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Analgesic effects of botulinum neurotoxin type A in a model of allyl isothiocyanate- and capsaicin-induced pain in mice

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ABSTRACT

We evaluate analgesic effects of BoNT/A in relation to the two main transient receptor potentials (TRP), the vanilloid 1 (TRPV1) and the ankyrin 1 (TRPA1), having a role in migraine pain. BoNT/A (15 pg/mouse) was injected in the inner side of the medial part of hindlimb thigh of mice, where the superficial branch of femoral artery is located. We chosen this vascular structure because it is similar to other vascular structures, such as the temporal superficial artery, whose perivascular nociceptive fibres probably contributes to migraine pain. After an interval, ranging from 7 to 30 days, capsaicin (agonist of TRPV1) or allyl isothiocyanate (AITC; agonist of TRPA1) were injected in the same region previously treated with BoNT/A and nocifensive response to chemicals-induced pain was recorded. In absence of BoNT/A, capsaicin and AITC induced extensive nocifensive response, with a markedly different temporal profile: capsaicin induced maximal pain during the first 5 min, while AITC induced maximal pain at 15–30 min after injection. Pretreatment with BoNT/A markedly reduced both the capsaicin- and AITC-induced pain for at least 21 days. These data suggest a long lasting analgesic effect of BoNT/A exerted via prevention of responsiveness of TRPV1 and TRPA1 toward their respective agonists.

1. Introduction

Botulinum neurotoxin serotype-A (BoNT/A)[1] proteolitycally cleaves synaptosomal-associated protein 25 (SNAP-25), a specific protein belonging to the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) responsible for presynaptic neurotransmitter release (Schiavo et al., 1993, 2000; Rossetto et al., 2001; Montecucco et al., 2004). Cleavage of SNAP-25 results in the formation of a non-functional SNARE complex and consequent block of synaptic transmission (Montecucco et al., 2005; Megighian et al., 2010; Pantano and Montecucco, 2013).

Besides the well-known action of BoNT/A of blocking acetylcholine release, many evidences demonstrate that BoNT/A inhibits the release of other neuromodulators and transmitters, such as glutamate, substance P and CGRP (Aoki and Francis, 2011; Francisco et al., 2012), which are critical for the neurotransmission of sensory pathways. Accordingly, particular interest has been devoted to use of BoNT/A for treating pain, both in animal (Pavone and Luvisetto, 2010; Dolly and O'Connell, 2012; Matak and Lackovic, 2014) and humans (Jabbari, 2008; Ashkenazi, 2010; Guo et al., 2013; Wheeler and Smith, 2013), including chronic migraine treated by injecting BoNT/A in scalp tissues (Diener et al., 2010, 2012; Durham and Cady, 2011; Frampton, 2012). Although the efficacy of BoNT/A injected into superficial cranial musculature as a treatment for migraine has been proved and the prophylactic treatment in adult chronic migraine has been approved in many countries, the mechanism of action of BoNT/A in migraine is still not completely elucidated and several hypotheses have been proposed (Ramachandran and Yaksh, 2014).

Recent studies demonstrates that BoNT/A inhibits CGRP release from trigeminal ganglion neurons and eliminated the excitatory effects of this peptide in brain stem sensory neurons evoked by capsaicin, which activates the transient receptor potential (TRP) vanilloid receptor type 1 (TRPV1) (Morenilla-Palao et al., 2004;

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[1] Abbreviations: AITC, allyl isothiocyanate; BoNT/A, botulinum neurotoxin serotype A; CGRP, calcitonin gene related protein; SNAP-25, synaptosomal-associated protein 25; SNARE, soluble *NSF* (*N*-ethylmaleimide-sensitive factor) attachment protein receptor; TRPA1, transient receptor potential ankyrin 1; TRPV1, transient receptor potential vanilloid type 1.

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Gazerani et al., 2006, 2009; Tugnoli et al., 2007; Meng et al., 2009; Ha et al., 2011; Yiangou et al., 2011; Matak et al., 2014). TRPV1 is a non-selective ligand-gated cation channel, member of TRPV1-4 family (Szallasi et al., 2007; Vennekens et al., 2008) which belongs to the TRP channels superfamily (Owsianik et al., 2006; Nilius et al., 2007; Eid and Cortright, 2009). TRPV1 channels respond to noxious heat, protons, and chemicals such as capsaicin, and it is preferentially expressed in small sensory neurons (Caterina et al., 1997), where it plays critical role in pain and neurogenic inflammation associated with tissue injury, inflammation, and nerve lesions. BoNT/A reduces pain and neurogenic inflammation induced by capsaicin because, through the cleavage of SNAP-25, it interacts with SNARE-dependent trafficking of TRPV1 (Apostolidis et al., 2005; Camprubí-Robles et al., 2009; Shimizu et al., 2012).

Transient receptor potential ankyrin 1 (TRPA1), another non−selective cation channel belonging to TRP channels superfamily, is normally coexpressed with TRPV1 (Story et al., 2003; Garcia-Anoveros and Nagata, 2007; Spahn et al., 2014). TRPA1 is directly activated by compounds causing burning sensation, such as allyl isothiocyanate (AITC) the main component of mustard oil, horseradish and wasabi (Jordt et al., 2004) and, indirectly, by mediators of inflammation, including bradykinin, prostaglandins, NO and others, eliciting nociceptor excitation and pain hypersensitivity (Bautista et al., 2006; Baraldi et al., 2010). Unlike TRPV1, the effect of BoNT/A on the TRPA1 has never been studied.

It is widely known that, in addition to the regulation of neuronal activity, the activation of TRP channels is implicated in a variety of non-neuronal processes, for example many endothelial functions, ranging from control of vascular tone and regulation of vascular permeability to angiogenesis and vascular remodelling (review in Zhang and Gutterman, 2011). Amongst TRP channels, TRPV1 and TRPA1 are widely expressed in neurovascular tissues (Pozsgai et al., 2010; Tóth et al., 2014), where they act as vasodilator component of neurogenic inflammation. As for example the vasodilation of meningeal arteries contributes to trigger migraine attacks (Geppetti et al., 2012).

In this study, for the first time, we analysed the effect of the pretreatment with BoNT/A on the pain evoked by injection of capsaicin or AITC in proximity of a neurovascular structure in mice. As model of neurovascular structures, easily accessible in mice, we chosen the superficial branch of femoral artery, vein and nerve. In mice, these structures are closely together, forming a single neurovascular fascia that lies under the skin on the inner side of the medial aspect of the thigh of the hindlimbs. Using such pain model we have easily tested the efficacy of BoNT/A as analgesic against pain evoked by agonists of TRPV1 and TRPA1 located in neurovascular structures. Our results have important implications for treatment of headache/migraine pain which in part depends from vasodilation of vascular structures similar to those analysed in the present report. Some of these results have been presented previously in abstract form (Luvisetto et al., 2014).

2. Methods

2.1. Animals

Adult (3−5 months) male mice, obtained by breeding mice with C57BL6/J as founders background, were maintained in normal diurnal lighting (2/12-h light/dark cycle, 07:00AM/07:00PM) and allowed access to food (Harlan Global Diet 2018; Harlan Italy, Italy) and tap-water *ad libitum*. Care and handling of the animals were in accordance with National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised, 1996), with the Italian National Law (DL116/92 as application of the European Communities Council Directive 86/609/EEC), and with the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain (Zimmermann, 1983). All the experiments were performed in a sound-attenuate cabin between 10:00 AM and 15:00 PM and mice were moved into the behavioral testing room at least 1-h prior to testing. The minimal number of animals necessary for each experiment were used. Experimenter was blind as for treatment groups. Mice were used only once and were euthanized by CO_2 immediately after the end of pain experiments.

2.2. Drugs and treatments

BoNT/A (150 kDa purified protein without accessory proteins) was a kind gift of Prof. C. Montecucco (Department of Biomedical Sciences, University of Padova, Italy). The toxin was frozen in liquid nitrogen and stored at −80 °C in 10 mM NaHepes, 150 mM NaCl, pH 7.2. Stock solutions of BoNT/A were tested for activity in the ex vivo mouse hemidiagphram model and in the *in vitro* cleavage of SNAP-25 (Schiavo and Montecucco, 1995). Injectable solutions of BoNT/A were freshly made in saline (0.9% NaCl) as vehicle. Capsaicin and AITC were purchased from Sigma−Aldrich (Milan, Italy). Injectable solutions of capsaicin or AITC were freshly made in a vehicle composed of 5% ethanol, 5% Tween-80, and 90% saline (0.9% NaCl).

Injection of BoNT/A was performed subcutaneously (sc) in the inner side of the medial part of hindlimb thigh, in correspondence of the femoral neurovascular fascia, while saline (0.9%NaCl) was injected in control mice. Particular attention was given to avoid injection of the toxin directly to the artery, vein or femoral nerve. BoNT/A (15 pg/paw; 5 μl) was chosen on the basis of previous neurotoxicity (Luvisetto et al., 2003) and behavioral studies (Luvisetto et al., 2006, 2007; Marinelli et al., 2010), and it is the maximal effective dose that can be peripherally injected in mice without causing side effects including neuroparalysis. Absence of motor deficits was checked by rotarod test. On the day of testing, capsaicin or AITC were sc-injected (10 μl) in the same site of pretreatment with BoNT/A, at dose of 2 μg/mouse for capsaicin (Caterina et al., 1997) and 1% (v/v) for AITC. Dose of AITC was chosen after a preliminary experiment with increasing amount of AITC in the range from 0.01% to 1%. In order to individuate in which structures BoNT/A was locally acting, in other mice groups AITC was sc injected into dorsal surface of the right hindpaw, distant from the site of BoNT/A injection.

2.3. Assays of chemical-induced inflammatory pain

Capsaicin- or AITC-induced pain was tested at day 7, 15, 21, or 30 after BoNT/A in different groups of mice. Time points of incubation with toxin were chosen on the basis of previous results, e.g. formalin test (Cui et al., 2004), demonstrating that analgesic effects of BoNT/A were still present at least for two weeks after toxin injection. One hour before testing, mice were individually placed in transparent plastic cage (30 × 12 × 13 cm). Then, mice were s.c.-injected with capsaicin or AITC. Immediately after injection, the cage was transferred to a sound-proofed cabin and a video camera, linked to a computer placed outside the cabin, was used for remote observation and recording. As pain-related response, or nocifensive behavior, we considered the cumulative amount of time the animal spent licking, scratching, flicking, or biting the injected hindlimb/hindpaw as extensively reported in literature (Sakurada et al., 1992; Kwan et al., 2006; Weng et al., 2012). Recording time was 25 (capsaicin) or 45 (AITC) min, chosen on the basis of preliminary trials. Nocifensive response was calculated in blocks of 5-min.