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REVIEW

Therapeutic use of botulinum toxin in migraine: mechanisms of action

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Migraine pain represents sensations arising from the activation of trigeminal afferents, which innervate the meningeal vasculature and project to the trigeminal nucleus caudalis (TNC). Pain secondary to meningeal input is referred to extracranial regions innervated by somatic afferents that project to homologous regions in the TNC. Such viscerosomatic convergence accounts for referral of migraine pain arising from meningeal afferents to particular extracranial dermatomes. Botulinum toxins (BoNTs) delivered into extracranial dermatomes are effective in and approved for treating chronic migraine pain. Aside from their well-described effect upon motor endplates, BoNTs are also taken up in local afferent nerve terminals where they cleave soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, and prevent local terminal release. However, a local extracranial effect of BoNT cannot account for allthe effects of BoNT upon migraine. We now know that peripherally delivered BoNTs are taken up in sensory afferents and transported to cleave SNARE proteins in the ganglion and TNC, prevent evoked *afferent* release and downstream activation. Such effects upon somatic input (as from the face) likewise would not alone account for block of input from converging meningeal afferents. This current work suggests that BoNTs may undergo transcytosis to cleave SNAREs in second-order neurons or in adjacent afferent terminals. Finally, while SNAREs mediate exocytotic release, they are also involved in transport of channels and receptors involved in facilitated pain states. The role of such post-synaptic effects of BoNT action in migraine remains to be determined.

Abbreviations

BoNT, botulinum toxin; DRG, dorsal root ganglion; HC, heavy chain; IPLT, ipsilateral intraplantar; LC, light chain; TG, trigeminal ganglion

Table of Links

TARGETS	LIGANDS
AMPA (GluA2) receptors	CGRP
NK_1 receptors	PACAP
NMDA (GluN2C) receptors	Substance P
TRPV1 channels	

This Table lists the protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a, b, c).

Introduction

Migraine is characterized by recurrent episodes of unilateral throbbing head pain lasting for 4–72 h accompanied by aura and symptoms like nausea, photophobia and phonophobia (Olesen, 2013). Broadly speaking, migraine headache occurring with a frequency of less than 15 attacks per month is referred to as *episodic* migraine, and headache that occurs more frequently that is more than 15 attacks per month for more than 3 months, is referred to as *chronic* migraine (Olesen, 2013). An estimated 14% of the general population suffer from migraine, with the prevalence ratio of 1:3 (male : female) (Russell *et al.*, 1995; Lipton *et al.*, 2007) making it one of the most common debilitating neurological disorders. Migraine costs more than $20 billion each year in the USA and thus represents a heavy socio-economic burden to the society primarily because of decreased working efficiency and workdays lost (Serrano *et al.*, 2013). Most of the prophylactic drugs currently available have some ameliorating effect on headache with frequent treatment-limiting side effects (Evers, 2008). Initial anecdotal reports in patients receiving botulinum toxin (BoNT) for facial cosmetic purposes (Binder *et al.*, 1998) noted the effects of these injections on headache (Wheeler, 1998) and trigger point-initiated pain syndromes (Acquadro and Borodic, 1994; Cheshire *et al.*, 1994), which appeared to be independent of its effects upon muscle tone. This led to clinical trials that resulted in the approval of a BoNT-A (BoTox®) injected into superficial cranial musculature as a treatment for migraine (Diener *et al.*, 2010; Dodick *et al.*, 2010). BoNT-A is currently approved for the prophylactic treatment of adult chronic migraine in approximately 67 countries, including the USA, all countries in the European Economic Area as well as Australia, Brazil, Canada, India, Korea and Russia (http://www.sec.gov/Archives/edgar/data/850693/000085069314000002/agn10-k2013.htm).

This reported efficacy of extracranial BoNT in treating migraine is surprising given the current thinking that migraine pain may not be the result of increased tone in cranial musculature nor does it represent a cutaneous trigger point-initiated syndrome. As will be reviewed later, current thinking is that migraine pain results from activation of intracranial meningeal perivascular afferents (Strassman *et al.*, 1994; 1996; Burstein *et al.*, 1998) with some studies suggesting the role of extracranial afferents (Schueler *et al.*, 2013; Burstein *et al.*, 2014). In this review, we will consider the literature reflecting the clinical efficacy of BoNT in migraine; outline the anatomical organization believed to underlie migraine; and then review the actions of BoNTs that appear to underlie the ability of extracranially applied BoNT to block nociceptive inputs arising from peripheral afferents.

Clinical history of BoNT in migraine

The A serotype of BoNT (BoNT-A: onabotulinumtoxin A: BoTox) is approved for cosmetic delivery to the face. Such injections lead to a local relaxation of the musculature secondary to the local block of ACh release at the neuromuscular junction. As reviewed later, this block reflects the persistent cleavage of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins complexes which includes synaptobrevin/vesicle-associated membrane proteins (VAMP), synaptosomal-associated protein 25 (SNAP-25) and syntaxin, that are the key components involved in vesicular fusion during exocytosis) (Rothman, 1994) with the recovery of releasing function and re-occurrence of muscle tone typically occurring over a period of 3–6 months (Flynn, 2010). A variety of early studies addressed the proposition that BoNT would have ameliorative effects on headache (as suggested by the potential role of aberrant muscular contraction in the cranial pain) and this speculation was extended to include migraine.

Systematic clinical trials using BoNT-A (BoTox) as a prophylaxis for migraine have resulted in a mix of positive and negative findings (Table 1). These systematic studies were double-blind, placebo-controlled randomized trials where participants suffered either from episodic or chronic migraine. The injection paradigm differed from one study to another. Some employed a standard fixed injection site approach, with typically the therapeutic dose (155U) divided into 31 spaced injections over the forehead, while others used a 'follow-the-pain' treatment (e.g. inject the total dose into divided doses into the cutaneous region to which the pain is referred); and few, a combination of both (Elkind *et al.*, 2006; Hamdy *et al.*, 2009; Lipton *et al.*, 2011). Out of 11 trials performed with episodic migraine, only two studies showed a significant effect of BoNT-A in reducing the frequency of episodic migraine as compared with the placebo (Barrientos and Chana, 2003). A meta-analysis performed on these studies concluded that BoNT-A had no effect on episodic migraine (Jackson *et al.*, 2012). In contrast, BoNT was associated with a greater likelihood of a 50% or greater improvement in the patient suffering from chronic migraine (Jackson *et al.*, 2012). From the pooled analysis of two phase III trials (Research Evaluating Migraine Prophylaxis Therapy studies of BoNT-A in chronic migraineurs), it emerged that efficacy increased over time (up to 56 weeks), and this was paralleled by a self-reported improvement in quality of life (Lipton *et al.*, 2011). A study also suggested that the effectiveness of BoNT was associated with perception of headache and that imploding/ocular migraine headache is more likely to be prevented by prophylactic BoNT than exploding headache (Jakubowski *et al.*, 2006). In general, based on the trials thus far reported (see Table 1), the properties of the therapeutic effects of local BoTox in chronic migraneurs may be summarized as follows.

BoTox doses between 150 U and 195 U are efficacious with limited side effects.

Time of onset of the therapeutic effect on migraine is observed from week 12 with meaningful reduction in headache at 56 weeks.

The duration of action of BoNT reflects the time course of loss of SNARE cleavage activity. This loss is due to the degradation of the intracellular light chain (LC), the component of the toxin responsible for SNARE protein cleavage. Practically, this time course is manifested by the recovery of muscle tone, a marker of neuromuscular junction function. As noted in Table 2, each serotype has a different half-life depending upon the stability of the LC. For BoNT-A the relapse period is in 4–5 months, which in the case of migraine should reflect the time course of the return of the frequency of headache days back to baseline. The PREEMPT trials have used single injections of BOTOX every 12 weeks and has proven to be effective in meeting the primary and secondary endpoints.

Table 1

A summary of the clinical findings of BoNT in acute migraine, chronic migraine and chronic migraine with medication overuse

Number	Study	Dose of BoNT	Number of participants	Length of the study (including follow ups)	Results		Comments
					Primary end points	Secondary endpoints	
Acute migraine (<15 attacks per month)							
1	Silberstein *et al.*, 2000	25 U or 75 U	123 (vehicle = 41, BoNT = 82)	3 months	Positive	NA	Both doses showed greater reduction of migraine severity
2	Barrientos and Chana, 2003	50 U	30 (BonT = 15, placebo = 15)	3 months	Positive	Positive	Greater reductions in headache frequencies and attack on day 90
3	Evers *et al.*, 2004	16 U or 100 U	60 (placebo = 20, 16 U BoNT = 20, 100 U BoNT = 20)	3 months	Negative	Negative	Only sum score of all accompanying symptoms showed some difference
4	Anand *et al.*, 2006	50 U	32 (placebo = 16, BoNT = 16)	3 months	Positive	Positive	75 % patients reported complete relief to mild headache
5	Elkind *et al.*, 2006	7.5 U–50 U	182 (placebo = 100, BoNT = 82)	120 days	Negative	Negative	No differences was observed in any efficacy variable
6	Relja *et al.*, 2007	75 U–225 U	*n* = 515	270 days	Negative	Negative	Both placebo and BoNT group showed improvement
7	Saper *et al.*, 2007	25 U	232 (placebo = 45, BoNT = 187)	3 months	Negative	Negative	No significant effect of BoNT was observed
9	Vo *et al.*, 2007	205 U	32 (placebo = 17, BoNT = 15)	3 months	Negative	Negative	Headache pattern index suggested a protective effect of BoNT against headache severity.
10	Petri *et al.*, 2009	80 U–120 U	127 (placebo = 63, BoNT = 64)	3 months	Negative	Negative	A trend in reduction of headache however not significant
11	Chankrachang *et al.*, 2011	120 U–240 U	128 (placebo = 37, BoNT = 82)	8–12 weeks	Negative	Positive	BoNT showed significant benefit over placebo at some end points
Chronic migraine (>15 attacks per month)							
1	Mathew *et al.*, 2005	105 U–260 U	*n* = 571	11 months	Negative	Positive	Secondary endpoint was met at 180 days
2	Freitag *et al.*, 2008	100 U	*n* = 86	4 months	Positive	Positive	BoNT showed superiority to placebo for both endpoints
3	Mathew and Jaffri, 2009	upto 200 U	60 (topiramate = 29 and BoNT = 26)	9 months	Positive	Positive	Both BoNT and topiramate showed similar efficacy
4	Diener *et al.*, 2010	155 U–195 U	679 (BoNT = 341 and placebo = 338)	32 weeks	Positive	Positive	Favoured all secondary endpoints
5	Aurora *et al.*, 2010	155 U–195 U	679 (BoNT = 341 and placebo = 338)	24 weeks	Negative	Positive	PREEMPT 1 trial
6	Dodick *et al.*, 2010	155 U–195 U	1384 (BoNT = 688 and placebo = 696)	24 weeks	Positive	Positive	Pooled results of PREEMPT trial
7	Magalhaes *et al.*, 2010	250 U	72 (BoNT = 35 and amitriptyline = 23)	90 days	Positive	Positive	BONT was as effective as amitriptyline
8)	Lipton *et al.*, 2011	155 U	1384 (placebo = 696, BoNT = 698)	56 weeks	Positive	Positive	Significant and clinically meaningful reduction in headache
9)	Aurora *et al.*, 2011	155 U–195 U	1384 (Placebo = 696, BoNT = 698)	56 weeks	Positive	Positive	Significant reduction in headache days
10)	Cady *et al.*, 2011	300 U	59 (topiramate = 30 and BoNT = 29)	26 weeks	Positive	Positive	Topiramate and BoNT showed similar efficacy
Chronic migraine with medication overuse							
1	Sandrini *et al.*, 2011	100 U	68 (BoNT = 33 and placebo = 35)	12 weeks	Negative	Positive	Suggested the tenderness of pericranial muscles in patients with MOH
2	Silberstein *et al.*, 2013	155 U–195 U	904 (placebo = 459, BoNT = 445)	24 weeks	Positive	Positive	Change in frequency of acute headache medication intakes was not statistically significant
3	Grazzi, 2013	100 U	*n* = 10	1 year	Negative		Results confirmed the efficacy of BoNT when used at 150 U
		150 U	*n* = 8		Positive		

NA, not applicable; MOH, medication overuse headache; PREEMPT, Phase III Research Evaluating Migraine Prophylaxis Therapy.

Table 2

Classification of BoNT serotypes A (Schiavo *et al.*, 2000), B (Sloop *et al.*, 1997; Eleopra *et al.*, 1998), C (de Paiva *et al.*, 1999; Jurasinski *et al.*, 2001), D (Yamamoto *et al.*, 2012), E (Foran *et al.*, 2003), F (Kauffman *et al.*, 1985; Ludlow *et al.*, 1992)

				Half-life ($t^{1/2}$)	
Number	**BoNT serotype**	**Targeted SNARES**	**Cleavage sites**	**Humans**	**Rodents**
1	BoNT type A (onabotulinumtoxin A)	SNAP-25	(Gln^{197}-Arg^{198}) 9 residues	>4 months	1–2 months
2	BoNT type B (rimabotulinumtoxin B)	Synaptobrevin II or VAMP 2	Gln^{59}-Phe^{60}	~2 months	21 days
3	BoNT type C (C1)	SNAP-25	Arg^{198}-Ala^{199}	<3 months	<25 days
4	BoNT type D	Synaptobrevin II or VAMP 2	Lys^{42}-Leu^{43}	NA	NA
5	BoNT type E	SNAP-25	(Arg^{180}-lie^{181}) 25 residues	<4 weeks	4 days
6	BoNT type F	Synaptobrevin II or VAMP 2	Gin^{41}-Lys^{42}	2 months	7 days
7	BoNT type G	Synaptobrevin II or VAMP 2	NA	NA	NA

NA, not applicable; SNAP-25, synaptosomal-associated protein 25; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor.

Even in patients reporting improved pain scores, there are no effects on associated migraine symptomatology (aura, phonophobia, photophobia, nausea).

No difference in efficacy/outcome observed if BoNT is injected in a fixed series of site or into the specific extracranial sites to which the pain is referred.

Regarding comparative efficacy, four clinical trials compared the therapeutic efficacy of BoNT-A with different prophylactic drugs. Although BoNT-A was more effective than steroids, prednisolone (Porta, 2000), it was not better than topiramate, valproate or amitriptyline in reducing the frequency of headache (Blumenfeld *et al.*, 2008; Magalhaes *et al.*, 2010; Cady *et al.*, 2011). According to the International Association for the Study of Pain (IASP), it is presently suggested that the best scientific evidence supports the use of systemic topiramate or local BoNT injections for the prevention of chronic migraine (IASP, 2013).

While the use of BoNT in migraine may only apply to a subpopulation of migraine patients, these clinical studies point to the advantageous use of BoNTs over other prophylactic strategies with respect to reduced side effect, persistent efficacy and tolerability in the treatment of migraine (Samton and Mauskop, 2006).

Migraine: referred pain

Despite many studies, the origin of migraine pain is controversial. However, considerable evidence suggests that the pain sensation in migraine arises from activation of meningeal perivascular afferents (Strassman *et al.*, 1996; Olesen *et al.*, 2009; Zhang *et al.*, 2013).

Meningeal afferents convey nociceptive information. The meningeal vasculature is innervated by small afferents, which project through trigeminal ganglia (TG) to the trigeminal nucleus caudalis (TNC) (Strassman *et al.*, 1986; Levy and Strassman, 2002; Levy *et al.*, 2007; Olesen *et al.*, 2009). These afferents have been shown to display epitopes common to C poly-modal nociceptors, for example TRPV1 receptors, and peptidergic neurotransmitters (Bove and Moskowitz, 1997; Eftekhari *et al.*, 2013). These axons are activated by the terminal application of a variety of proinflammatory substances including PGs, histamine, NO and TNF (Strecker and Messlinger, 2003; Zhang *et al.*, 2007; 2011). In the superficial dorsal horn, these afferents terminate in superficial TNC laminae, where they activate lamina I (marginal) and deep laminae (wide dynamic range) neurons (Strassman *et al.*, 1994; Roch *et al.*, 2007). As with other small-afferent input, this afferent input activates the second-order neurons in a frequency-dependent fashion. Importantly, high-frequency small-afferent traffic can initiate a facilitated state, wherein the second-order neurons will display an enhanced response (wind up) subsequent to afferent input. In humans, mechanical and electrical stimulation of dural and cerebral arteries in patients undergoing open cranium operative procedures, resulted in nausea and headache-like pain sensations referred to specific extracranial regions (Ray and Wolff, 1940; McNaughton and Feindel, 1977). These studies also suggested a possible contribution of extracranial structures like pericranial muscles and arteries in headache generation. While the involvement of extracranial tissues in migraine has been a subject of debate, recent observations argue for this possibility (Jakubowski *et al.*, 2006; Burstein *et al.*, 2014). Neuronal tracing and electrophysiological recordings suggest that the nerve fibres that innervate the extracranial structures (pericranial muscles) may represent functional collaterals passing though cranial sutures from the innervation of intracranial structures (meninges), and that these collaterals can deliver sensory information from the outside of the cranium (Schueler *et al.*, 2013). It should be noted that if this extracranial innervation contributed to the migraine pain states that the actions of topical agents should be effective in treating migraine when delivered at that site only (e.g. over the scalp to which the migraine pain is referred). Given these issues, it is evident to the degree that migrane represents pain originating from the meninges, this referral to extracranial strutures makes this a referred pain state and as with other pain states of a visceral origin, we may consider the potential role of convergence between intracranial and extracranial input.

Viscerosomatic convergence of meningeal and extracranial cutaneous afferents. As noted, an important property of migraine pain is that it is often referred to a specific extracranial region and is an example of referred pain mechanism. In humans, stimulation of the meninges and meningeal vessels results in painful sensation in supraorbital, retrobulbar and occipital region (areas to which the pain component is referred during migraine) (Ray and Wolff, 1940). Such referral of pain of a visceral origin to specific somatic regions is a common property of visceral pain states and is considered to reflect a convergence of the sensory afferents arising from the visceral site (here the meninges) onto second-order neurons, which also receive input from somatic regions (here the face or forehead) (Foreman, 2000; Brumovsky and Gebhart, 2010). Anatomical and electrophysiological studies have indeed reported that nocisponsive neurons in TNC and upper cervical spinal cord dorsal horn (Strassman *et al.*, 1994; Piovesan *et al.*, 2001; Morch *et al.*, 2007) receive *convergent* input from poly-modal nociceptive afferents that arise from localized regions of the head and face and others from the meninges (Strassman *et al.*, 1994; Burstein *et al.*, 1998; Noma *et al.*, 2008). Thus, as described further later, activation of nociceptive meningeal afferents believed to underlie migraine project to TNC neurons, which also receive trigeminal afferent input from homologous area of skin.

Properties of meningeal and somatic afferent activation. Both the extracranial (somatic) and intracranial originating small poly-modal nociceptive afferents have their respective cell bodies located in the ipsilateral TG. These afferent cell bodies are distributed in the TG according to their respective trigeminal divisions, for example V1 originating from the ipsilateral supraorbital regions and from the meninges overlying the ipsilateral frontal poles are both found in the first division region of the ipsilateral TG (Steiger and Meakin, 1984). These afferents are activated by the exposure of their respective peripheral terminals to a variety of proinflammatory products, such as 5-HT, prostanoids, H^+ and bradykinin (Ebersberger, 2001; Strassman and Levy, 2006). Upon activation of their distal terminals, two events transpire. At the local site (in the skin or the meninges), an action potential is generated that travels orthodromically to the spinal terminals (here the nucleus caudalis) and antidromically along collaterals of the parent axon to the local peripheral terminals. At the central and peripheral terminals, the local depolarization opens voltage-gated calcium channels that activate SNARES that mobilize synaptic vesicles to release their contents. These terminals contain and release glutamate and a variety of peptides including calcitonin gene-related peptide (CGRP), pituitary adenylate cyclase activating peptide (PACAP) and substance P (Uddman *et al.*, 1985; 2002; Jansen *et al.*, 1992). Release of these peptides in the periphery (meningeal vasculature) triggers a neurogenic inflammatory response (Markowitz *et al.*, 1988; Ramachandran *et al.*, 2014), for example vasodilatation, plasma protein extravasation and mast cell degranulation (Dimitriadou *et al.*, 1991; Brain and Grant, 2004; Peroutka, 2005). At the central terminals, such release of glutamate and peptides acting upon eponymous excitatory receptors activate second-order neurons.

Visceral afferent sensitization and somatic afferent input. Electrophysiological studies have shown that application of inflammatory products to dural afferents served not only to initiate depolarization, but also to sensitize the peripheral terminals and enhance the response of the second-order TNC neurons to subsequent stimulation (e.g. a central sensitization of the TNC neurons receiving the meningeal input) (Burstein *et al.*, 1998; Bartsch and Goadsby, 2003). Importantly, this activation of meningeal afferents is accompanied by increased sensitivity to stimuli applied to the skin (e.g. a cutaneous tactile allodynia) (Burstein *et al.*, 1998; Oshinsky and Gomonchareonsiri, 2007). The enhanced response to somatic input is believed to reflect the enhanced response of the second-order neuron to the somatic input initiated by the meningeal input into that same pool of second-order neurons. Throbbing pain of migraine is thus likely mediated through both peripheral sensitization of the meningeal afferent axon and the central (TNC) sensitization resulting from the ongoing small-afferent traffic. Such central sensitization and the convergence of somatic upon second-order neurons receiving meningeal afferent traffic may mechanistically account for the migraineurs enhanced sensitivity to light touch applied to the skin (e.g. in effect a secondary tactile allodynia) (Burstein *et al.*, 2000a,b; Jakubowski *et al.*, 2005). Again it should be noted that this motif is similar to that reported in other visceral organ systems where local bowel inflammation will lead to an enlarged area of tactile sensitivity (Laird *et al.*, 2001; Eijkelkamp *et al.*, 2007; Robinson and Gebhart, 2008).

BoNT

BoNTs are synthesized by *Clostridium botulinum* bacteria (Peck, 2009). There are seven serotypes (A–G). BoNTs are synthesized as single chain sequences and undergo cleavage to generate toxins that consist of a heavy chain (HC) and a LC joined by a single disulphide linkage. The HC (~100 kDa) is responsible for uptake in the cytosol. The uptake may occur by several mechanisms:

The HC binds the toxin to presynaptic gangliosides on the cell surface and promotes LC (~50 kDa) translocation into cytosolic endosomes (Fischer and Montal, 2007; Fischer *et al.*, 2009; Montal, 2010). A component of this uptake into neurons is enhanced by membrane depolarization, stimulating BoNT endocytosis by its HC (Keller *et al.*, 2004; Dong *et al.*, 2006; 2007; Rummel *et al.*, 2007).

Internalization by mechanisms that are independent of vesicle recycling involving gangliosides such as synaptic vesicle protein 2 on the plasma membrane (Fotinou *et al.*, 2001; Stenmark *et al.*, 2008).

The acidic environment in the endosome cleaves the disulphide bond and the LC moves into the cytosol. The LC is a zinc (Zn2+) endopeptidase with proteolytic activity located at the N-terminal end, targeting consensus sites on the SNARE proteins (Montecucco and Schiavo, 1994). There are seven different serotypes of BoNT, each acting on different components of SNARE protein. BoNT-A, C and E specifically target SNAP-25, which is largely expressed on plasma membranes (Schiavo *et al.*, 2000), whereas BoNT-B, -D, -F and -G explicitly act on vesicular protein isoforms of synaptobrevin (also called VAMP). BoNT-C also cleaves the plasmalemma

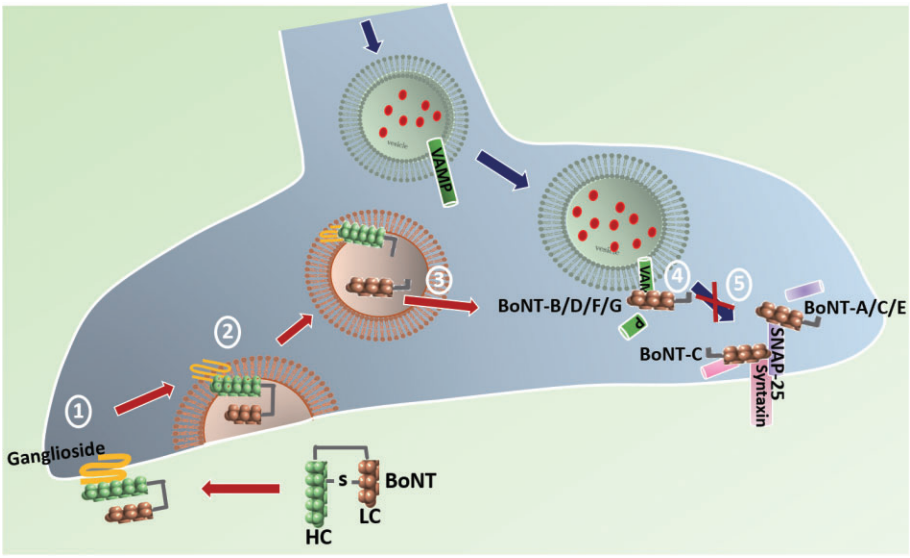

Figure 1

Uptake mechanism and targets of BoNT. HC of BoNT binds to the presynaptic ganglioside present on the plasma membrane (1) that promotes translocation of LC into endosomes (2). Acidic milieu in the endosome cleaves the disulphide linkage between the HC and LC releasing LC into the cytosol (3). The LC of BoNT-B/D/F/G specifically cleaves VAMP on the vesicle, BoNT-A/C/E cleaves SNAP-25 and BoNT-C also cleaves syntaxin on plasma membrane (4), thus inhibiting the vesicular fusion and blocking the neurotransmitter release (5).

protein syntaxin (Rummel, 2013). Cleavage of any one SNARE results in block of vesicle mobilization and release (Figure 1). Types A and B represent the most commonly studied serotypes of this family (Brin *et al.*, 1987; Aoki, 2003; Cui *et al.*, 2004; Huang *et al.*, 2011; Chinnapongse *et al.*, 2012; Marino *et al.*, 2014). Given locally into the musculature the property of BoNT in inhibiting exocytosis of ACh has been strategically used in clinical practice for treating several muscular disorders including cervical dystonia and blepharospasm (Mahant *et al.*, 2000; Dolly *et al.*, 2009). As reviewed earlier, current evidence based on clinical studies now supports the therapeutic potential of BoNT in treatment of chronic migraine (Diener *et al.*, 2010; Dodick *et al.*, 2010; Lipton *et al.*, 2011). Later, we will consider possible mechanisms whereby the peripherally delivered BoNT may alter central processing.

Mechanisms of actions of BoNT in migraine

Several hypotheses have been tendered and will be considered later.

Muscle tone. Not unreasonably, it was earlier proposed that the effectiveness of BoNT in treating pain conditions was due to the relaxation of pathological muscle tension, an effect suggested by its potent and efficacious ability to block local neuromuscular transmission. This hypothesis, however, does not garner support as: (i) Pain alleviation occurred in conditions of heightened muscle tone, like cervical dystonia prior to muscle relaxation (Brin *et al.*, 1987); and (ii) The effectiveness of BoNT in migraine was observed in the areas with no reduction in muscle tension (Brin *et al.*, 1987), thus ruling out a primary role of aberrant muscle tone in the migraine pain.

Anti-inflammatory activity. Following local stimulation, the peripheral terminals of unmyelinated sensory afferents display calcium-dependent vesicularly mediated release of local transmitters such as substance P and CGRP (Gupta *et al.*, 2010). These products can lead to a cascade of local events including plasma extravasation, mast cell degranulation and chemotaxis of inflammatory cells (Markowitz *et al.*, 1988; Kowalski *et al.*, 1990; Dimitriadou *et al.*, 1991; Peroutka, 2005) that may jointly change the local chemical milieu and activate the peripheral nerve terminal (Zhang *et al.*, 2007)'. Not surprisingly, this local terminal release of peptide transmitters and the consequence of this release (e.g. vasodilatation and increased capillary permeability), typically imitated by the local delivery of the TRPV1 channel agonist capsaicin, can be blocked by the local (peripheral) delivery of BoNTs in humans (Kramer *et al.*, 2003; Voller *et al.*, 2003; Gazerani *et al.*, 2006; Tugnoli *et al.*, 2007) and in animals (Bach-Rojecky *et al.*, 2008; Carmichael *et al.*, 2010; Marino *et al.*, 2014). This effect occurs in visceral afferent as well, as indicated by the effects of BoNTs on peptide release for example from bladder afferents (Rapp *et al.*, 2006; Lucioni *et al.*, 2008). The local block by local BoNTs typically occurs with a short latency, within hours (see Marino, *et al.*, 2014), and is achieved at low doses, resulting in an action limited to the tissue close to the site of delivery (e.g. ipsilateral and not a result of a systemic redistribution). Similar local effects have been observed in humans examining the evoked flare after BoNT (Gazerani *et al.*, 2006; 2009a,b; Tugnoli *et al.*, 2007). These effects are consistent with the ability of toxins to prevent afferent transmitter release, as measured by effects on evoked release in dorsal root ganglion cell culture systems (Meng *et al.*, 2007; 2009; Dolly and O'Connell, 2012). While there is little doubt that local BoNTs can alter release from

local nerve terminals, the role of such a local effect in the skin and superficial musculature to changes in migraine activity is unclear. It should be noted that, while controversial, the onset of migraine does not typically present itself with a change in extracranial blood flow or with signs of local peripheral inflammation (Zwetsloot *et al.*, 1991).

Central (spinopetal) afferent transport. The paralytic effect of local BoNTs emphasizes that local BoNT uptake occurs in motor neuron terminals, while the effects upon neurogenic antidromic vasodilatation emphasizes that peripheral uptake also occurs in sensory afferents, certainly in C poly-modal peptidergic nociceptors.

The current work increasingly emphasizes that the local sensory (and motor) terminal will not only take up local BoNTs, but move them in an active form via fast axonal transport to central terminals. Such transport can be demonstrated by movement of radiolabelled toxins as shown in early studies (Habermann, 1974; Wiegand *et al.*, 1976; Black and Dolly, 1986); and by the cleavage of the respective SNARE protein in the dorsal root ganglion (DRG) and TG or motor neurons in ventral horn (Aoki, 2003; Antonucci *et al.*, 2008; Matak *et al.*, 2011; Restani *et al.*, 2011; 2012; Lawrence *et al.*, 2012; Simpson, 2013; Marino *et al.*, 2014). Thus, in the mouse, ipsilateral intraplantar (IPLT) delivery of BoNT-B into the hind paw cleaves VAMP in ipsi- but not in contralateral DRG within 24 h after IPLT delivery (Marino *et al.*, 2014). For the BoNT-A serotype, truncated (cleaved) SNAP-25 products were observed in the ipsilateral TNC at most by day 3 following the injection of BoNT-A into the whisker pad. Importantly, such central effects can be blocked by treatment of the nerve with blockers of axon transport (Matak *et al.*, 2012; Marino *et al.*, 2014). These results showing homotopic cleavage in the respective sensory ganglia argue that the peripherally delivered BoNT-A and B serotypes in an active form had gained access to and were cleaving SNARES in the DRGs of axons that were innervating the injected skin region. While early work suggested that the transported toxin may be non-functional (Lawrence *et al.*, 2012), the physiological relevance of this cleavage is indeed suggested by three observations. (i) IPLT BoNT blocked not only the release of substance P from the ipsilateral primary C fibre afferent, but prevented the ipsilateral constitutive Finkel-osteogenic sarcoma (cFOS) expression (marker of neuronal activation) otherwise evoked by unilateral IPLT formalin (Marino *et al.*, 2014). In the trigeminal system, activation of trigeminal sensory neurons by capsaicin evokes CGRP release, which is attenuated by BoNT (Durham *et al.*, 2004; Meng *et al.*, 2009). (ii) IPLT BoNT-B prevented the dorsal horn release of substance P and cFOS activation otherwise evoked by the intrathecal delivery of capsaicin (e.g. direct stimulation of the spinal terminals of the TRPV bearing, substance P positive, C fibres, emphasizing that the block of central release by the peripheral BoNT was not due to a block by the peripheral BoNT of afferent activation by the formalin) (Marino *et al.*, 2014). (iii) BoNTs (A and B) delivered unilaterally in the paw and/or the whisker pad will produce a homotopic anti hyperpathic (defined as 'a painful syndrome characterized by an abnormally painful reaction to a stimulus, especially a repetitive stimulus, as well as an increased threshold') effect in mouse and rat models such as Phase 2 flinching evoked by formalin (Cui *et al.*, 2004; Aoki, 2005; Marino *et al.*, 2014) and after inflammation (Bach-Rojecky and Lackovic, 2005; Bach-Rojecky *et al.*, 2008; Matak *et al.*, 2011; 2013) and in mouse and/or rats with mononeuropathies (nerve ligation) or poly neuropathies (diabetes, chemotherapeutics (Bach-Rojecky and Lackovic, 2005; 2009; Park *et al.*, 2006; Luvisetto *et al.*, 2007; Ma *et al.*, 2012; Marinelli *et al.*, 2012). Moreover, the block of formalin-evoked flinching produced by pretreatment with BoNT in the whisker pad was prevented by block of axonal transport using colchicine applied to the infraorbital nerve (Matak *et al.*, 2011).

Importantly, comparable results have been reported in human studies where local BoNTs reduce hyperesthesia (defined as 'increased sensitivity to stimulation, excluding the special senses') in post-herpetic neuralgia (Liu *et al.*, 2006; Ruiz and Bermejo, 2008; Xiao *et al.*, 2010), diabetic neuropathy (Yuan *et al.*, 2009), nerve injury (Piovesan *et al.*, 2005; Ranoux *et al.*, 2008; Fabregat *et al.*, 2013) and as reviewed in certain forms of migraine (Diener *et al.*, 2010; Dodick *et al.*, 2010). Such results suggest that the peripheral BoNT is taken up in the afferent axon and transported in an active form to the DRG of the axons innervating the injection site (Dolly and O'Connell, 2012).

While the earlier commentary emphasizes that peripherally delivered BoNTs can indeed gain access to the homolateral sensory ganglion and dorsal horn and cleave SNARE proteins, preventing release from that terminal and downstream activation otherwise produced by that cleavage, this central action on the presynaptic afferent terminal does not appear to provide a tenable explanation for the ability of BoNT transported spinopetally in somatic afferents (arising from the injection site in the extracranial skin and musculature) to alter the input from the intracranial meninges.

Trans-synaptic movement of BoNT. As reviewed earlier, it is now certain that BoNTs are taken up and undergo fast axonal transport in brain to the spinal cord after peripheral application. It has long been considered that such transport, in contrast to that displayed by tetanus toxin, if occurred, was largely limited to the axon in which the uptake occurs (Rossetto and Montecucco, 2008). This was initially based on the classic observations that intramuscular tetanus toxin would induce spasticity, a sign that the toxin had moved from the motor neurons to a local inhibitory (glycinergic) interneurons whereas BoNT yielded only a flaccid paralysis (Rossetto and Montecucco, 2008). However, it seems clear now that BoNT induces an immediate flaccidity whereas the local tetanus paralytic action is delayed. Central transport occurs quickly, but the potential central, trans-synaptic, effects of BoNT are obscured by the peripheral block of muscle function (Restani *et al.*, 2012). There is now evidence that BoNTs may in fact also undergo a transcytotic movement in neurons (Lalli *et al.*, 2003; Antonucci *et al.*, 2008; Restani *et al.*, 2011; Torii *et al.*, 2011; Akaike *et al.*, 2013; Marchand-Pauvert *et al.*, 2013) and glia (Marinelli *et al.*, 2012). Several specific studies may be cited to show such transport in the afferent systems. (i) In recent work, we observed that dorsal horn cFOS activation otherwise initiated bilaterally by intrathecally delivered substance P is reduced in the dorsal horn, ipsilateral to the paw that received IPLT BoNT-B (Marino *et al.*, 2014). As the receptors for substance P (NK_1) are largely postsynaptic to the primary afferent

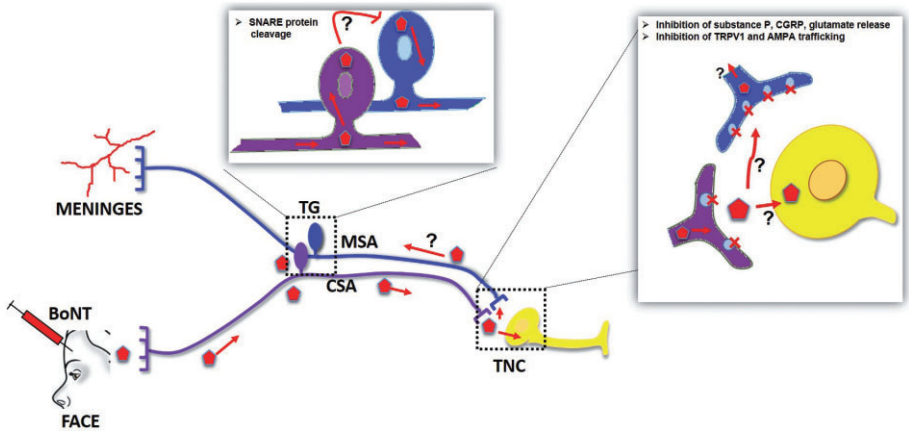

Figure 2

Speculative mechanism(s) of BoNT action. BoNT injected into cranial dermatomes will be taken up by the cutaneous sensory afferents (CSA) and transported retrogradely to the TG where it cleaves SNAREs and is then transported centrally to TNC. Transported BoNT may undergo a trans-synaptic movement either at the second-order neuron (which receives convergent input from the meningeal afferent) or the terminal of the converging activated meningeal afferent. Such a trancytosis may also hypothetically occur in TG sensory neurons and block the activated meningeal afferent release. Future experiments are required to address these options.

(Littlewood *et al.*, 1995) and because the ipsilateral block of bilateral activation by IPLT BoNT is considered to represent an effect postsynaptic to the primary afferent terminal, these findings suggest that BoNT is not only transported to the central terminal, but may also undergo trans-synaptic movement from the central afferent terminal to second-order neurons. (ii) The presence of cleaved SNARE (Filipovic *et al.*, 2012) (specifically SNAP-25) has been found in dorsal horn astrocytes after IPLT delivery, suggesting a transcytotic movement of the BoNT from the afferent to the proximal glial membrane (Marinelli *et al.*, 2012). (iii) In an elegant study, it was found that unilateral injection of BoNT in to the vibrissae pad reduced dural extravasation (Filipovic *et al.*, 2012), suggesting that BoNT-A in the somatic afferent reached the meningeal afferents responsible for the local release of pro-inflammatory substances leading to dural extravasation.

Other studies have provided support for transcytotic movement of BoNTs in motor axons (Akaike *et al.*, 2013). Similar studies in animals (Antonucci *et al.*, 2008; Torii *et al.*, 2011; Restani *et al.*, 2012) and humans (Marchand-Pauvert *et al.*, 2013) support this hypothesized trans-synaptic action.

The mechanisms of the transcytotic BoNT movement are not certain and controversial. Several uptake pathways have been identified including one mediated by local vesicle recycling and a distal retrograde endosome-based trafficking pathway. Different fractions of the BoNT being taken up may enter either pathway with the predominant fraction thought to be in the local pool. It has been speculated that the intact BoNT may be transported, depending on its HC component, and the transport of active toxin depends upon a lack of exposure of the transported BoNT to an acidic environment (which serves to cleave the heavy and light chains) (Restani *et al.*, 2012). While there are no direct data supporting the role of such trans-synaptic movement in mediating the effects of extracranial BoNT on the migraine phenomena, the ability of BoNT to undergo central transport along primary afferents and its apparent ability to move to a second-order membrane to reduce SNARE mediated functions, presents an intriguing if speculative hypothesis for how BoNT moving in the somatic afferent can act upon the input arising from the meningeal afferent. It can either be by transport between adjacent cell bodies in the TG or between spatially contiguous terminals in the nucleus caudalis or between the somatic afferent and the second-order nucleus caudalis neurons receiving convergent input from the trigeminovascular and somatic afferents. Accordingly, we hypothesize that BoNT injected into cranial dermatomes will be transported retrogradely in the primary afferents and cleave its target (SNAREs) in TG and further, will alter by a presynaptic effect upon the meningeal afferent or the convergent second-order neurons excitation arising from the meningeal afferent and underlying the aversive component of the migraine state (Figure 2).

Pre- versus postsynaptic effects of BoNTs

As reviewed, BoNTs exert their principal effect through the cleavage of SNARES. Accordingly, the effects of BoNTs reflect upon the role played by SNAREs in cellular function.

Regulation of exocytotic release. The principal discussion in the preceding sections has been based on the defined relevance of SNARES to the mobilization of neurotransmitter vesicles. As reviewed above BoNTs block evoked release from sensory and motor systems as well as from central excitatory and inhibitory neurons (see Marino *et al.*, 2014). However, such effects have been reported in virtually every exocytotic release system studied including parasympathetic axons (Ikeda *et al.*, 2012; Shan *et al.*, 2013), post-ganglionic sympathetics (Smyth *et al.*, 2006), chromaffin cells (Lawrence *et al.*, 2002) enterocromaffin cells (Zanner *et al.*, 2002), pancreatic Islet cells (He *et al.*, 2008) pituitary hormone secretion (Leggett *et al.*, 2013) and mast cells (Bottinger *et al.*, 1987; Park, 2013). In the present context, one might speculate that the analgesic effects of afferent transported BoNT would be

represented by this well-defined effect upon afferent terminal release. It is interesting to note, however, that peripheral BoNTs are largely ineffective in models of *acute* nociception, for example lack of effect upon acute thermal threshold in both animals (Marino *et al.*, 2014) and humans (Gazerani *et al.*, 2009a); but are most efficacious in models where there is the activation of a facilitated states, for example the second, but not first phase of the formalin flinching model for both paw and whisker injections (Matak *et al.*, 2013; Marino *et al.*, 2014). Were the principal effects of BoNTs on mechanisms of primary afferent transmission, we would anticipate a potent effect upon acute thresholds (much as agents such as morphine) which act presynaptically on the primary afferent and are well known to alter acute threshold response (Yaksh *et al.*, 1999). Other actions of SNAREs may thus be in play to account for the antihyperalgesic effects of peripheral BoNTs.

Intracellular transport. An alternative BoNT target relates to the appreciation of the role of SNAREs in the trafficking of several ionotropic and metabotropic receptors, such as the subunits of TRPV1 channels (Montell, 2004) and AMPA (Steinberg *et al.*, 2004) and NMDA receptors (Lau *et al.*, 2010), that are actively involved in the process of spinal sensitization. Several specific examples will be noted.

Central sensitization process involves rapid trafficking of the glutamate receptors to the membrane. During the phase of repetitive afferent stimulation, there is an increase in the transport and insertion of subunits into the membrane, for the calcium permeable AMPA receptor (Schenk *et al.*, 2003; Steinberg *et al.*, 2004; Lau *et al.*, 2010; Ahmad *et al.*, 2012) and block of these spinal receptors has a powerful antihyperpathic action (Choi *et al.*, 2010; Tao, 2012). Strong evidence suggests that SNAREs play a mediating role in this vesicular transport of AMPA receptors to the membrane. BoNTs can alter such trafficking, although this has been primarily assessed in cerebellum (Kakegawa and Yuzaki, 2005). Interventions by BoNT in glutamate ionophore trafficking would unquestionably influence facilitated processing thereby having profound effects upon facilitated states in migraine.

The TRPV1 channel is expressed both centrally and peripherally in the trigeminal afferent system. In trigeminal models, activation of TRPV1 channels evoke CGRP release from trigeminal afferents (Akerman *et al.*, 2003) . Inhibition of TRPV1 channels can both prevent and reverse established allodynia (clinicaltrials.gov, 2014). Subcutaneous BoNT-A into the face decreased TRPV1 positive neurons in the ophthalmic division of the rat TG, secondary to an inhibition of their trafficking to the plasma membrane (Shimizu *et al.*, 2012). This property of BoNT is likely to hold true for most of the ionotropic and metabotropic receptors involved in pain facilitation.

Finally, while there is only limited data thus far, we suggest that the potential role of BoNTs in regulating cell surface expression of a variety of receptors and channels is extensive. Most examples of intracellular protein trafficking involves formation of a coated vesicle that displays tethering and fusion proteins and is moved as cargo via actin- or tubulin-based filaments. Upon approximation to the target compartment, the vesicle tethering proteins utilize SNAREs to initiate binding and fusion to deliver the contents to the target membrane (Angers and Merz, 2011; Juliano *et al.*, 2013). It is thus likely that BoNTs can regulate trafficking of a wide range of targets, including catalytic receptors, such as receptor tyrosine kinases and GPCRs. Importantly, SNARES are greatly enriched in lipid rafts (Lang, 2007) and this organization may be important for membrane trafficking of these receptor and channel proteins as well as the spatial organization of the secretory machinery required for exocytosis (Chamberlain *et al.*, 2001). Given the ubiquitous role of SNARES in cell function, the relative specificity and lack of evident toxicity suggests a high degree of targeting, which is only just beginning to be appreciated.

Therapeutic potential of other BoNT serotypes

The work outlined earlier characterizing the action of BoNTs in pain processing has typically been limited and restricted to one or two BoNT serotypes (typically A and B). As noted, the seven different serotypes of BoNT have a similar function of inhibiting neurotransmitter release, but by targeting different machineries involved in the process of exocytosis (Table 2). However, the individuality of each BoNT is in part defined by the activity of the LC-protease. For example, the LC-protease of BoNT-A cleaves only nine residues from the C-terminus of SNARE protein SNAP-25 and thus partially blocks its participation in the exocytosis process (Molgo *et al.*, 1990; Meng *et al.*, 2009). BoNT-E, also targeting SNAP-25, cleaves a total of 26 residues from the C-terminus, completely blocking neuroexocytosis (Wang *et al.*, 2011; Lawrence *et al.*, 2012). However, BoNT-A/LC has a longer half-life of 3–4 months as compared with the very short half-life of BoNT-E (Keller *et al.*, 1999; Foran *et al.*, 2003). This therapeutic disadvantage was overcome by engineering the toxins to form a recombinant molecule providing relief in inflammatory pain models (Dolly *et al.*, 2009). A study reported that BoNT-A showed only limited inhibition of capsaicin-induced CGRP release as compared with K+ and bradykinin-evoked release. As capsaicin was found to require 180–197 residues of SNAP-25 for exocytosis (Meng *et al.*, 2009), BoNT-A/LC that targets only nine residues was unable to completely abolish the capsaicin-induced CGRP release. This property may contribute to the mixed findings observed in clinical trials for BoTox. However, recombinant chimeras of BoNT-A and BoNT-E successfully inhibited capsaicin-induced CGRP release from trigeminal sensory neurons, highlighting the potential of other serotypes (Meng *et al.*, 2009). Another study generated an active catalytic conjugate by coupling BoNT-A to lectin from *Erythrina crystagalli*. This derivative shows selectivity for nociceptive afferents neurons with little effect on neighbouring spinal neurons *in vitro* suggesting that the properties of BoNT can be tailored to selectively target the cells of interest (Duggan *et al.*, 2002). Furthermore, in rat TG cultures, BoNT-C1 incompletely cleaves both SNAP-25 and syntaxin I and partially inhibits Ca^{2+}-dependent CGRP release evoked by capsaicin, K^+ and bradykinin (Meng *et al.*, 2007). BoNT–C-sensitive syntaxins 2 and 3 (Schiavo *et al.*, 1995) were present in low levels in TG, potentially accounting for the minimal reduction in CGRP release. Treatment of cells with BoNT-D cleaved all the isoforms of synaptobrevin and completely abolished the capsaicin and K^+-induced CGRP release. BoNT-D also affected bradykinin induced release, however,

with a lower potency (Meng *et al.*, 2007). Effectiveness of BoNT-F in treating torticollis and oromandibular dystonia has been reported, however, with reduced length of benefit and side effects (Ludlow *et al.*, 1992). As the serotypes differ in catalytic targets potential duration of action, and importantly, terminal uptake and transport, future studies should focus on characterization of these properties and manipulate them for the development of pain pharmaceuticals targeted for chronic pain conditions in general and migraine (Borodic *et al.*, 2001; Pellett, 2012).

Concluding remarks

The triptans are currently considered to be the gold standard for the treatment of migraine as it is the only class of specific anti-migraine drugs in clinical use (Buzzi *et al.*, 1991; Nilsson *et al.*, 1997; Burstein and Jakubowski, 2004; Jakubowski *et al.*, 2005; Olesen and Ashina, 2011). Evidence suggests that, triptans act on $5HT_{1B/1D/1F}$ receptors on the peripheral and central terminals of the meningeal afferents blocking, respectively, peptide neurotransmitter release and thereby reducing the local neuro-inflammatory event in the meninges and attenuating the signals transmitted from primary afferents to second-order neurons (Burstein and Jakubowski, 2004; Burstein *et al.*, 2004; Ramachandran *et al.*, 2012). This property of triptans in migraine correlates with the mechanism of BoNT that is blocking the neurotransmitter release from primary afferents, although each acts by different targeting mechanisms. This property of BoNT if therapeutically modified by improving the pharmacological properties and reducing the unwanted side effects can be a promising approach towards the development of therapies for migraine as well as other debilitating craniofacial disorders including trigeminal neuralgia and temperomandibular joint syndrome.

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Conflict of interest

We declare that there is no conflict of financial interest with regard to our paper.

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