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

Jianghui Meng*, Jiafu Wang*, Gary Lawrence and J. Oliver Dolly‡

International Centre for Neurotherapeutics, Dublin City University, Glasnevin, Dublin 9, Ireland

*These authors contributed equally to this work

‡Author for correspondence (e-mail: oliver.dolly@dcu.ie)

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 exocytotic SNAREs [SNAP25, syntaxin 1 and the synaptobrevin (Sbr, also known as VAMP) isoforms] and synaptotagmin. Ca^{2+}-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 I, 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 hyper-excitability 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 Ca^{2+}-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 Ca^{2+}-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-convalent interactions. Each serotype binds with high affinity to distinct acceptors on susceptible motor nerves (Dolly et al.,

Journal of Cell Science

Journal of Cell Science

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 immuno-staining of BR2 – a G-protein-coupled receptor known to occur in nociceptive neurons (Steranka et al., 1988) – was

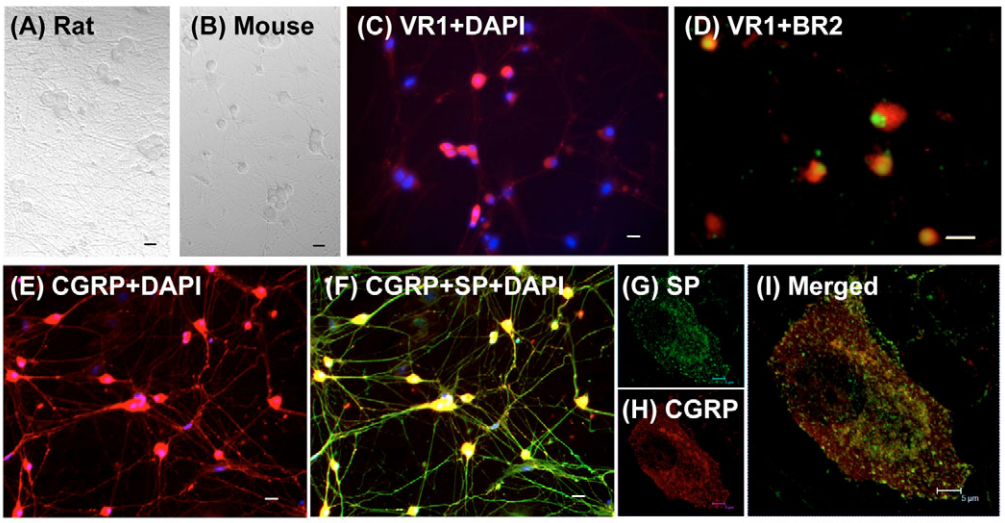

Fig. 1. Visualisation of the morphology and markers of sensory neurons in cultured TGNs. Samples were viewed in an inverted microscope by phase contrast (A,B) or in fluorescence mode (C-F), and by confocal microscopy (G-I). Bright-field views of neurons from (A) rat postnatal day 5 (P5) and (B) mouse (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).

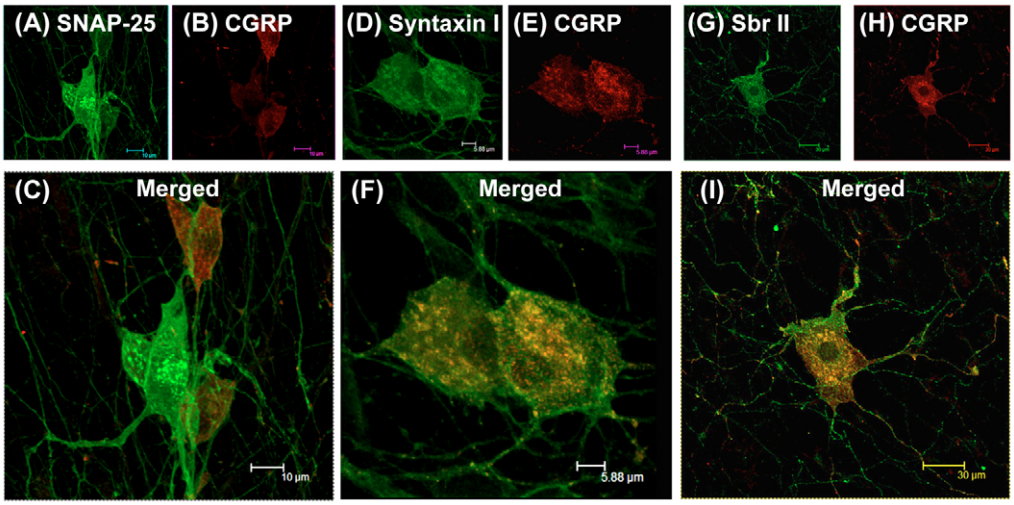

Fig. 2. Microscopic demonstration of the presence of SNAREs and CGRP in rat TGNs. Cells were grown on coverslips for 7 DIV, fixed and permeabilised prior to labelling with specific primary and secondary antibodies (as described for Fig. 1). Confocal images after labelling with antibodies against SNAP25 (1:500), CGRP (1:500), syntaxin I (1:500) and Sbr II (1:1000) demonstrated punctate staining of these proteins and the neuropeptide in cell bodies and/or their processes. Merged images are shown below each pair of singly stained micrographs.

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

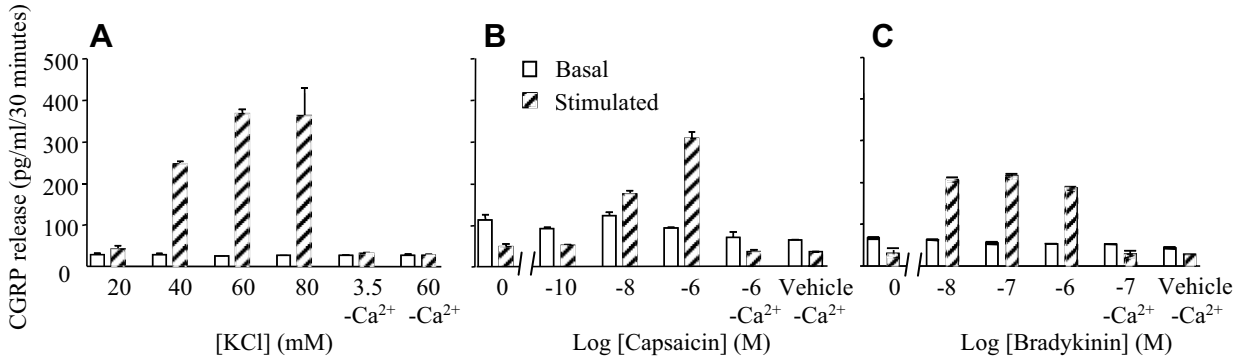

Fig. 3. CGRP release evoked by K^+, capsaicin or bradykinin from TGNs is Ca^{2+}-dependent. (A-C) After 7 DIV, neurons were incubated for 30 minutes at 37°C in BR-HBS alone or with vehicle added (see Materials and Methods) for measurement of basal release of CGRP (white bars), and then incubated in BR-HBS in the presence of varying concentrations of (A) K^+, (B) capsaicin or (C) bradykinin to determine stimulated release of CGRP (hatched bars). Release stimulated by K^+ (60 mM), capsaicin (1 μM) or bradykinin (0.1 μM) represents 30, 20 and 15% of the total content, respectively. Likewise, Ca^{2+}-independent release was determined, except in Ca^{2+}-free BR-HBS containing 2 mM EGTA. Buffer in which cells were bathed was carefully removed and the concentration of CGRP determined by enzyme immuno-assay (mean ± s.e.m., n=4). Note that the basal efflux is higher when either 0.1% ethanol or dimethyl sulphoxide was included (Fig. 3B,C) than when absent (Fig. 3A). Note that each stimulus gave significant increments of evoked release over their basal level.

Journal of Cell Science

Journal of Cell Science

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

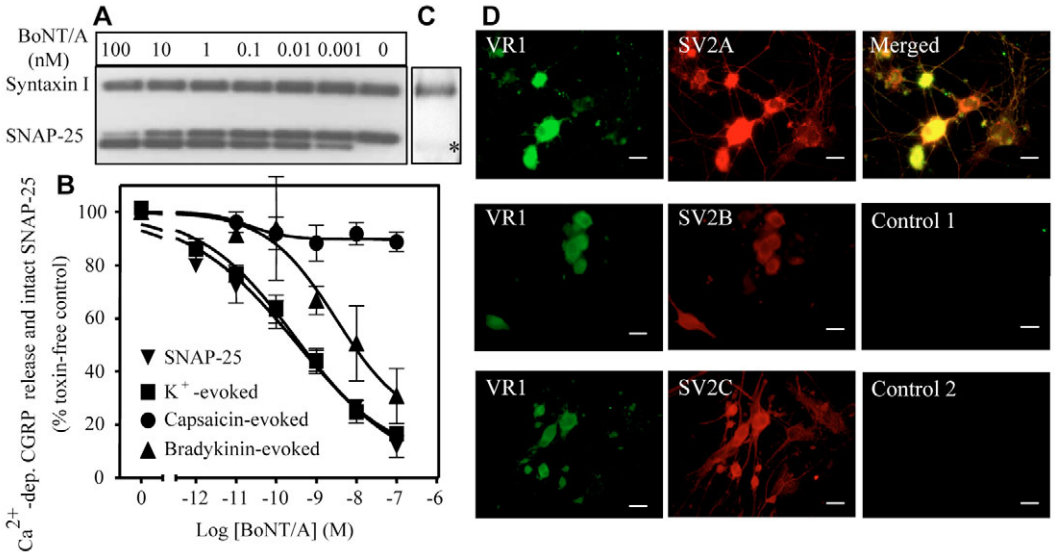

Fig. 4. BoNT/A differentially inhibits Ca^{2+}-dependent CGRP release evoked from rat TGNs by three stimuli and cleaves SNAP25: receptors of BoNT/A occur on VR1-positive cells. TGNs were exposed to BoNT/A and release of CGRP was assayed over 30 minutes. Cells were then solubilised in SDS-sample buffer 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.

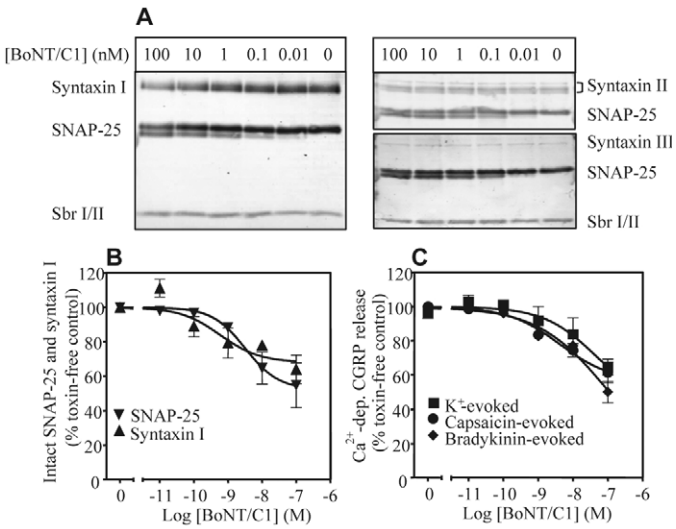

Fig. 5. BoNT/C1 incompletely cleaves SNAP25 and syntaxin I, and partially inhibits Ca^{2+}-dependent CGRP release evoked by three stimuli. TGNs were treated with BoNT/C1, and release of CGRP was assayed and western blotting performed. Results were calculated as described for Fig. 4 relative to Sbr I and Sbr II control. (A) Partial cleavage of SNAP25 by BoNT/C1, visualised with the IgG used in Fig. 4. Decrease in syntaxin I was revealed with an antibody only reactive with this intact SNARE. Syntaxin II and, especially, syntaxin III, proved difficult to quantify and, thus, cleavage by toxin was not detectable. (B) Dose-response curves for BoNT/C1-induced cleavage of SNAP25 (▼) and syntaxin I (▲). (C) BoNT/C partially inhibits CGRP release evoked by 60 mM K^+ (■), 0.1 μM bradykinin (◆) or 1 μM capsaicin (●). Data plotted are the mean ± s.e.m.; $n \geqslant 3$.

(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 (EC_{50} 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

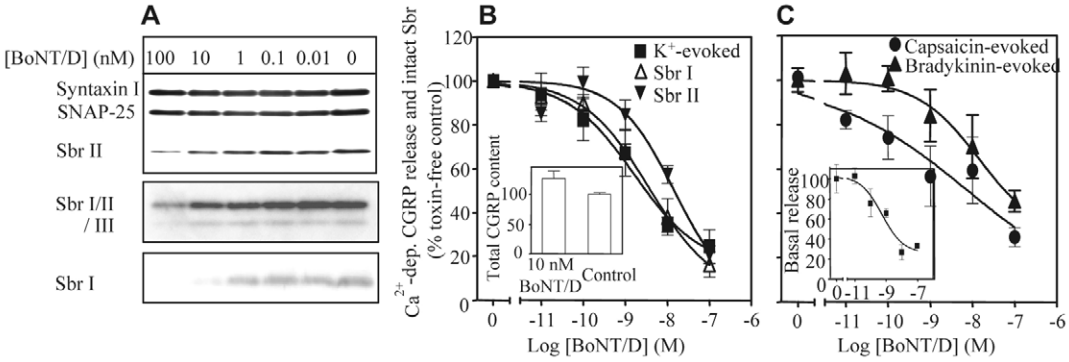

Fig. 6. BoNT/D blocks evoked Ca^{2+}-dependent CGRP release and cleaves three Sbr isoforms. The amount of Ca^{2+}-dependent basal and evoked release of CGRP for each stimulus was measured in cells treated with BoNT/D, as described for Fig. 4. (A) Three Sbr isoforms were detected using antibodies against Sbr I or Sbr II, or Sbr I, Srb II and Srb III together (HV-62); Srb I and Srb II co-migrate. (B) Dose-response curves for the remaining intact Sbr II (▼) and Sbr I (Δ), and for inhibition of release evoked by 60 mM K^+ (■); inset shows the amount of CGRP in TGNs after overnight incubation with or without BoNT/D. (C) Dose-response curves for BoNT/D-induced inhibition of CGRP release evoked by 1 μM capsaicin (●) or 0.1 μM bradykinin (▲). Inset shows the reduction of basal release by BoNT/D. The values are the mean ± s.e.m.; n=8.

Journal of Cell Science

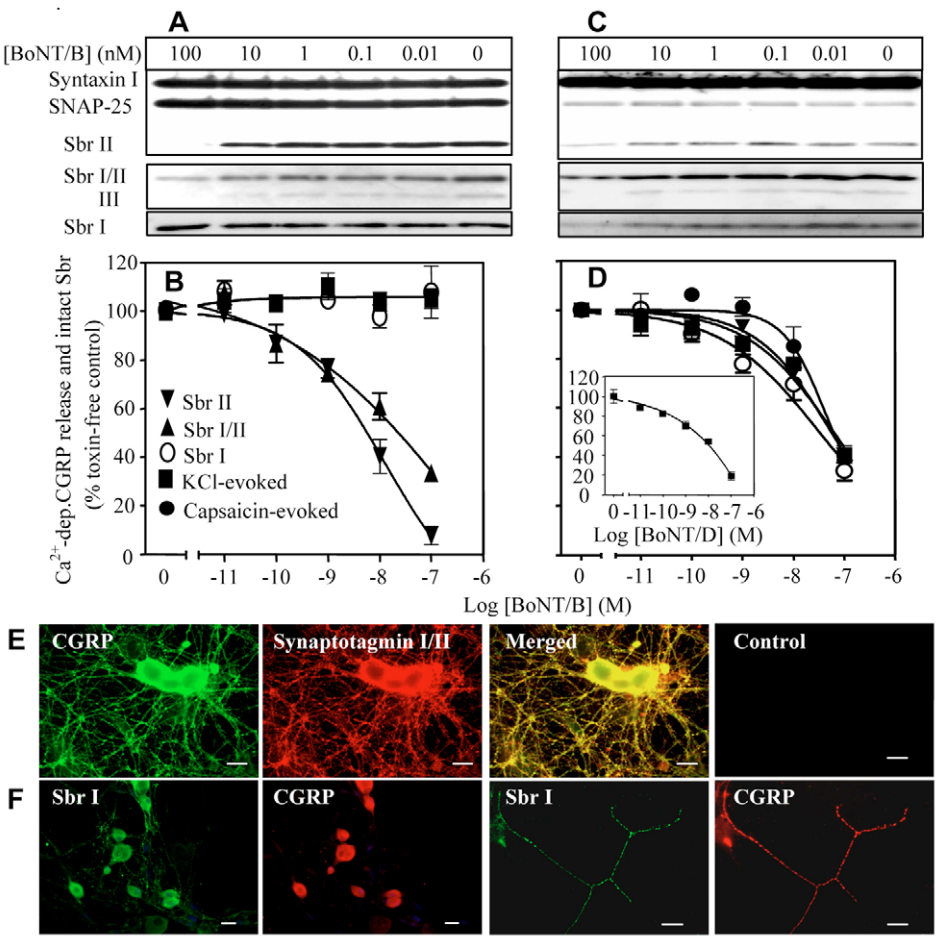

Fig. 7. BoNT/B proteolyses Sbr II and SbrIII in rat TGNs but does not reduce K^+-evoked CGRP release despite the presence of its receptor; in mouse TGNs, Sbr I is also cleaved and exocytosis is blocked. TGNs were cultured from rat (left panels) and mouse (right panels) for 7 DIV before exposure to BoNT/B; 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 (Srb II and Srb III) for rat and three (Srb I, Srb II and Srb 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 Srb 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.

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 (Fig. 7B). The possibility of BoNT/B-resistant CGRP release occurring from a sub-population of neurons that are unable to bind and internalise this toxin seems unlikely because complete cleavage of Sbr II is observed. Moreover, synaptotagmins I and II, the acceptors of the toxin, were detected in all TGNs containing CGRP (Fig. 7E). Thus, the lack of blockade of CGRP release by BoNT/B raised the question whether the insensitive Sbr isoform I is invoved. This idea was enforced by the co-occurrence of Sbr I and CGRP on the cell bodies and, particularly, their striking punctate location on the neurites (Fig. 7F). As murine Sbr I is susceptible to BoNT/B, experiments were repeated with TGNs prepared from P5 mice; the resultant cultured cells displayed similar morphology and

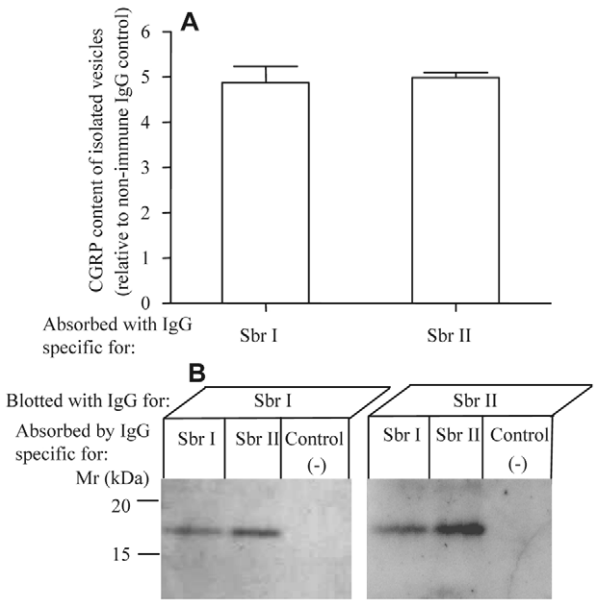

Fig. 8. Immuno-absorption of vesicles from TGNs by antibodies against Sbr I and Sbr II. Equal amounts of the total membrane fraction from lysed TGNs were incubated overnight at 4°C with protein A beads coupled to IgGs specific for Sbr I or SbrII, or rabbit non-immune IgG (control). After extensive washing, equivalent aliquots of beads were sedimented and pellets dissolved in SDS sample buffer for SDS-PAGE and western blotting. Alternatively, 2 M acetic acid with 0.1% TFA was added to replicate samples for CGRP determination. (A) CGRP content (± s.e.m.) measured in two separate preparations show the large enrichment in vesicles obtained using beads containing Sbr I or Sbr II relative to the control. (B) Western blots of the two vesicle preparations and the control, using antibodies specific for Sbr I or Sbr II.

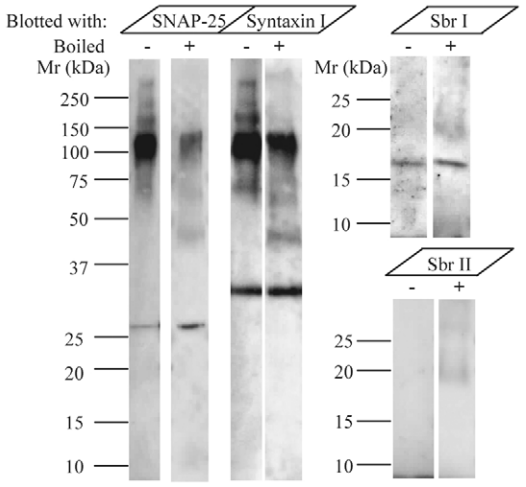

Fig. 9. Sbr I complexes with SNAP25 and syntaxin 1 in TGNs. The pelleted cells were extracted in 1 ml of buffer containing 1% (v/v) Triton X-100 (see Materials and Methods) for 1 hour at 4°C, followed by centrifugation. The supernatant was incubated at 4°C overnight with anti-Sbr I IgG coupled to protein A agarose. After sedimentation and extensive washing with the extraction buffer, beads were suspended in SDS sample buffer for SDS-PAGE (with and without boiling the samples for 10 minutes) under non-reducing conditions, followed by western blotting using antibodies specific for each SNARE. Only the lower halves of the gels blotted for Sbr I or Sbr II are shown because of excessive staining of the rabbit IgG that overlapped the SNARE complex. Note that boiling raised the proportions of dissociated SNAP25, syntaxin I and Sbr I; this corresponds to the decrease in the complex.

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 and syntaxin 1 while decreasing the relative amounts of the complex, although amounts of the complex remained substantial (Fig. 9). As expected, some free Sbr I was found in the unheated sample but its level increased after boiling (Fig. 9); this accords with the corresponding decreased intensity of the main immuno-reactive protein complexes (>100 kDa) upon boiling (not shown). Notably, Sbr II was not detectable in the non-boiled or boiled samples (Fig. 9). Likewise, SNARE complexes were successfully precipitated from TGN lysate extract with antibodies specific for syntaxin 2 or 3 and shown, by western

Journal of Cell Science

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 being 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 staining of neurites is striking. On the other hand, the intensity of the staining for SNAP25 and CGRP in some adjacent neurons was different. It proved possible to establish the requirement for exocytosis of each SNARE, or a particular isoform, by using BoNTs. The evidence obtained for SNAP25 being essential for K^+-evoked CGRP release is clear-cut because BoNT/A caused near-complete inhibition (~90%) and an equivalent extent of cleavage. This accords with its putative acceptors — synaptic vesicle proteins 2A, B and C (Dong et al., 2006; Mahrhold et al., 2006) — being found in all of the TGNs. By contrast, capsaicin-elicited exocytosis of CGRP from TGNs proved largely non-susceptible to BoNT/A; this corresponds to the minimal inhibition of substance P release by this toxin from rat dorsal root ganglionic neurons (Purkiss et al., 2000). As VR1-positive cells represent a main fraction of the SV2-expressing TGNs, BoNT/A should be able to enter the capsaicin-responsive neurons. Although rat SNAP23 is non-susceptible to BoNT/A, only low levels were detected in TGNs; therefore, this seems inadequate to explain the lack of inhibition of CGRP exocytosis from capsaicin-sensitive neurons. Thus, the observed lower efficiency of BoNT/A in blocking capsaicin-evoked release of CGRP might be due to the known ability of elevated intra-neuronal Ca^{2+} concentration to partially reverse BoNT/A-induced inhibition (Sakaba et al., 2005; Verderio et al., 2004); this could result from a large, capsaicin-triggered Ca^{2+} influx through the non-selective cation channel of the VR1 receptor (Caterina et al., 2000). Contrary to the consistent outcome of this study and that of (Purkiss et al., 2000), there is a single report that a haemagglutinin–toxin complex of type A blocks capsaicin-evoked CGRP release from TGNs (Durham and Cady, 2004); this different finding might be because of the use of such a large (~900 kDa), multi-component complex and/or the measurement being made after 1 DIV rather than 7 DIV, as employed in our study. Unlike SNAP25, determination of the contribution of syntaxin 1 to exocytosis from TGNs was not possible because a near-equal cleavage of syntaxin 1 and SNAP25 by BoNT/C1 precluded assessment of their individual contributions to its partial inhibition of CGRP release.

Evidence for Sbr being essential for CGRP release evoked by K^+, capsaicin or bradykinin was provided by the inhibition of each by BoNT/D, together with cleavage of Sbr I, II and III. Moreover, basal efflux seemed to be reduced, which accords with its ability to reduce spontaneous release at crayfish motor synapses (Hua et al., 1998). As this neurotoxin cleaved all three isoforms of Sbr, assessment of their relative contributions to exocytosis necessitated additional experiments with BoNT/B, which is unable to cleave Sbr I in rat (Fig. 7A). Despite near-complete cleavage of Sbr II (and Sbr III) by 100 nM BoNT/B, its observed inability to cause any detectable inhibition of K^+-evoked CGRP release indicated that these two isoforms are either not essential or their roles can be taken by Sbr I. The possibility of BoNT/B-resistant CGRP release occurring from a sub-population of neurons unable to internalise this toxin was excluded by the complete cleavage of Sbr II and, especially, the demonstrated presence of synaptotagmin I and II in all of the CGRP-positive neurons, because these proteins act as acceptors for BoNT/B (Chai et al., 2006; Dong et al., 2003; Jin et al., 2006). Participation of Sbr I in CGRP exocytosis is an interesting and new feature of TGNs, also seen with K^+-evoked Ca^{2+}-dependent release of substance P from rat TGNs, which proved non-susceptible to BoNT/B but were inhibited by BoNT/D, BoNT/C1 or BoNT/A (data not shown). Importantly, BoNT/B did cleave Sbr I and blocked exocytosis of CGRP from mouse TGNs; accordingly, preliminary observations from initial experiments on knock-down of Sbr I indicate a substantial reduction in CGRP release (our unpublished data). Thus, it is reasonable to conclude that isoform Sbr I can mediate the release of CGRP from LDCVs – at least in sensory neurons. This is supported by co-immunoprecipitation experiments on TGNs showing that Sbr I occurs in SNARE complexes that contain SNAP25 and syntaxin 1. A large proportion of these complexes proved resistant to SDS-denaturation and were shown to be >100 kDa on SDS-PAGE unless the samples were boiled, in which case the signals for the individual SNARE components were increased due to complex disassembly. These features are characteristic of neuronal SNARE complexes (Hayashi et al., 1994; Otto et al., 1997) Moreover, CGRP-containing vesicles that were immuno-isolated by SbrI-specific IgGs possessed Sbr I and II. Accordingly, LDCVs isolated by density-gradient centrifugation were shown to contain CGRP as well as the

Journal of Cell Science

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-amino-butyrate, 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 Ca^{2+}-dependent exocytosis from SCSVs in these neurons (at least 80% of glutamate release from rat cerebellar neurons) requires Sbr II and/or Sbr III; thus, it seems Sbr I is not essential for the latter. Likewise, a major role for Sbr II in exocytosis from SCSVs (Takamori et al., 2006) accords with its predominance therein and correlates with a 100-times reduction in Ca^{2+}-triggered fast transmitter release in knockout mice that lack this isoform (Schoch et al., 2001). Hence, the ability of Sbr I to support exocytosis in TGNs is not replicated in all neuron types, possibly because of the reported differential expression of Sbr I and Sbr II (Aguado et al., 1999; Trimble et al., 1990). Unfortunately, the release of detectable levels of classical SCSV transmitters could not be elicited from the TGNs with 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 (Lomneth 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), Ca^{2+}- and Mg^{2+}-free Hank's balanced salt solution (CMF-HBSS), Dulbecco's phosphate buffered saline (lacking Mg^{2+} and Ca^{2+}), antibiotics and mouse nerve growth factor (NGF-2.5S) were purchased from GibcoBRL. Dispase II, collagenase I and DNase I were supplied by Roche Inc. Cytosine-β-D-arabinofuranoside, capsaicin, bradykinin, protease inhibitor cocktail, poly-L-lysine, laminin, Trypzean, 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 antisera against SNAP23, Sbr I, Sbr II, or SV2A or B and syntaxin 2 or 3 were purchased from Synaptic Systems. Goat anti-synaptotagmin I and II and SV2C were bought from Santa Cruz Biotechnology. 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 affinity purified (Foran et al., 2003). Guinea pig and rabbit antisera against rat VR1 were supplied by Chemicon and Neuromics, respectively. Anti-species secondary antibodies fluorescently labelled or conjugated to horseradish peroxidase were obtained from Invitrogen and Jackson ImmunoResearch or Sigma Aldrich, respectively. ECL reagents were from Amersham. Homogeneous, fully-active di-chain BoNT/A was obtained from B. DasGupta (Food Research Institute, University of Wisconsin, MA). Metabiologics Inc. supplied BoNT/B single-chain, which was converted (>95%) to the di-chain form by nicking with Trypzean (200 μg of toxin and 2 μg of enzyme in 0.2 ml 50 mM HEPES with 50 mM NaCl, pH 7.4) at 27°C for 40 minutes, followed by addition of soyabean trypsin inhibitor. BoNT/C1 and BoNT/D (obtained from Metabiologics Inc.) contained ~60% di-chain form.

Isolation and culturing of rat and mouse TGNs

The procedures described in Eckert et al. (Eckert et al., 1997) were used with a number of modifications. Briefly, TGs were dissected from postnatal day 5 (P5) female Wistar rats or Tyler's Ordinary mice after being deeply-anesthetised with intraperitoneal injection of Dolethal (50 mg/kg body weight). The tissue was placed in ice-cold L15 medium, washed twice in ice-cold sterile CMF-HBSS before centrifugation at 170 ***g*** for 1 minute. After chopping into small pieces and passing through 10-ml Falcon pipettes pre-coated with L15 medium, the tissue was incubated while shaking at 37°C for 30 minutes in a 1:1 mixture of CMF-HBSS (containing 2.4 U/ml dispase II) and collagenase I (1 mg/ml). The suspension was then gently triturated through 10-ml Falcon pipettes pre-coated with L15 medium until cloudy, before adding 1 mg/ml DNase I for 15 minutes. Following centrifugation at 170 ***g*** for 5 minutes, the pellet was suspended and washed thrice in culture medium [Ham's F12 solution containing 10% (v/v) heat-inactivated FBS, 100 IU/ml penicillin and 100 μg/ml streptomycin]. Cells were seeded onto 24-well plates precoated with poly-L-lysine (0.1 mg/ml) and laminin (20 μg/ml) in F12 medium supplemented with NGF (50 ng/ml) and maintained in a CO_2 incubator at 37°C. After 24 hours and every other day thereafter, the culture supernatant was replaced with medium containing the anti-mitotic agent cytosine-β-D-arabinofuranoside (10 μM).

Cytochemical staining and microscopic recording of images

TGNs, cultured on coverslips coated with poly-L-lysine- and laminin, were washed thrice with Dulbecco's phosphate buffer saline (lacking $Mg^{2+}$and Ca^{2+}), fixed for 20 minutes with 3.7% paraformaldehyde in the latter buffer. The cells were then washed with PBS three times, followed by permeabilisation for 5 minutes with 0.2% Triton X-100 in PBS before blocking with 1% bovine serum albumin in PBS for 1 hour. Primary antibodies were applied in the same solution and left overnight at 4°C; after extensive washing, fluorescent secondary antibodies were added for 1 hour at room temperature. In some cases, counterstaining of nuclei was carried out with 4′,6′-diamidino-2-phenylindole (DAPI) (1 μg/ml in water) added before the final wash. Immuno-fluorescence pictures were taken with an inverted confocal (Leica Dmire 2) or an Olympus IX71 microscope equipped with a CCD camera. Images were analysed using Leica confocal software and Image-Pro Plus 5.1, respectively. Omission of primary antibody from the staining procedure gave the fluorescence background for secondary antibody; signals above this very low level were used.

Quantification of CGRP release and total cellular content

At 7 days in vitro (DIV), medium was gently aspirated from the TGNs, 0.5 ml of basal release buffer (BR-HBS, mM; 22.5 HEPES, 135 NaCl, 3.5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 3.3 glucose and 0.1% BSA, pH 7.4) was added into each well, followed by a 30-minute incubation at 37°C. After brief centrifugation at 4°C, the supernatants were stored at –20°C until subjected to enzyme immuno-assay. Ca^{2+}-dependent CGRP release stimulated with 60 mM KCl in HBS (isotonically balanced with NaCl) was performed in the same way. For stimulation with capsaicin or bradykinin, stocks (1 mM) were prepared in ethanol or dimethyl sulphoxide, respectively, and diluted in BR-HBS to the required concentrations. In all cases, the final concentration of vehicle was kept at 0.1%; this was also included in BR-HBS when

Journal of Cell Science

measuring basal efflux. Quantification of Ca^{2+}-independent basal release and that evoked by K^+, capsaicin or bradykinin was carried out as above except for Ca^{2+} being replaced by 2 mM EGTA. The values obtained for each were subtracted from the requisite totals to yield the Ca^{2+}-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 were added to 96-well plates coated with a mAb against CGRP, and enzyme immuno-assay was performed following instructions for the kit. Total CGRP content was determined on randomly selected wells from each culture, as detailed 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/C1 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 BR-HBS buffer, Ca^{2+}-dependent basal release and that evoked by 60 mM K^+, 1 μM capsaicin or 0.1 μM bradykinin were measured as above. Non-toxin-treated samples were processed 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). Each SNARE was detected with specific IgGs and anti-species secondary antibodies, as detailed in Figure legends. After ECL development, the lanes were analysed using the G BOX Chemi-16 gel documentation system and intensities quantified with Image J software, ensuring that the values fell within a linear standard curve. To determine the fraction of each cleaved SNARE, the ratios calculated for intact substrate and the requisite internal standard (i.e. a SNARE not susceptible to the toxin in use) for BoNT-treated samples were expressed relative to those for controls.

Immuno-absorption of vesicles from TGNs

TGNs were washed and homogenised in 0.32 M sucrose, 1 mM EDTA, 4 mM Hepes pH 7.2, and a cocktail of protease inhibitors using a Potter homogeniser, followed by passage through a 25G needle. After removal of nuclei and cell debris by centrifugation at 1200 ***g*** for 15 minutes, the resultant supernatant was subjected to centrifugation at 100,000 ***g*** for 2 hours. The suspended pellets were lysed for 45 minutes on ice in 40 mM sucrose, 1 mM EDTA, 4 mM HEPES pH 7.2 and protease inhibitors. After centrifugation at 100,000 ***g*** for 2 hours, the pellets were resuspended in homogenisation buffer. Aliquots of the suspension (~144 μg protein) were incubated overnight at 4°C, with rabbit antibodies (10 μg) specific for Sbr I or Sbr II, or rabbit non-immune control IgG, all coupled to protein A agarose; the beads were washed five times with homogenisation buffer. Equal aliquots (0.5 ml) were sedimented and pellets dissolved in 2× SDS sample buffer for SDS-PAGE, or in 2 M acetic acid with 0.1%TFA. Solvents were removed from the latter buffer by vacuum drying and the residues dissolved in enzyme immuno-assay buffer for CGRP quantification.

Co-immunoprecipitation of SNAREs from detergent-solubilised TGNs

The pellets of washed TGNs were dissolved in 1 ml of extraction buffer [140 mM KCl, 2 mM EDTA, 20 mM HEPES-KOH pH 7.3 and 1% (v/v) Triton X-100]. After extraction for 1 hour at 4°C and centrifugation for 3 minutes at 700 ***g***, the supernatant was incubated overnight at 4°C with 10 μg of rabbit IgG against Sbr I or SbrII coupled to protein A agarose beads. After sedimentation, beads were washed five times in extraction buffer and proteins eluted using 2×SDS-PAGE sample buffer. A control sample was treated similarly except rabbit non-immune IgG was used. SNAREs were detected by SDS-PAGE and western blotting, as described above.

Statistical analysis

Data were calculated and graphs generated by GraphPad Prism 4.0; each point represents the mean ± s.e.m. from at least three independent experiments.

This work has been kindly supported by a research professorship grant (to J.O.D.) from the Science Foundation Ireland and a research contract from Allergan Inc. We thank T. Zurawski, A. Sasse and F. O'Sullivan for help and with capture and analysis of images.

References

Aguado, F., Majo, G., Ruiz-Montasell, B., Llorens, J., Marsal, J. and Blasi, J. (1999). Syntaxin 1A and 1B display distinct distribution patterns in the rat peripheral nervous system. *Neuroscience* **88**, 437-446.

Aoki, K. R. (2003). Evidence for antinociceptive activity of botulinum toxin type A in pain management. *Headache* **43** Suppl. 1, S9-S15.

Baccaglini, P. I. and Hogan, P. G. (1983). Some rat sensory neurons in culture express characteristics of differentiated pain sensory cells. *Proc. Natl. Acad. Sci. USA* **80**, 594-598.

Bergquist, F., Niazi, H. S. and Nissbrandt, H. (2002). Evidence for different exocytosis pathways in dendritic and terminal dopamine release in vivo. *Brain Res.* **950**, 245-253.

Bernardini, N., Neuhuber, W., Reeh, P. W. and Sauer, S. K. (2004). Morphological evidence for functional capsaicin receptor expression and calcitonin gene-related peptide exocytosis in isolated peripheral nerve axons of the mouse. *Neuroscience* **126**, 585-590.

Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I. and Julius, D. (2000). Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288**, 306-313.

Chai, Q., Arndt, J. W., Dong, M., Tepp, W. H., Johnson, E. A., Chapman, E. R. and Stevens, R. C. (2006). Structural basis of cell surface receptor recognition by botulinum neurotoxin B. *Nature* **444**, 1096-1100.

De Biasi, S. and Rustioni, A. (1988). Glutamate and substance P coexist in primary afferent terminals in the superficial laminae of spinal cord. *Proc. Natl. Acad. Sci. USA* **85**, 7820-7824.

Dolly, J. O., de Paiva, A., Foran, P., Lawrence, G., Daniels-Holgate, P. and Ashton, A. C. (1994). Probing the process of transmitter release with botulinum and tetanus neurotoxins. *Semin. Neurosci.* **6**, 149-158.

Dong, M., Richards, D. A., Goodnough, M. C., Tepp, W. H., Johnson, E. A. and Chapman, E. R. (2003). Synaptotagmins I and II mediate entry of botulinum neurotoxin B into cells. *J. Cell Biol.* **162**, 1293-1303.

Dong, M., Yeh, F., Tepp, W. H., Dean, C., Johnson, E. A., Janz, R. and Chapman, E. R. (2006). SV2 is the protein receptor for botulinum neurotoxin A. *Science* **312**, 592-596.

Durham, P. L. and Russo, A. F. (1999). Regulation of calcitonin gene-related peptide secretion by a serotonergic antimigraine drug. *J. Neurosci.* **19**, 3423-3429.

Durham, P. L. and Cady, R. (2004). Regulation of calcitonin gene-related peptide secretion from trigeminal nerve cells by botulinum toxin type A: implications for migraine therapy. *Headache* **44**, 35-42; discussion 42-43.

Eckert, S. P., Taddese, A. and McCleskey, E. W. (1997). Isolation and culture of rat sensory neurons having distinct sensory modalities. *J. Neurosci. Methods* **77**, 183-190.

Edvinsson, L. (2004). Blockade of CGRP receptors in the intracranial vasculature: a new target in the treatment of headache. *Cephalalgia* **24**, 611-622.

Foran, P., Lawrence, G. and Dolly, J. O. (1995). Blockade by botulinum neurotoxin B of catecholamine release from adrenochromaffin cells correlates with its cleavage of synaptobrevin and a homologue present on the granules. *Biochemistry* **34**, 5494-5503.

Foran, P. G., Mohammed, N., Lisk, G. O., Nagwaney, S., Lawrence, G. W., Johnson, E., Smith, L., Aoki, K. R. and Dolly, J. O. (2003). Evaluation of the therapeutic usefulness of botulinum neurotoxin B, C1, E, and F compared with the long lasting type A. Basis for distinct durations of inhibition of exocytosis in central neurons. *J. Biol. Chem.* **278**, 1363-1371.

Gazerani, P., Staahl, C., Drewes, A. M. and Arendt-Nielsen, L. (2006). The effects of Botulinum Toxin type A on capsaicin-evoked pain, flare, and secondary hyperalgesia in an experimental human model of trigeminal sensitization. *Pain* **122**, 315-325.

Guo, A., Vulchanova, L., Wang, J., Li, X. and Elde, R. (1999). Immunocytochemical localization of the vanilloid receptor 1 (VR1): relationship to neuropeptides, the P2X3 purinoceptor and IB4 binding sites. *Eur. J. Neurosci.* **11**, 946-958.

Gupta, V. K. (2005). Botulinum toxin type A therapy for chronic tension-type headache: fact versus fiction. *Pain* **116**, 166-167; author reply 167.

Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Sudhof, T. C. and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. *EMBO J.* **13**, 5051-5061.

Hua, S. Y., Raciborska, D. A., Trimble, W. S. and Charlton, M. P. (1998). Different VAMP/synaptobrevin complexes for spontaneous and evoked transmitter release at the crayfish neuromuscular junction. *J. Neurophysiol.* **80**, 3233-3246.

Jin, R., Rummel, A., Binz, T. and Brunger, A. T. (2006). Botulinum neurotoxin B recognizes its protein receptor with high affinity and specificity. *Nature* **444**, 1092-1095.

Kummer, W. (1992). Ultrastructure of calcitonin gene-related peptide-immunoreactive nerve fibres in guinea-pig peribronchial ganglia. *Regul. Pept.* **37**, 135-142.

Lomneth, R., Martin, T. F. and DasGupta, B. R. (1991). Botulinum neurotoxin light chain inhibits norepinephrine secretion in PC12 cells at an intracellular membranous or cytoskeletal site. *J. Neurochem.* **57**, 1413-1421.

Mahrhold, S., Rummel, A., Bigalke, H., Davletov, B. and Binz, T. (2006). The synaptic vesicle protein 2C mediates the uptake of botulinum neurotoxin A into phrenic nerves. *FEBS Lett.* **580**, 2011-2014.

McMahon, H. T., Foran, P., Dolly, J. O., Verhage, M., Wiegant, V. M. and Nicholls, D. G. (1992). Tetanus toxin and botulinum toxins type A and B inhibit glutamate, gamma-aminobutyric acid, aspartate, and met-enkephalin release from synaptosomes. Clues to the locus of action. *J. Biol. Chem.* **267**, 21338-21343.

Nystuen, A. M., Schwendinger, J. K., Sachs, A. J., Yang, A. W. and Haider, N. B. (2007). A null mutation in VAMP1/synaptobrevin is associated with neurological defects and prewean mortality in the lethal-wasting mouse mutant. *Neurogenetics* **8**, 1-10.

O'Connor, T. P. and van der Kooy, D. (1988). Enrichment of a vasoactive neuropeptide (calcitonin gene related peptide) in the trigeminal sensory projection to the intracranial arteries. *J. Neurosci.* **8**, 2468-2476.

Otto, H., Hanson, P. I. and Jahn, R. (1997). Assembly and disassembly of a ternary complex of synaptobrevin, syntaxin, and SNAP-25 in the membrane of synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **94**, 6197-6201.

Price, T. J., Louria, M. D., Candelario-Soto, D., Dussor, G. O., Jeske, N. A.,

Patwardhan, A. M., Diogenes, A., Trott, A. A., Hargreaves, K. M. and Flores, C. M. (2005). Treatment of trigeminal ganglion neurons in vitro with NGF, GDNF or BDNF: effects on neuronal survival, neurochemical properties and TRPV1-mediated neuropeptide secretion. *BMC Neurosci.* **6**, 4.

Purkiss, J., Welch, M., Doward, S. and Foster, K. (2000). Capsaicin-stimulated release of substance P from cultured dorsal root ganglion neurons: involvement of two distinct mechanisms. *Biochem. Pharmacol.* **59**, 1403-1406.

Rummel, A., Karnath, T., Henke, T., Bigalke, H. and Binz, T. (2004). Synaptotagmins I and II act as nerve cell receptors for botulinum neurotoxin G. *J. Biol. Chem.* **279**, 30865-30870.

Sakaba, T., Stein, A., Jahn, R. and Neher, E. (2005). Distinct kinetic changes in neurotransmitter release after SNARE protein cleavage. *Science* **309**, 491-494.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B. R. and Montecucco, C. (1992). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. *Nature* **359**, 832-835.

Schiavo, G., Shone, C. C., Bennett, M. K., Scheller, R. H. and Montecucco, C. (1995). Botulinum neurotoxin type C cleaves a single Lys-Ala bond within the carboxyl-terminal region of syntaxins. *J. Biol. Chem.* **270**, 10566-10570.

Schoch, S., Deak, F., Konigstorfer, A., Mozhayeva, M., Sara, Y., Sudhof, T. C. and Kavalali, E. T. (2001). SNARE function analyzed in synaptobrevin/VAMP knockout mice. *Science* **294**, 1117-1122.

Silberstein, S. D. and Aoki, K. R. (2003). Botulinum toxin type A: myths, facts, and current research. *Headache* **43** Suppl. 1, S1.

Steranka, L. R., Manning, D. C., DeHaas, C. J., Ferkany, J. W., Borosky, S. A., Connor, J. R., Vavrek, R. J., Stewart, J. M. and Snyder, S. H. (1988). Bradykinin as a pain mediator: receptors are localized to sensory neurons, and antagonists have analgesic actions. *Proc. Natl. Acad. Sci. USA* **85**, 3245-3249.

Takamori, S., Holt, M., Stenius, K., Lemke, E. A., Gronborg, M., Riedel, D., Urlaub, H., Schenck, S., Brugger, B., Ringler, P. et al. (2006). Molecular anatomy of a trafficking organelle. *Cell* **127**, 831-846.

Trimble, W. S., Gray, T. S., Elferink, L. A., Wilson, M. C. and Scheller, R. H. (1990). Distinct patterns of expression of two VAMP genes within the rat brain. *J. Neurosci.* **10**, 1380-1387.

Vedder, H. and Otten, U. (1991). Biosynthesis and release of tachykinins from rat sensory neurons in culture. *J. Neurosci. Res.* **30**, 288-299.

Verderio, C., Pozzi, D., Pravettoni, E., Inverardi, F., Schenk, U., Coco, S., Proux-Gillardeaux, V., Galli, T., Rossetto, O., Frassoni, C. et al. (2004). SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. *Neuron* **41**, 599-610.

Welch, M. J., Purkiss, J. R. and Foster, K. A. (2000). Sensitivity of embryonic rat dorsal root ganglia neurons to Clostridium botulinum neurotoxins. *Toxicon* **38**, 245-258.

Woolf, C. J. (2004). Pain: moving from symptom control toward mechanism-specific pharmacologic management. *Ann. Intern. Med.* **140**, 441-451.

Yamasaki, S., Baumeister, A., Binz, T., Blasi, J., Link, E., Cornille, F., Roques, B., Fykse, E. M., Sudhof, T. C., Jahn, R. et al. (1994). Cleavage of members of the synaptobrevin/VAMP family by types D and F botulinal neurotoxins and tetanus toxin. *J. Biol. Chem.* **269**, 12764-12772.

Journal of Cell Science