Synaptobrevin I mediates exocytosis of CGRP from sensory neurons and inhibition by botulinum toxins reflects their anti-nociceptive potential

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Accepted 21 June 2007
Journal of Cell Science 120, 2864-2874 Published by The Company of Biologists 2007
doi:10.1242/jcs.012211

Summary
Calcitonin-gene-related peptide (CGRP), a potent vasodilator that mediates inflammatory pain, is elevated in migraine; nevertheless, little is known about its release from sensory neurons. In this study, CGRP was found to occur in the majority of neurons from rat trigeminal ganglia, together with the three exocytic SNAREs [SNAP25, syntaxin 1 and the synaptobrevin (Sbr, also known as VAMP) isoforms] and synaptotagmin. Ca2+-dependent CGRP release was evoked with K+-depolarisation and, to lower levels, by capsaicin or bradykinin from neurons that contain the vanilloid receptor 1 and/or bradykinin receptor 2. Botulinum neurotoxin (BoNT) type A cleaved SNAP25 and inhibited release triggered by K+ > bradykinin >> capsaicin. Unlike BoNT type D, BoNT type B did not affect exocytosis, even though the neurons possess its receptor and Sbr II and Sbr III got proteolysed (I is resistant in rat) but, in mouse neurons, it additionally cleaved Sbr I and blocked transmitter release. Sbr I and II were found in CGRP-containing vesicles, and each was shown to separately form a SNARE complex. These new findings, together with punctate staining of Sbr I and CGRP in neurites, implicate isoform Sbr I in exocytosis from large dense-core vesicles together with SNAP25 (also, probably, syntaxin 1 because BoNT type C1 caused partial cleavage and inhibition); this differs from Sbr-II-dependent release of transmitters from small synaptic vesicles. Such use of particular Sbr isoform(s) by different neurons raises the functional implications for other cells previously unrecognised.

Key words: Sbr I/II, SNAP25, Syntaxin 1, Nociception, Trigeminal ganglion, Capsaicin, Bradykinin

Introduction
Pain of various kinds (nociceptive, neuropathic, inflammatory) pose substantial medical challenges, with 21% of worldwide adults suffering from persistent pain (WHO report 2004). A number of sensory nerve types are involved in pain propagation (Woolf, 2004). Fast-conducting, myelinated A-beta fibres mediate the synaptic release of transmitters such as excitatory amino acids from small clear synaptic vesicles (SCSVs), which cause cortical spreading depression, neuronal hyperexcitability and central sensitisation. However the slow transfer of signals via small thinly myelinated A-delta and unmyelinated C fibres elicit the secretion of calcitonin-gene-related peptide (CGRP), substance P, neurokinin A and glutamate (Silberstein and Aoki, 2003). CGRP and substance P serve as the main mediators of inflammatory pain; notably, these are released from large dense-core vesicles (LDCVs) at sites away from the active zones where exocytosis of SCSVs occurs (Kummer, 1992). Their more diffuse secretion enables cellular communication over wide areas, causing dilation of intracranial blood vessels and transmission of nociceptive signals from this vasculature to the central nervous system. Despite the importance of such peptides, limited molecular information is available on their release process compared with the in-depth knowledge on Ca2+-regulated, SNARE [soluble NSF (N-ethylmaleimide sensitive factor) attachment protein receptor]-dependent exocytosis of fast-acting neurotransmitters from SCSVs.

In this study, a molecular basis for CGRP release was examined in trigeminal ganglionic neurons (TGNs) because of their role as a pain relay centre and the fact that these neurons in culture provide a good model for such biochemical investigations (Baccaglini and Hogan, 1983). Moreover, subpopulations of these nociceptive neurons can be distinguished by selective stimulation with capsaicin or bradykinin, and the responsive cells stained for the requisite receptors. Capsaicin from chilli peppers causes a variety of inward currents and produces pain by acting on the vanilloid receptor type 1 (VR1), an integrator of inflammatory pain pathways (Caterina et al., 2000). Bradykinin, a mediator produced by tissue damage or inflammation, activates sensory neurons by acting on the bradykinin receptor type 2 (BR2), causing acute sensation of pain (Steranka et al., 1988). This investigation also exploited the unique abilities of botulinum neurotoxin (BoNT) serotypes to inhibit Ca2+-regulated exocytosis from LDCVs or SCSVs (Foran et al., 1995; Foran et al., 2003; McMahon et al., 1992). BoNT types A-G have molecular mass of ~150 kDa and consist of a binding and translocating heavy chain, and an enzymatic light chain, linked through a disulphide bond and non-covalent interactions. Each serotype binds with high affinity to distinct acceptors on susceptible motor nerves (Dolly et al.,
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1994); synaptic vesicle protein 2 has been identified as a putative binding component for BoNT type A (BoNT/A) (Dong et al., 2006; Mahrhold et al., 2006), and synaptotagmins I and II for BoNT types B and G, respectively (BoNT/B and BoNT/G, respectively) (Chai et al., 2006; Dong et al., 2003; Jin et al., 2006; Rummel et al., 2004). After gaining access into the neuronal cytosol, their metalloprotease activities selectively proteolyse and disable SNARE proteins which mediate vesicular transmitter release. SNAP25 (synaptosomal-associated protein, molecular mass 25 kDa), syntaxin 1A or syntaxin 1B that predominantly reside on the plasma membrane are cleaved by BoNT/A, BoNT/C1, BoNT/E and BoNT/C1, respectively. BoNT/B, BoNT/D, BoNT/F and BoNT/G act on the vesicular protein isoforms I, II and III of synaptobrevin [hereafter referred to as Sbr, but also known as vesicle-associated membrane protein (VAMP)] – Sbr I, Sbr II and Sbr III. Sbr I in rat is unusual in being resistant to BoNT/B owing to a mutation at the fission site (Foran et al., 2003; Schiavo et al., 1992; Yamasaki et al., 1994). Gaining data on the action of the toxins in sensory neurons should yield insights into the basis of toxin A therapy for certain types of pain (Gazerani et al., 2006; Gupta, 2005). Initial investigations using animal pain models have indicated that inhibition of the release of transmitters from nerves by BoNT/A in the periphery can attenuate peripheral sensitisation (Aoki, 2003).

Here, certain SNARE isoforms found in TGNs were demonstrated to contribute to the exocytosis of CGRP, using four serotypes of BoNT, thereby, extending the inhibition reported for the BoNT/A complex (Durham and Cady, 2004). BoNT/A cleaved SNAP25 and blocked K+-stimulated efflux of CGRP but gave only limited inhibition of that evoked by capsaicin. However, the effects of BoNT/B and BoNT/D on rat and mouse TGNs revealed that CGRP release can be mediated by Sbr I, whereas this isoform is not essential for SCSV exocytosis because Sbr II and Sbr III are sufficient (Foran et al., 1995; Foran et al., 2003; McMahon et al., 1992). Notably, Sbr I and Sbr II were localised in the same CGRP-containing vesicles and each formed distinct SNARE complexes. These collective findings highlight possible advantages of certain SNARE isoforms being used in exocytosis from different neurons, and raise the exciting prospect of tailoring toxins by protein engineering to act preferentially on pain-mediating peptidergic neurons.

Results

Sensory peptidergic neurons cultured from rat trigeminal ganglia contain exocytotic machinery and release CGRP in response to depolarisation or pain stimuli

Demonstration of the co-occurrence of CGRP, substance P, nociceptive receptors and SNAREs in TGNs.

Neurons in the trigeminal ganglia from neonatal rats or mice, dissociated and maintained in culture, provide a good source of sensory, peptide-containing cells (Durham and Russo, 1999; Eckert et al., 1997). Light microscopic examination after 7 days in vitro (DIV) (Fig. 1A,B) and staining with an antibody for neurofilament 200 (data not shown) demonstrated that the neurons were enriched (>95%). Most of the TGNs possessed bipolar or multi-polar neurite extensions, whereas some had a pseudo-unipolar shape. The characteristics of sensory neurons in brain sections (Guo et al., 1999) are reflected by the staining patterns observed for specific markers in these cultured cells. An antibody specific for VR1, which is responsive to capsaicin and occurs in peptidergic C fibres (Caterina et al., 2000), labelled the majority of the rat neurons (Fig. 1C); unipolar immunostaining of BR2 – a G-protein-coupled receptor known to occur in nociceptive neurons (Steranka et al., 1988) – was

![Fig. 1. Visualisation of the morphology and markers of sensory neurons in cultured TGNs. Samples were viewed in an inverted microscope by phase contrast (A,B) or in fluorescence mode (C-F), and by confocal microscopy (G-I). Bright-field views of neurons from (A) rat postnatal day 5 (P5) and (B) mouse (P5) after 7 DIV. Antibodies specific for VR1 (1:1000) stained the majority of rat TGNs (C); note that BR2 (1:500) was detected at one pole of VR1-positive cells (D); CGRP (1:500) and substance P (1:1000) appeared highly colocalized when visualised under low-magnification microscopy (E, F) but showed some distinct distribution in confocal microscopy (G-I). Fluorescently labelled secondary antibodies (goat anti-mouse Alexa Fluor-488, 1:200 or goat anti-rabbit Alexa Fluor-546, 1:200) were used. In some cases, the specimens were counterstained with DAPI. Bars, 20 µm (A-F) and 5 µm (G-I).]
seen in many of the VR1-positive cells (Fig. 1D). Isolectin B4 (IB4), which binds small nociceptive neurons in C fibres (Guo et al., 1999), stained a fraction of the cells (not shown). CGRP was visualised in most of the cultured cells (Fig. 1E) and co-occurred with substance P (Fig. 1F). Confocal microscopy confirmed their co-expression (Fig. 1G,H), although the merged picture highlights overlapping, but to some extent distinct, subcellular locations of CGRP and substance P (Fig. 1I). With regard to the molecular mechanism underlying their release, it was important to identify the SNAREs present in the sensory neurons. SNAP25 was visualised by confocal immuno-microscopy (Fig. 2A,C); lower magnification micrographs showed that the majority of neurons were stained (not shown). Labelling appeared on the membrane of cell bodies, axons (broad) and dendrites (fine); also, punctate staining was visible in the cytoplasm. In some cases, immunoreactivity seemed high in certain cells and low in others (Fig. 2A,C), regardless of the focal plane. Labelling of the other t-SNARE, syntaxin 1, gave a pattern (Fig. 2D,F) resembling that for SNAP25; isoforms 2 and 3 of syntaxin showed similar distributions (data not shown). The v-SNARE, Sbr II, was visualised predominantly in the cell body with ‘vesicular-like’ staining in the perinuclear area, as well as labelling of the extensive network of neurites (Fig. 2G,I). Importantly, dual labelling with the requisite antibodies demonstrated that CGRP occurs together with SNAP25, syntaxin 1 and Sbr II in TGNs (Fig. 2B,C,E and F,H,I).

**Ca**²⁺-dependent CGRP release from TGNs is elicited by **K**⁺-depolarisation, capsaicin or bradykinin but, to different extents

After culturing TGNs for 7 days, the amount of CGRP released under basal and stimulated conditions was quantified by enzyme immuno-assay. Elevated [**K**⁺] (60 mM proved optimal) gave a ~14 times increase over the basal level (in 3.5 mM **K**⁺/2.5 mM **Ca**²⁺), and this required **Ca**²⁺ (Fig. 3A). Consistent with TGNs containing the VR1 and BR2 proteins (Fig. 1C,D), capsaicin or bradykinin triggered **Ca**²⁺-dependent CGRP release to a maximum of 3.3 times or 3.9 times over basal, respectively (Fig. 3B,C), levels that accord with their

![Fig. 2. Microscopic demonstration of the presence of SNAREs and CGRP in rat TGNs.](image)

![Fig. 3. CGRP release evoked by **K**⁺, capsaicin or bradykinin from TGNs is **Ca**²⁺-dependent.](image)
Efficiencies in elevating the efflux of peptidergic transmitters in brain (Vedder and Otten, 1991).

Evidence for the involvement of SNAREs, especially Sbr I, in CGRP release evoked by different stimuli gained from differential inhibition by BoNT serotypes

Although BoNTs have proved instrumental in demonstrating that all three SNAREs are essential for Ca\(^{2+}\)-regulated exocytosis in a number of neuron types, this remains to be established for cultured TGNs. As stimulation of neurotransmitter release by K\(^+\), capsaicin and bradykinin relies on different signalling mechanisms, and in the two latter cases could occur in sub-populations possessing VR1 or BR2, determining their susceptibilities to BoNT serotypes is a prerequisite for the attractive prospect of engineering a variant that could be targeted and, thus, preferentially inhibit CGRP secretion in certain types of sensory neurons.

Truncation of SNAP25 by BoNT/A gives distinct inhibition of CGRP release evoked by three stimuli

TGNs were incubated overnight at 37°C with BoNT/A and Ca\(^{2+}\)-dependent CGRP secretion was measured in response to different stimuli, before the same cells were subjected to SDS-PAGE and western blotting of the SNAREs. SNAP25 was detected with an antibody exhibiting equal reactivity with the intact and the toxin-truncated SNARE (Fig. 4A). Increasing BoNT/A concentrations cleaved SNAP25 as reflected by appearance of a faster-migrating product, giving an EC\(_{50}\) value of 0.3 nM, derived from densitometric scanning of five replicate gels (Fig. 4B). Only trace amounts of the BoNT/A-resistant homologue SNAP23 could be visualised (Fig. 4C). K\(^+\)-evoked CGRP release was inhibited by BoNT/A with a concentration dependence identical to that for SNAP25 cleavage (Fig. 4B). This accords with the demonstrated presence by immunostaining of synaptic vesicle proteins 2A, B and C (Fig. 4D) on all the TGNs, which serve as the receptors for BoNT/A (Dong et al., 2006; Mahrhold et al., 2006). By contrast, it proved less potent in blocking exocytosis elicited by bradykinin (Fig. 4B). The CGRP release elicited by capsaicin was least susceptible to BoNT/A, with only ~15% reduction seen even at 100 nM toxin (Fig. 4B); this minimal sensitivity is not attributable to lack of the receptors on these particular neurons because synaptic vesicle proteins 2A, B and C were detected in VR1-positive cells (Fig. 4D; see Discussion). Such disparate BoNT/A susceptibilities of neuro-exocytosis triggered by various stimuli differ from the rank order observed for type D (see below).

Limited cleavage of syntaxin 1 and SNAP25 by BoNT/C1 partially blocks exocytosis induced by all the stimuli

Incubation of TGNs with BoNT/C1 (under the above outlined conditions for BoNT/A) resulted in partial cleavage of syntaxins 1A and/or 1B. This is indicated by the decreasing intensities on western blots stained with a monoclonal antibody

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**Fig. 4.** BoNT/A differentially inhibits Ca\(^{2+}\)-dependent CGRP release evoked from rat TGNs by three stimuli and cleaves SNAP25: receptors of BoNT/A occur on VR1-positive cells. TGNs were exposed to BoNT/A and release of CGRP was assayed over 30 minutes. Cells were then solubilised in SDS-sample buffer and equal volumes subjected to SDS-PAGE and western blotting, using an antibody that recognises intact and truncated SNAP25. The proportion of remaining substrate was calculated relative to an internal syntaxin control, using digital images of the gels. (A) Immunoblot showing the cleavage by the neurotoxin of SNAP25 but not syntaxin I. (B) Dose-response curve for BoNT/A-induced blockade of CGRP release evoked by 60 mM K\(^+\) ( ), which correlates with the percent of remaining SNAP25 ( ). Lesser extents of inhibition by BoNT/A were observed for release evoked by 0.1 μM bradykinin ( ) and, especially, 1 μM capsaicin ( ). Data plotted are the mean ± s.e.m.; n=5. (C) Western blot of TGNs visualised with antibodies specific against syntaxin I or SNAP23 ( ). (D) Representative micrographs demonstrating VR1 and SV2A, SV2B and SV2C in rat TGNs. Fluorescent images were obtained after labelling the cells with antibodies raised in guinea pig specific for VR 1 (1:1000) and in rabbit for SV2A or SV2B (1:1000) or in goat for SV2C (1:100). The controls were treated similarly except in the absence of primary antibodies but incubated with fluorescently labelled secondary IgGs against rabbit (as in Fig. 1) and guinea pig (goat anti-guinea-pig Alexa Fluor-488, 1:200) (1) or goat (donkey anti-goat Cy3, 1:800) and guinea pig (donkey anti-guinea-pig Cy2, 1:200) (2). Bars, 20 μm. Note that all the SV2 isoforms are present in VR1-positive neurons.
(mAb) against both isoforms of intact syntaxin 1 that were not resolved (Fig. 5A). Moreover, the toxin also truncated SNAP25 (Fig. 5A); the dose-response curves derived from analysis of several blots are similar for the partial cleavage of both substrates by the toxin (Fig. 5B). BoNT/C1 caused a minimal reduction in CGRP exocytosis with little discrimination between the stimuli used (Fig. 5C). Syntaxins 2, 3 and 4 were also detected in TGNs by western blotting but the two BoNT/C1-sensitive isoforms 2 and 3 (Schiavo et al., 1995) are apparently present in low amounts which probably underlies their lack of cleavage (Fig. 5A); this situation was accentuated by the limited uptake of the toxin, as reflected in the partial inhibition of CGRP release and minimal cleavage of both substrates (Fig. 5). The simplest interpretations of the results are that, BoNT/C1 enters a fraction of the responsive neurons or all the cells with poor efficiency; the contribution of its cleavage of syntaxin to CGRP inhibition could not be determined because SNAP25 also gets truncated.

BoNT/D cleaves all Sbr isoforms and inhibits CGRP release: the importance of Sbr I is unveiled by BoNT/B-induced blockade of exocytosis from mouse but not rat TGNs even though they possess its receptor. TGNs were treated with BoNT/D as above, before visualising Sbr isoforms on western blots with specific antibodies. Increasing concentrations of BoNT/D gave a progressive reduction in the staining for Sbr II or Sbr I and Sbr III bands (Fig. 6A), indicative of their cleavage. When Sbr II or Sbr I were individually labelled with isoform-specific antibodies, and the averaged intensities of each band normalised to an internal control (SNAP25), the resultant plots (Fig. 6B) demonstrated that BoNT/D cleaves Sbr I somewhat more effectively than Sbr II (EC50 values of 3.6 nM and 14.6 nM, respectively). Such treatment of cells with BoNT/D blocked K+- and capsaicin-evoked CGRP release (Fig. 6B,C), and the dose-dependence for K+-evoked CGRP release is very close to that for Sbr I cleavage. Likewise, CGRP exocytosis elicited by bradykinin was reduced by the toxin but with a lower potency (Fig. 6C). Basal efflux was also decreased (Fig. 6C inset), as found with this toxin in other neurons (Hua et al., 1998). This differential inhibition of evoked release may relate to distinct BoNT/D susceptibilities of sensory neuron populations that respond to capsaicin or bradykinin. It is noteworthy that the toxin-induced diminution of CGRP exocytosis is not due to
death of the TGNs, because no decrease in the cellular contents of CGRP (Fig. 6B inset), SNAP25 or syntaxin 1 (Fig. 6A) resulted from overnight exposure of the neurons to 10 nM BoNT/D.

It was necessary to ascertain whether cleavage of one or more isoforms of Sbr is required for the blockade of exocytosis. Involvement of Sbr II was addressed using BoNT/B because it does not cleave Sbr I in rat (Fig. 7A) (Schiavo et al., 1992). Incubation of the TGNs with BoNT/B decreased the intensity of Sbr II and Sbr III bands (Sbr I remained unchanged, consistent with it being resistant) that were detected with the broad-specificity HV-62 antibody (Fig. 7A).

Notably, the mAb specific for Sbr II showed a more complete cleavage of this isoform compared with HV-62 because the latter would have labelled the persisting BoNT/B-resistant Sbr I (Fig. 7A). Despite this extensive cleavage of Sbr II and III, BoNT/B failed to inhibit K⁺-evoked CGRP release despite the presence of its receptor; in mouse TGNs, Sbr I is also cleaved and exocytosis is blocked. TGNs were cultured from rat (left panels) and mouse (right panels) for 7 DIV before exposure to BoNT/B; enzyme immuno-assay of CGRP release and western blotting were carried out as described for Fig. 4. Values are the mean ± s.e.m., n=8. (A, C) Immuno-bLOTS showing the disappearance of Sbr isoforms [two (Sbr II and Sbr III) for rat and three (Sbr I, Sbr II and Sbr III) for mouse] relative to the internal standard (SNAP25) that remained unchanged. (B, D) Dose-response curves for inhibition of CGRP release evoked by 60 mM K⁺ (■), capsaicin (▲) and remaining Sbr II (▼), Sbr I and Sbr II (▲), and Sbr I (○). Inset in D illustrates the inhibition by BoNT/D of K⁺-evoked CGRP release from mouse TGNs, for comparison. (E, F) Representative fluorescence micrographs showing that the putative protein receptors of BoNT/B, synaptotagmin I and II, are present in CGRP-positive neurons; as well as CGRP and Sbr I are highly colocalized in cell bodies and their fine fibres in rat TGNs. Specimens were stained using (E) rabbit anti-CGRP (1:500) (and donkey anti-rabbit IgG Cy2, 1:200) and goat anti-synaptotagmin I/II (1:100) (and donkey anti-goat IgG Cy3, 1:800) or (F) rabbit anti-Sbr I (1:1000) (and donkey anti-rabbit IgG Cy2, 1:200) and mouse anti-CGRP (1:500) (and donkey anti-mouse IgG Cy3, 1:800). Notice the striking punctate co-staining in the two right hand panels in F. The control was treated in the absence of primary antibodies but incubated with secondary fluorescently labelled IgGs against goat and rabbit. Bars, 20 μm.

Fig. 7. BoNT/B proteolyses Sbr II and Sbr III in rat TGNs but does not reduce K⁺-evoked CGRP release despite the presence of its receptor; in mouse TGNs, Sbr I is also cleaved and exocytosis is blocked. TGNs were cultured from rat (left panels) and mouse (right panels) for 7 DIV before exposure to BoNT/B; enzyme immuno-assay of CGRP release and western blotting were carried out as described for Fig. 4. Values are the mean ± s.e.m., n=8. (A, C) Immuno-bLOTS showing the disappearance of Sbr isoforms [two (Sbr II and Sbr III) for rat and three (Sbr I, Sbr II and Sbr III) for mouse] relative to the internal standard (SNAP25) that remained unchanged. (B, D) Dose-response curves for inhibition of CGRP release evoked by 60 mM K⁺ (■), capsaicin (▲) and remaining Sbr II (▼), Sbr I and Sbr II (▲), and Sbr I (○). Inset in D illustrates the inhibition by BoNT/D of K⁺-evoked CGRP release from mouse TGNs, for comparison. (E, F) Representative fluorescence micrographs showing that the putative protein receptors of BoNT/B, synaptotagmin I and II, are present in CGRP-positive neurons; as well as CGRP and Sbr I are highly colocalized in cell bodies and their fine fibres in rat TGNs. Specimens were stained using (E) rabbit anti-CGRP (1:500) (and donkey anti-rabbit IgG Cy2, 1:200) and goat anti-synaptotagmin I/II (1:100) (and donkey anti-goat IgG Cy3, 1:800) or (F) rabbit anti-Sbr I (1:1000) (and donkey anti-rabbit IgG Cy2, 1:200) and mouse anti-CGRP (1:500) (and donkey anti-mouse IgG Cy3, 1:800). Notice the striking punctate co-staining in the two right hand panels in F. The control was treated in the absence of primary antibodies but incubated with secondary fluorescently labelled IgGs against goat and rabbit. Bars, 20 μm.
purity (Fig. 1B). Importantly, type B toxin caused a pronounced inhibition of K⁺-elicited CGRP release from the mouse TGNs (Fig. 7D), similar to that seen with BoNT/D although higher doses were required (Fig. 7D inset). Moreover, capsaicin-evoked CGRP release was inhibited. Accordingly, BoNT/B cleaved Sbr I in mouse TGNs as revealed by the concentration-dependent reduction in labelling with an antibody exclusively reactive with this isoform (Fig. 7C,D). Therefore, it is reasonable to deduce that CGRP release from LDCVs can use Sbr I.

CGRP-containing vesicles possess Sbr I and II, and each can form separate SNARE complexes

The BoNT/B-induced cleavage of Sbr II (and Sbr III) in rat TGNs failed to reduce K⁺-evoked CGRP exocytosis, whereas the additional cleavage of Sbr I in the mouse neurons resulted in blockade. This indicates that isoform Sbr I can mediate exocytosis from these peptidergic LDCVs. Evidence to support this hypothesis was obtained by selectively precipitating CGRP-containing vesicles from a TGN lysate with IgG that is known to be exclusively reactive with Sbr I (cf. Fig. 7A). The resultant vesicles were found to be enriched in CGRP (Fig. 8A) relative to the level observed in the control sample (prepared with non-immune IgG beads). Moreover, analysis of these isolated vesicles by SDS-PAGE and western blotting revealed the expected presence of Sbr I, which was absent from the control (Fig. 8B). Notably, the immuno-isolated vesicles also contained isoform II (Fig. 8B). Clearly, Sbr II co-exists with isoform I on CGRP-containing vesicles because both were found in the immuno-isolates irrespective of whether IgGs specific for Sbr II (Fig. 8) or Sbr I were used.

The functionality of Sbr I in these vesicles was confirmed by its demonstrated presence in a characteristic SDS-resistant SNARE complex separated from an extract of TGNs in non-denaturing detergent with IgG specific for Sbr I, coupled to protein A beads. The sedimented proteins were solubilised in SDS sample buffer and subjected to SDS-PAGE and western blotting (one aliquot of each sample was boiled before the analysis). Before boiling, antibodies specific for SNAP25 or syntaxin 1 stained a broad band corresponding to the main SNARE complexes (>100 kDa); much lower levels of these free constituents were present (Fig. 9). Boiling raised the proportions of dissociated SNAP25, syntaxin 1 and Sbr I; this corresponds to the decrease in the complex.
blotting, to contain SNAP25 plus Sbr I and II. Boiling these samples increased the amount of free SNAREs detected (data not shown). Sbr II also formed SNARE complexes, but were not co-isolated with those containing Sbr I (not shown). In conclusion, the collective evidence presented here shows, for the first time, that Sbr I can mediate regulated CGRP exocytosis from TGNs.

**Discussion**

Deciphering the molecular details of transmitter release from sensory neurons has been neglected so far, despite its great importance in understanding the mechanisms for the propagation of painful stimuli. Lack of progress is partly owing to difficulties in obtaining adequate quantities of pain-propagating cells for biochemical work, a problem overcome by using cultured TGNs. This model was validated by showing that the TGNs retain the histochemical properties of sensory neurons in situ (Baccaglini and Hogan, 1983; Guo et al., 1999; Price et al., 2005), and release peptidergic transmitters in a Ca\(^{2+}\)-dependent manner in response to different stimuli. However, the proportions of cultured TGNs found to contain VR1 and CGRP exceed those in the ganglia, a difference attributed to their upregulation by added NGF (Price et al., 2005). Virtually all of the neurons containing CGRP also possessed substance P but the confocal micrographs revealed that these two peptides do not wholly reside in the same vesicles; this accords with CGRP having been found only in LDCVs, whereas substance P can also exist in smaller vesicles from spinal cord sensory neurons (De Biasi and Rustioni, 1988; O’Connor and van der Kooy, 1988).

A prime objective of this investigation was to identify the SNAREs underlying the release of CGRP because of its pivotal role as a pain mediator. For the first time, all three SNAREs were visualised in the sensory neurons by confocal microscopy with each displaying some characteristic distribution features. Most importantly, a co-occurrence of CGRP with each of the exocytotic proteins was unveiled although, again, confocal images revealed similarities as well as differences in their subcellular locations. On one hand, Sbr I and CGRP exhibited a notable degree of colocalisation; in particular, their punctuate subcellular locations. On one hand, Sbr I and CGRP exhibited a notable degree of colocalisation; in particular, their punctuate

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SNAREs (Sbr I, II and III together with SNAP25 and syntaxin 1; data not shown). In rat, where Sbr I is non-susceptible to BoNT/B, this toxin can nevertheless block exocytosis from central neurons of several transmitters (e.g. glutamate, γ-aminobutyrate, dopamine) (Bergquist et al., 2002; Foran et al., 2003; Schoch et al., 2001; Verderio et al., 2004). These published data indicate that the majority of Ca2+-dependent exocytosis from SCVs in these neurons (at least 80% of published data indicate that the majority of Ca2+-dependent BoNT/B, this toxin can nevertheless block exocytosis from other varieties of secretory cells, and pinpointing the inherent processes and/or their fine control in eukaryotic cells. Sbr II preferentially in exocytosis from different vesicle types in 2007). Undoubtedly, identifying SNARE isoforms used contribute to the neurological defects found in mice with a lack of statistically significant inhibition of substance P (data not shown). Furthermore, our proposal is supported by peptide exocytosis from LDCVs in sensory neurons might be a characteristic of sensory neurons because studies on the stimulation methods used for CGRP and, thus, it was not possible to investigate the use of Sbr I. Utilisation of Sbr I may be a characteristic of sensory neurons because studies on rat-derived preparations, in which this isoform is BoNT/B-resistant, have shown that cleavage of Sbr II and Sbr III blocks exocytosis of catecholamines from large dense-core granules as well as the release of noradrenaline from large, dense core-like vesicles in cerebrocortical synaptosomes and PC12 cells (Lonneth et al., 1991; McMahon et al., 1992). Although this is the first demonstration of Sbr I mediating regulated exocytosis in TGNs, it is noteworthy that BoNT/B reduces dopamine release from LDCVs in rat brain nerve terminals but not the somatodendritic release (Bergquist et al., 2002). Considering this, together with our direct evidence, leads to the deduction that Sbr I participates in toxin-B-resistant release from rat TGNs at sites remote from the active zones in the presynaptic membrane where CGRP exocytosis has been shown to occur (Bernardini et al., 2004). Indeed, as BoNT/B was completely ineffective in reducing CGRP release (despite cleaving all Sbr II and Sbr III in rat TGNs), it must largely arise from vesicles that contain Sbr I. Based on all these consistent findings, it is apparent that Sbr I can support this special type of exocytosis that allows the released CGRP to activate its receptor on blood vessels in the vicinity (Edvinsson, 2004). This also seems to apply to other sensory neurons because K+-evoked CGRP release from mouse (but not rat) dorsal root ganglionic neurons is blocked by BoNT/B (data not shown). Furthermore, our proposal is supported by the lack of statistically significant inhibition of substance P release by BoNT/B (unlike other serotypes) in cultured neurons from dorsal root ganglia of embryonic rats (Welch et al., 2000). In fact, the demonstrated involvement of Sbr I in peptide exocytosis from LDCVs in sensory neurons might contribute to the neurological defects found in mice with a SbrI-null mutation that die soon after birth (Nystuen et al., 2007). Undoubtedly, identifying SNARE isoforms used preferentially in exocytosis from different vesicle types in other varieties of secretory cells, and pinpointing the inherent functional advantages, should shed light on subtle dissimilarities that are likely to exist in the exocytotic processes and/or their fine control in eukaryotic cells.

Materials and Methods

Materials
Leibowitz’s L15 and Ham’s F12 culture medium, foetal bovine serum (FBS), Ca2+- and Mg2+-free Hank’s balanced salt solution (CMF-HBSS), Dulbecco’s phosphate buffered saline (lacking Mg2+ and Ca2+), antibiotics and mouse nerve growth factor (NGF-2.5S) were purchased from GibcoBRL. Disperse II, collagenase I and DNase I were supplied by Roche Inc. Cytosine-β-D-arabinofuranoside, capsaicin, bradykinin, protease inhibitor cocktail, poly-L-lysine, laminin, Trypspean, trypsin inhibitor, rabbit non-immune IgG, rabbit anti-CGRP antibody, mAbs against syntaxin 1A and 1B (HPC1) and CGRP (CD8) and protein A agarose were obtained from Sigma Aldrich. CGRP enzyme immuno-assay kit was bought from SPI-BIO. A mAb specific for SNAP25 (SMI-81) was from Sternberger Monoclonals, Inc., whereas Sbr II mAb (CL 69.1) and rabbit antiserum against SNAP25, Sbr I, Sbr II, or SV2A or B and syntaxin 2 or 3 were purchased from Synaptic Systems. Goat anti-synaptogamin I and II and SV2C were bought from Santa Cruz Biotechnology, Inc. mAbs specific for BR2 and substance P were obtained from Fitzgerald and Abcam, respectively. Polyclonal IgGs reactive against Sbr I, Sbr II and Sbr III (anti-HV62 reactive with a 62-mer peptide residues 32-94 of human Sbr II) were generated and purified (Foran et al., 1993), (diluted in BR-HBS to the required concentrations. In all cases, the final wash. Immuno-fluorescence pictures were taken with an inverted confocal

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measuring basal efflux. Quantification of Ca²⁺-independent basal release and that evoked by K⁺. Capsaicin or bradykinin was carried out as above except for Ca²⁺ being replaced by 2 mM EGTA. The values obtained for each were subtracted from the respective controls to yield the Ca²⁺-dependent component; expression of the evoked release relative to that for basal efflux gave the increment for each stimulus. To determine the amounts of CGRP released, 0.1 ml of sample was added to 96-well plates coated with a mAb against CGRP, and enzyme immuno-assay was performed following the instructions for the kit. Total CGRP content was determined on randomly selected wells from each tissue, as described elsewhere (Purkiss et al., 2000).

Treatment of TGNs with BoNTs: monitoring of effects on CGRP release and SNARE cleavage

After 7 DIV, fresh medium, or medium containing BoNT/A, BoNT/B, BoNT/C or BoNT/D was added to TGNs for 24 hours at 37°C, at the concentrations specified. After removal of the unbound toxin and washing twice with 1 ml of toxin-free BRB buffer, HBS buffer, Ca²⁺-dependent basal release and that evoked by 60 mM K⁺, 1 μM capsaicin or 0.1 μM bradykinin was measured as above. No-toxin-treated samples were treated similarly. Stimulated release was calculated as before; expression of the resultant values for BoNT-treated samples relative to those of controls gave the remaining CGRP release in percent. Cells in each well were then lysed by 0.2 ml of 2×SDS sample buffer, heated for 5 minutes at 95°C and separated by SDS electrophoresis, using pre-cast NuPAGE 12% Bis-Tris gels (Foran et al., 2003). The remaining CGRP release was measured by ELISA (12% Bis-Tris gels (Foran et al., 2003)).


