Neuropharmacology 118 (2017) 223–232

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

A novel therapeutic with two SNAP-25 inactivating proteases shows long-lasting anti-hyperalgesic activity in a rat model of neuropathic pain

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ARTICLE INFO

Article history:
Received 22 December 2016
Received in revised form
16 March 2017
Accepted 23 March 2017
Available online 24 March 2017

Keywords:
Neuropathic pain
Botulinum neurotoxin
SNAREs
Analgesics

ABSTRACT

A pressing need exists for long-acting, non-addictive medicines to treat chronic pain, a major societal burden. Botulinum neurotoxin type A (BoNT/A) complex – a potent, specific and prolonged inhibitor of neuro-exocytosis – gives some relief in several pain disorders, but not for all patients. Our study objective was to modify BoNT/A to overcome its inability to block transmitter release elicited by high $[Ca^{2+}]_i$ and increase its limited analgesic effects. This was achieved by fusing a BoNT/A gene to that for the light chain (LC) of type/E. The resultant purified protein, LC/E-BoNT/A, entered cultured sensory neurons and, unlike BoNT/A, inhibited release of calcitonin gene-related peptide evoked by capsaicin. Western blotting revealed that this improvement could be due to a more extensive truncation by LC/E of synaptosomal-associated protein of Mr = 25 k, essential for neuro-exocytosis. When tested in a rat spared nerve injury (SNI) model, a single intra-plantar (IPL) injection of LC/E-BoNT/A alleviated for ~2 weeks mechanical and cold hyper-sensitivities, in a dose-dependent manner. The highest non-paralytic dose (75 U/Kg, IPL) proved significantly more efficacious than BoNT/A (15 U/Kg, IPL) or repeated systemic pregabalin (10 mg/Kg, intraperitoneal), a clinically-used pain modulator. Effects of repeated or delayed injections of this fusion protein highlighted its analgesic potential. Attenuation of mechanical hyperalgesia was extended by a second administration when the effect of the first had diminished. When injected 5 weeks after injury, LC/E-BoNT/A also reversed fully-established mechanical and cold hypersensitivity. Thus, combining advantageous features of BoNT/E and/A yields an efficacious, locally-applied and long-acting anti-hyperalgesic.

1. Introduction

Major healthcare and economic problems arise from ~20% of the population suffering from chronic pain (Volkow and McLellan, 2016), with 21% of such patients experiencing depression and 19% losing their jobs as a result of their pain (Breivik et al., 2006). Conventional analgesics (typically opiates) and anti-inflammatory drugs (steroidal and non-steroidal) are short-lived, usually exert adverse effects or may be associated with tolerance/dependence, and some patients are non-responsive (Volkow and McLellan, 2016). Due to severe side-effects or lack of efficacy, 23% of patients stop taking medication for their pain (Breivik et al., 2006). Clearly, there is an urgent unmet need for effective, long-lasting, well-tolerated and non-addictive analgesics to overcome these limitations. Nerve/tissue damage is a major cause of chronic pain due to abnormal signalling from peripheral nociceptors that elevates the release of excitatory transmitters and neuropeptides, as well as increasing the surface trafficking of signal transducing channels (Ellis and Bennett, 2013). This, together with increased secretion of pro-inflammatory cytokines, leads to neuronal hyper-

Abbreviations: BoNT, botulinum neurotoxin; BoTIM/A, protease-inactive mutant of BoNT/A; CGRP, calcitonin gene-related peptide; DAS, digit abduction score; DC, di-chain; DTT, dithiothreitol; HC, heavy chain; H_N, translocation domain, N-terminal half of HC; IMAC, immobilised metal affinity chromatography; IPL, intra-plantar; LC, light chain; LC/E-BoNT/A, a recombinant chimera of LC/E and BoNT/A; mLD_{50}, median lethal dose; SC, single chain; SNAP-25, synaptosomal-associated protein of Mr = 25 k; SNAP-25A and SNAP-25E, BoNT/A- /E-cleaved products; SNAREs, soluble N- ethylmaleimide sensitive factor attachment protein receptors; SNI, spared nerve injury; TGNs, trigeminal ganglionic neurons; TRP, transient receptor potential; TRPV1, TRP vanilloid 1.

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http://dx.doi.org/10.1016/j.neuropharm.2017.03.026
0028-3908/

sensitisation and consequential neuropathic pain (Basbaum et al., 2009). Lowering such over-excitability should be achievable by inhibiting the release of pain mediators and lowering the elevated level of transient receptor potential (TRP) channels on the plasmalemma of pain-sensing nerves (Meng et al., 2016). Such an intervention could provide an attractive alternative to current drugs that mainly act centrally.

Notably, botulinum neurotoxin type A (BoNT/A)-haemagglutinin complex from *Clostridium botulinum* is a selective, prolonged inhibitor of transmitter release, and has been extensively used for treating the hyper-activity of neurons innervating muscles or secretory glands (Naumann et al., 2013; Thenganatt and Fahn, 2012). Encouragingly, the toxin complex relieves chronic headache in a restricted number of patients (reviewed by (Diener et al., 2011; Whitcup et al., 2014)), and has shown promise for the treatment of certain neuropathic pain conditions in clinical research investigations (Jabbari, 2015; Oh and Chung, 2015; Whitcup et al., 2014). Thus, it is notable that BoNT/A inhibits regulated exocytosis and alters the surface trafficking of cation channel transducers in sensory neurons (Meng et al., 2016; Zhang et al., 2016). Each of the 7 serotypes of BoNT possesses a Zn^{2+}-dependent light chain (LC) protease that is linked via a disulphide and non-covalent bonds to a heavy chain (HC); the latter binds neuronal ecto-acceptors and leads to endocytotic uptake (Dolly et al., 1984; Meng et al., 2013). Then, LC is translocated via the N-terminal half of HC (H_N domain) into the cytoplasm (Pirazzini et al., 2015) where it cleaves and inactivates soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), which are essential for the exocytotic release of transmitters and pain mediators (Sudhof, 2014). Despite the advantageous long duration of action of LC/A, its limited effectiveness against pain may arise from it being unable to block capsaicin-evoked neuronal release of a representative peptide, CGRP (calcitonin gene-related peptide) (Meng et al., 2007). Although BoNT/A cleaves off 9 C-terminal residues from SNAP-25, the ability of the product (SNAP-25_A) to form stable SNARE complexes with other partners might support CGRP secretion elicited by a large and sustained elevation of $[Ca^{2+}]_i$ arising from activation of the TRP vanilloid 1 receptor (TRPV1) by capsaicin (Meng et al., 2009, 2014). Attaching BoNT/E protease (LC/E), which instead truncates 26 residues from SNAP-25, to a protease-inactive mutant of /A (BoTIM/A) generated LC/E-BoTIM/A; this enters sensory neurons, produces SNAP-25_E and reduces capsaicin-evoked CGRP release (Meng et al., 2009; Wang et al., 2011).

Herein, two active SNAP-25 proteases were incorporated into a new fusion toxin, LC/E-BoNT/A. This protein showed greater solubility and stability than LC/E-BoTIM/A, and retained biological activity. Accordingly, a single intra-plantar (IPL) injection of LC/E-BoNT/A attenuated for up to 2 weeks both mechanical and cold hyper-sensitivities induced in a rat spared nerve injury (SNI) model of peripheral neuropathic pain (Decosterd and Woolf, 2000). It proved more efficacious than BoNT/A or repeated systemic administration of pregabalin, a clinically-used short-acting pain modulator (Verma et al., 2014), and its benefit was prolonged by a second injection. A single administration of LC/E-BoNT/A at a time when the pain behaviour had become fully established still proved effective.

2. Materials and methods

2.1. Animals and study approval

Adult male Sprague-Dawley rats for behavioural tests were purchased from Envigo (formerly Harlan, UK) and weighed 200–250 g at the time of surgery. The rats (for neuronal cultures), and Tuck-Ordinary mice were bred in an approved Bio-Resource Unit at Dublin City University. Animals were group-housed in individually ventilated cages, with food and water available *ad libitum*. Room temperature was maintained between 18 and 22 °C and relative humidity between 40 and 60%; a 12 h light/12 h dark schedule (lights on from 08:00–20:00) was operated. All procedures were approved by the University Research Ethics Committee and authorised by the Health Products Regulatory Authority, Ireland, thereby, fulfilling the requirements of Directive (2010)/63/EU and the National Statutory Instrument S.I. No 543 of 2012 (as amended by S.I. No. 434 of 2013 and S.I. No. 174 of 2014) on the protection of animals used for scientific purposes.

2.2. Production of LC/E-BoNT/A

A synthetic gene for LC/E (residues 1–411) was cloned into pET29a vector (Millipore, Ireland) between *Nde*I and *Eco*RV sites. Subsequently, a BoNT/A gene was amplified by polymerase chain reaction, using a reported synthetic construct as template (Wang et al., 2011), and sub-cloned into the above vector at *Eco*RV and *Xho*I sites with LC/E; this yielded a plasmid encoding LC/E-BoNT/A. The sequence-verified plasmid was transformed into *E. coli* BL21.DE3 strain (Millipore, Ireland) for expression of the His_6-tagged single-chain (SC) protein, using auto-induction medium (Studier, 2005). Briefly, bacteria grown overnight in Luria-Bertani broth medium, containing 50 μg/ml of kanamycin sulphate, were inoculated (1:1000 v/v) into ZYP-5052 auto-induction medium containing kanamycin and further cultured at 37 °C for ~5 h with agitation (220 rpm/min). Cells were then incubated at 22 °C for 20 h with continuous shaking before centrifugation at 5000 g for 30 min. Cell pellets from 2 L of culture were homogenously re-suspended in 80 ml of lysis buffer (20 mM HEPES, 145 mM NaCl, pH 8.0). After adding lysozyme (to a final concentration of 2 mg/ml), a protease inhibitor cocktail III (1:200 v/v, Millipore, Ireland) and 1500 units of benzonase nuclease (Millipore, Ireland), cells were lysed at 4 °C for 1 h on a roller before storage at −30 °C. Following one freeze-thaw cycle, the thawed lysate was centrifuged at 18,000 g for 1 h to remove debris; the resultant supernatant was subjected to immobilised metal affinity chromatography (IMAC) on Talon super-flow resin (Aquilant Scientific, local distributor for Clontech), with elution by 500 mM imidazole in lysis buffer. The eluate was buffer-exchanged into 20 mM sodium phosphate (pH 6.5) using a PD10 column (Fisher Scientific Ireland, local distributor for GE Healthcare Life Sciences), and loaded onto a Resource S column (Fisher Scientific Ireland), followed by washing with 100 mM NaCl before elution by a stepwise gradient up to 1 M NaCl in the above-mentioned phosphate buffer. The pooled pure sample was buffer exchanged into 20 mM HEPES, 145 mM NaCl, pH 7.4 buffer before conversion of SC to dichain (DC) by incubation with thrombin (Millipore, Ireland) (1 unit/mg of toxin) at 22 °C for 3 h and stopping of the reaction with phenylmethylsulfonyl fluoride at 1 mM final concentration. The expression, purification and nicking of LC/E-BoNT/A were monitored by SDS-PAGE on precast NuPAGE 4–12% Bis-Tris gels, followed by protein staining with Coomassie Blue. In some cases, purified samples separated by SDS-PAGE were electro-transferred to polyvinylidene difluoride membranes, probed by Western blotting with a His tag monoclonal antibody (Millipore, Ireland), rabbit antibodies against LC/E (Custom antibodies prepared by Zymed Laboratories Inc.) (Wang et al., 2008) or rabbit antibodies against 13 C-terminal residues of BoNT/A (a gift from Dr. Leonard Smith, United States Army Medical Research Institute of Infectious Diseases, Maryland). After incubation with horseradish peroxidase-conjugated anti-mouse or -rabbit secondary antibodies and chemiluminescent development, samples were visualized using the G:BOX Chemi-16 gel documentation system