostated to 35 ° C.

A stock solution (SS) for the mobile phases was prepared: 1 mol/L NaH<sub>2</sub>PO<sub>4</sub>, 1 mmol/L EDTA, 6.5 mmol/L OSA, 5 mmol/L TEA, in highly purified water. From the SS mobile phases A (10 % SS + 90 % H<sub>2</sub>O) and B (10 % SS + 40 % H<sub>2</sub>O + 50 % MeOH) were prepared. Mobile phases were adjusted to pH 4 using H<sub>3</sub>PO<sub>4</sub>. The eluent flow rate was set to 0.8 ml/min. The method is based on a previously published one using isocratic elution [39]. A binary HPLC-pump was used for gradient elution. The starting composition of the mobile phase was 88 % A and 12 % B and was kept for 10 min. During the following 20 min gradual adjustment to 30 % A and 70 % B was performed and this percentage kept for 10 min. For column reequilibration, percentages of A and B were returned to initial conditions within 4 min and kept for 6 min before the next sample injection. Total acquisition time was 50 min per sample. Analytes were detected applying an oxidation potential of 0.72 V versus the reference electrode. Chromatograms were acquired with Agilent ChemStation for LC 3D software.



### **2.5.5. Quantification, validation and statistical analysis**

The calibration standards for the quantification validation contained NA, MHPG, DA, DOPAC, HVA, 5-HT and 5-HIAA. Calibration plots were generated by plotting the peak height versus the standards' solution concentration (20 ng/ml, 40 ng/ml, 80 ng/ml). Analyte concentrations in biological samples were finally expressed as ng/g of brain tissue. The retention times were the following: 5.8 min for NA, 12.6 min for DA, 14.3 min for MHPG, 20.9 min for DOPAC, 24.0 min for 5-HT, 26.7 min for 5-HIAA and 30.2 min for HVA.

The limit of detection (LOD) was 1.3 ng/ml for NA, MHPG, DA as well as for DOPAC and 2.5 ng/ml for 5-HT, 5-HIAA as well as for HVA.

The results are presented as mean  $\pm$  standard error mean (SEM). ANOVA test was conducted and between-group differences were analyzed by Newman–Keuls post hoc test.  $P < 0.05$  was considered significant.

## **RESULTS**

Similarly to previous experiments [28], BTX-A peripheral injection into the whisker pad reduced the duration of second phase of the nocifensive behavior induced by orofacial formalin in rats (saline + formalin treatment =  $457 \pm 40$  seconds; BTX-A + formalin treatment =  $310 \pm 27$  seconds,  $p < 0.01$  (two-tailed unpaired t-test)). In amygdala and trigeminal nucleus caudalis (TNC), formalin induced pain caused a statistically significant rise of HVA and DOPAC, respectively, however the BTX-A effect remained insignificant. In hypothalamus, the 5-HT levels were higher in rats treated with BTX-A (BTX-A + saline) compared to the control (saline + saline) group. In brain regions related to nociceptive transmission we did not observe statistically significant changes of monoamines or their metabolites after formalin induced pain and BTX-A administration. In all the remaining brain regions BTX-A has not caused significantly different changes of monoamines or their metabolites, except for striatum where BTX-A increased NA concentrations. All results are presented in Table 1.

## **DISCUSSION AND CONCLUSION**

The natural target of BTX is represented by the neuromuscular junction, where BTX poisoning results in flaccid paralysis due to blockade of acetylcholine release [6]. Beside the peripheral effects, BTX-A is known for its central effects, too. Studies in rodents show that the toxin is retrogradely transported and transcytosed to second-order neurons in the central

nervous system [2, 3, 37]. Also, studies in humans support the idea that BTX-A injected at therapeutic doses induces distant spinal and cortical effects indirectly by promoting brain changes due to plastic rearrangements subsequent to denervation or alterations in sensory input [22]. An excellent clinical example is that BTX injected into the affected muscles in patients with cervical dystonia relieves pain [10]. In addition, possible direct central actions mediated through toxin axonal transport from periphery to the spinal cord ventral horn have been reported in patients treated for spasticity [26]. In the present study we examined the effects of BTX-A on concentrations of neurotransmitters and their metabolites in brain regions involved in pain transmission and processing, as well as motor regions considering that BTX-A is reaching the facial nucleus and trigeminal nucleus caudalis via motor and sensory neurons, respectively [2, 28]. Results showed that BTX-A did not cause a significant change in concentrations of monoamines and their metabolites in a model of formalin induced pain with the exception of DOPAC in the trigeminal nucleus caudalis and for HVA in the amygdala. Therefore, it would be reasonable to conclude that in this model the BTX-A antinociceptive effect is not related to central monoamines. Interestingly, in two brain regions we observed low but significant changes in monoamine concentrations in saline + BTX-A-treated animals. BTX-A caused a bilateral increase of 5-HT in hypothalamus and an ipsilateral increase of NA in striatum. These data would require further confirmation and elaboration through experimental work, however the current results and rise in 5-HT and NA concentrations may be useful in explaining the role of BTX-A in depression or depression associated pain [15] and sleep [19,12]. Biochemical and pharmacological studies have demonstrated that changes in neurotransmitter concentrations play an important role in the pathogenesis of depression [9, 41, 38]. The monoamine hypothesis postulates that depletion in the levels of 5-HT, NA, and/or DA in the CNS are the pathophysiologic basis of depression [1]. 5-HT, NA and DA are involved in mood regulation and states such as sleep, anxiety, alertness, attention and reward processes. The purpose of major antidepressant drug types is to enhance and prolong the action of NA and/or 5-HT and/or DA. That is achieved by various mechanisms including altering the reuptake, degradation or receptor pharmacodynamics of biogenic amines [21] or even influencing gene expression, synaptic plasticity, neurogenesis, and neuronal survival [23, 47]. Our experiment has shown that BTX-A caused a rise in 5-HT in hypothalamus, which is along with other limbic system regions investigated here (amygdala, thalamus and hippocampus) a brain region that plays a significant role in depression. Similarly, BTX-A peripheral injection has also raised the NA concentrations in striatum, a region implicated rather in retarded depression. Our previous experiments

suggested that BoNT/A enzymatic activity is not detectable in higher order brain regions projecting to trigeminal nucleus caudalis, however, it may affect pain-evoked neuronal activation in regions where its enzymatic activity is not detectable (locus coeruleus and periaqueductal gray) [28]. Similarly, BTX-A mediated up-regulation of serotonin and noradrenaline concentrations in hypothalamus and striatum probably results from indirect modulation of neural activity within distant brain regions rather than the direct BTX-A action. Our results may also be interpreted as to support the hypothesis of recent studies that associated BTX-A with significant improvement in depressive symptoms [14, 24, 25]. Converging lines of evidence suggested a role for facial expressions in the pathophysiology and treatment of mood disorders [14]. Finzi and Wasserman were the first to report cases of depression treated with BTX-A [15]. The studies that followed [18, 32, 13, 49, 16, 45, 46, 20, 17, 14, 29, 25] were also based on the facial feedback hypothesis and designed to evaluate antidepressant effects after injecting BTX-A into the corrugator and procerus muscles of the glabellar region. The hypothesis implies that the brain continuously assesses the extent of facial muscle contraction and muscle tension by proprioception. One can view the state of corrugator muscle tension as part of a neuronal circuit involving the brainstem, with motor input from the facial nerve and sensory afferents from facial and trigeminal cranial nerves. BTX-A treatment of the corrugator muscle, would interrupt the normal circuitry, reduce distress signals to the brain and thereby influence mood in a favorable way [14]. The interruption of this feedback loop by the paralysis of the respective muscles is caused by reversible inhibition of acetylcholine release. Some of the benefits of BTX-A as an antidepressant agent is that it is not absorbed systemically, the long-lasting effect of a single dose and the limited adverse reactions. As binding of [3H]-imipramine and [3H]-serotonin do not change after tetanus toxin as well as BTX-A in isolated brain synaptosomes, this indicates that these Clostridium neurotoxins do not act on the serotonin high-affinity site at SERT or at other serotonin high affinity sites [31]. Indeed, evidence suggest that the SNARE protein syntaxin 1A binds to the N-terminal tail of SERT thus regulating SERT-conducting states including cell excitability and drug mediated effects [35, 34]. Effects of BTX-C,D demonstrate inhibitory effects on 5-HT<sub>1C</sub> receptor induced Cl<sup>-</sup> current in *Xenopus* oocytes [42]. In addition, low molecule weight G-proteins ADP-ribosylated by botulinum ADP-ribosyltransferase are involved in phospholipase C activity [43]. Serotonin facilitates AMPA-type responses in isolated siphon motor neurons of *Aplysia* in culture [8]. Therefore, even if there are only mild changes in concentrations of biogenic amines and their metabolites, this does not rule out BTX-induced changes in transmitter responses. Syntaxin 1A also regulates

DA transporter activity, phosphorylation and surface expression [7] and SNARE proteins are involved in exocytosis of NA from synaptic vesicles at low frequencies of stimulation [30, 4] . It is evident that proteolysis of SNARE proteins alters facilitation and depression in a specific way [48, 50]. By this, changes in neurotransmission by BTX may be able to influence clinical phenotypes like depression, sleep and pain. Further studies are required to elucidate the role of the elevated brain concentrations of 5-HT and NA observed after BTX-A facial injection in our study and to elucidate the inhibitory effect of BTX-A on peripheral acetylcholine release, which also might contribute to antidepressant BTX-A activity.

### **Acknowledgement**

This work was realized with a grant from the German Academic Exchange Service (DAAD).

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## TABLE

Brain regions with significant changes in concentrations of biogenic amines and /or their metabolites.

BIOGENIC AMINE / METABOLITE	REGION	TREATMENT GROUPS	p
NA	Striatum (ipsi)	BTX-A + saline ipsi vs. saline + saline ipsi	p < 0.01
5-HT	Hypothalamus (ipsi)	BTX-A + saline ipsi vs. saline + saline ipsi	p < 0.05
DOPAC	Trigeminal nucleus caudalis (contra)	saline + formalin cont vs. saline + formalin ipsi	p < 0.05
HVA	Amygdala (contra)	saline + formalin cont vs. saline + formalin ipsi	p < 0.05