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Intrinsic calcium dynamics control botulinum toxin A susceptibility in distinct neuronal populations

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ABSTRACT

SNAP-25 is a SNARE protein implicated in exocytosis and in the negative modulation of voltage-gated calcium channels. We have previously shown that GABAergic synapses, which express SNAP-25 at much lower levels relative to glutamatergic ones, are characterized by a higher calcium responsiveness to depolarization and are largely resistant to botulinum toxin A. We show here that silencing of SNAP-25 in glutamatergic neurons, a procedure which increases KCl-induced calcium elevations, confers these synapses with toxin resistance. Since it is known that calcium reverts the efficacy of botulinum A, we investigated whether the lower effectiveness of the toxin in inhibiting GABAergic vesicle cycling might be attributable to higher evoked calcium transients of inhibitory neurons. We demonstrate that either expression of SNAP-25_{1-197} or BAPTA/AM treatment, both inhibiting calcium dynamics, facilitate block of GABAergic vesicle exocytosis upon toxin treatment. These data indicate that intrinsic calcium dynamics control botulinum A susceptibility in distinct neuronal populations.

1. Introduction

SNAP-25 belongs to the SNARE superfamily of membrane proteins that participate in synaptic vesicle (SV) exocytosis. It contributes two alpha-helices in the formation of the exocytotic fusion complex together with syntaxin-1 and synaptobrevin/VAMP [1,2]. Besides acting as a fundamental component of the SNARE complex, SNAP-25 has been shown to negatively modulate voltage gated calcium channels in various overexpression systems [3,4] and in cultured hippocampal neurons [5,6].

Botulinum neurotoxin A (BoNT/A) is a protease specific for SNAP-25. Although the BoNT/A-generated fragment, SNAP-25_{1-197}, retains the capability to form the exocytotic fusion complex [7,8], SNAP-25 proteolysis by the toxin reduces the probability of vesicle fusion and inhibits neurotransmitter release. It has been previously shown that BoNT/A preferentially inhibits SV recycling at glutamatergic terminals and is more efficient in impairing the release of excitatory than inhibitory neurotransmitters [5,9–11]. Lower susceptibility of GABAergic terminals to BoNT/A does not result from a defective toxin penetration, in line with the presence of the BoNT/A receptor SV2 in inhibitory neurons [11]. Notably the different susceptibility to BoNT/A of glutamatergic and GABAergic cells correlates with the levels of expression of the toxin substrate SNAP-25 in the two neuron types. Indeed, although most cultured GABAergic neurons use SNAP-25 for evoked exocytosis [12–14], they are characterized by lower levels of SNAP-25 as compared to glutamatergic terminals [5,15–17] and become sensitive to BoNT/A upon exogenous expression of the protein [11]. Why BoNT/A is highly efficient in inhibiting vesicle cycling selectively in neurons expressing high levels of SNAP-25 is still to be defined. The clarification of this point may have important implications, especially in consideration of the wide use of BoNT/A for the treatment of human diseases, and in view of its employment in CNS pathologies [18–20].

2. Materials and methods

2.1. Hippocampal neuronal cultures

Primary cultures of rat hippocampal neurons were prepared from the hippocampi of 18-day-old fetuses as previously described [11]. Neurons were transfected using the calcium phosphate precipitation method. Silencing of SNAP-25 was achieved via transfection of a pSUPER construct provided by T. Galli (Paris, France) [5]. The cDNA for SNAP-25_{1-197} and SNAP-25_{1-180} were generously provided O. Rossetto (Padua, Italy).

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2.2. Exo-endocytotic assay

BoNT/A was a kind gift of Prof. Eric Johnson, Madison, WI, purified and stored as previously described [11]. Control or transfected cultures were exposed to 125 nM BoNT/A for 2 h at 37 °C, before being assayed for SV recycling with monoclonal antibodies directed against the intravesicular domain of synaptotagmin I (Syt-ecto Abs; clone 604.2, kind gift of Dr. R. Jahn, Gottingen) as previously described [11]. Cultures were counterstained with polyclonal antibodies directed against vGLUT-1 (1:300, Synaptic Systems), vGAT (1:400–800; Synaptic Systems) or GAD (human sera recognizing GAD, kind gift of Dr. Solimena, Dresden). In a set of experiments recycling of GABAergic vesicles was evaluated using anti vGAT luminal antibodies (vGAT ecto, kind gift of Dr. H. Martens, Synaptic Systems). Cultures were co-stained with monoclonal anti-VGAT-N (Synaptic Systems). Images were acquired using Zeiss LSM 510 Meta confocal microscope and analyzed by Image J software. vGLUT positive recycling synapses, transfected with siRNA construct, were revealed by generating a binary mask of siRNA/vGLUT double-positive images. Total fluorescence (area per mean grey value) of Syt-ecto positive synapses expressing the siRNA construct, identified by the binary mask, were normalized to the total fluorescence of the corresponding v-GLUT staining. Syt-ecto/vGLUT fluorescence ratios of siRNA-transfected synapses were compared to those of non-transfected vGLUT synapses. vGAT positive recycling synapses, transfected with SNAP-25-HA fragments, were revealed by generating a binary mask of SNAP-25-HA/vGAT double-positive images. The number of Syt-ecto positive synapses expressing SNAP-25-HA, identified by the binary mask, was calculated. Synapses were scored as positive for Syt-ecto Ab internalization when the fluorescence intensity was at least 2.5–3 times higher compared with cultures exposed only to secondary antibodies. For BAPTA experiments, vGAT positive synapses were revealed by generating a binary mask of vGAT images. Total fluorescence (area per mean grey value) of GABAergic Syt-ecto synapses, identified by the binary mask, were normalized to the total fluorescence of v-GAT staining. At least 10 fields containing excitatory/inhibitory synapses were analyzed, in three independent experiments. Quantitative analysis of SNAP-25 downregulation and overexpression in hippocampal cultures transfected with SNAP-25/GFP or cotransfected with iSNAP25–GFP was carried out with monoclonal antibodies directed against SNAP-25 (SMI81, Sternberger Monoclonals, Baltimore MD). Images were acquired using Zeiss LSM 510 Meta confocal microscope and analyzed by Image J-1.4.3.67 NIH software. SNAP-25 expression was measured as mean grey value in neuronal processes of transfected and non-transfected cells.

2.3. Calcium measurements

Cultures were loaded with the ratiometric calcium dye FURA-2/AM as previously described [5]. Polychrome IV (TILL Photonics, Grafelfing, Germany) was used as a light source. Fura-2 fluorescence images were collected with a PCO Super VGA SensiCam (Axon Instruments, Forest City, CA, USA) and analyzed with TILLvision software. After excitation at 340 and 380 nm wavelengths, the emitted light was acquired at 505 nm at 1–4 Hz. The ratio values in discrete areas of interest were calculated from sequences of images to obtain temporal analyses. Calcium concentrations were expressed as F340/380 fluorescence ratios.

2.4. Statistical analysis

Results are presented as means ± S.E. Data were statistically compared using the Student's t test by using Origin software (Origin Lab, Northampton). Significance values are calculated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. Levels of SNAP-25 expression influence neuron sensitivity to BoNT/A

We have previously shown that GABAergic SV exocytosis, monitored by antibodies to the intravesicular domain of synaptotagmin I (Syt-ecto), is highly resistant to BoNT/A [5,11]. By monitoring GABAergic vesicle fusion with antibodies recognizing the C-terminus of the vesicular GABA transporter vGAT (vGAT ecto) [21], we confirmed that the majority of GABAergic terminals, intoxicated with 125 nM BoNT/A, internalized vGAT ecto antibodies upon exposure to 55 mM KCl for 4 min; exocytosis was instead efficiently blocked by tetanus toxin (20 nM, TeNT) which cleaves synaptobrevin/VAMP-2 (Fig. 1A). Conversely, BoNT/A treatment inhibited SV recycling at excitatory synapses, monitored by antibodies to the intravesicular domain of synaptotagmin I (Syt-ecto) (Fig. 1B). SNAP-25 overexpression in GABAergic terminals was reported to largely increase sensitivity to BoNT/A and prevent Syt-ecto internalization [11]. To complement this finding and confirm that the expression level of SNAP-25 is an important determinant in neuronal sensitivity to BoNT/A, we investigated the effect of reducing SNAP-25 expression in glutamatergic neurons by co-transfection of cDNA codifying nucleotides 321–339 of rat SNAP-25 together with GFP (siRNA sequence). SNAP-25 expression was reduced of about 60% at 1–2 days after transfection (Fig. 1C, quantitation) and progressively decreased to levels not reliably quantifiable (Fig. 1C, arrow). Downregulation of SNAP-25 did not impair Syt-ecto Abs internalization at excitatory terminals (Fig. 1D), thus implying that low levels of the protein are sufficient to mediate SV fusion, as already demonstrated in cultures derived from SNAP-25 heterozygous mice [13, Antonucci et al., unpublished observations]. Cultures co-transfected with SNAP-25 siRNA sequence and GFP were exposed to BoNT/A and SV recycling was assayed using Syt-ecto Abs. A larger resistance to BoNT/A was detected at SNAP-25-silenced excitatory synapses. Indeed, the percentage of excitatory synapses resistant to intoxication raised from about 20% in controls to 40% after siRNA treatment (Fig. 1E). Furthermore the extent of recycling in SNAP-25-silenced boutons was higher as compared to the BoNT/A resistant terminals, either non-transfected or GFP transfected (Fig. 1E and F). Overall these data support the concept that variations in the expression of the SNARE at nerve terminals underlie differences in BoNT/A sensitivity.

3.2. Reduction of neuronal calcium transients enhances sensitivity of GABAergic terminals to BoNT/A

BoNT/A cleaves SNAP-25 at residue 197 to generate a membrane bound NH_2-terminal SNAP-25_{1-197} fragment, while BoNT/E removes a larger C terminus region of the protein by cleaving SNAP-25 at residue 180. The 17 amino acids between the cleavage sites for BoNT/A and BoNT/E are known to be required for high affinity binding of SNAP-25 to syntaxin 1 [22], for ternary complex formation with VAMP-2 and syntaxin 1 [7], for Ca^{2+}-triggered membrane fusion [23] and Ca^{2+}-dependent synaptotagmin binding [24]. In addition, the C-terminal region of SNAP-25 between BoNT/A and BoNT/E cleavage sites is also required for SNAP-25 modulation of voltage gated calcium currents [5,6].

To get insights into the mechanisms by which overexpression of SNAP-25 confers GABAergic neurons with BoNT/A sensitivity [11], hippocampal cultures were transfected with plasmids encoding portions of SNAP-25 (SNAP-25_{1-180} and SNAP-25_{1-197}) fused to a HA-tag. SV fusion was monitored in transfected GABAergic terminals upon BoNT/A intoxication. While no significant reduction of SV exocytosis was detected at intoxicated GAD positive synapses expressing the SNAP-25_{1-180} fragment, SV fusion was