Neuroscience 352 (2017) 155–169

BOTULINUM NEUROTOXIN TYPE A-CLEAVED SNAP25 IS CONFINED TO PRIMARY MOTOR NEURONS AND LOCALIZED ON THE PLASMA MEMBRANE FOLLOWING INTRAMUSCULAR TOXIN INJECTION

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Abstract—The mechanism of action of botulinum neurotoxin type A (BoNT/A) is well characterized, but some published evidence suggests the potential for neuronal retrograde transport and cell-to-cell transfer (transcytosis) under certain experimental conditions. The present study evaluated the potential for these processes using a highly selective antibody for the BoNT/A-cleaved substrate ($SNAP25_{197}$) combined with 3-dimensional imaging. $SNAP25_{197}$ was characterized in a rat motor neuron (MN) pathway following toxin intramuscular injections at various doses to determine whether $SNAP25_{197}$ is confined to MNs or also found in neighboring cells or nerve fibers within spinal cord (SC). Results demonstrated that $SNAP25_{197}$ immuno-reactive staining was colocalized with biomarkers for MNs, but not with markers for neighboring neurons, nerve fibers or glial cells. Additionally, a high dose of BoNT/A, but not a lower dose, resulted in sporadic $SNAP25_{197}$ signal in distal muscles and associated SC regions without evidence for transcytosis, suggesting that the staining was due to systemic spread of the toxin. Despite this spread, functional effects were not detected in the distal muscles. Therefore, under the present experimental conditions, our results suggest that BoNT/A is confined to MNs and any evidence of distal activity is due to limited systemic spread of the toxin at higher doses and not through transcytosis within SC. Lastly, at higher doses of BoNT/A, $SNAP25_{197}$ was expressed throughout MNs and colocalized with synaptic markers on the plasma membrane at 6 days post-treatment. These data support previous studies suggesting that $SNAP25_{197}$ may be incorporated into SNARE-protein complexes within the affected MNs.

Key words: botulinum neurotoxin, onabotulinumtoxinA, SNAP25, neuromuscular junction, motor neuron, spinal cord.

INTRODUCTION

Botulinum neurotoxin type A (BoNT/A) is used clinically for a growing number of indications including disorders involving skeletal and smooth muscle, glands and nociceptive pain mechanisms (Brin, 2009; Aoki and Francis, 2011). The primary reasons for the increased therapeutic application of BoNT/A is attributed to its marked clinical efficacy and proven safety record (Simpson et al., 2016). The general mechanism of action (MoA) for BoNT/A has been established (Montal, 2010; Rossetto et al., 2014) and involves a four-step sequential process: (1) receptor-specific binding to the presynaptic nerve terminal, (2) internalization into the terminal via endocytosis, (3) translocation of the light-chain (L_C/A) portion into the cytosol, and (4) enzymatic cleavage of intracellular synaptosomal-associated protein of 25 kDa ($SNAP25_{206}$) to form the inactive $SNAP25_{197}$ cleaved product. This protein is part of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex involved in mediating neuroexocytosis and vesicular recycling. Functionally, this mechanism leads to the inhibition of neurotransmitter/neuropeptide release from nerve fiber endings and subsequent transient paralysis or quiescence of the associated end-organ being innervated (Dolly and Lawrence, 2014). Results from decades of clinical usage have confirmed this peripheral action of BoNT/A (Simpson et al., 2016)

Recent studies have proposed that BoNT/A catalytic activity is not strictly confined to presynaptic terminals at the peripheral site of injection. These investigations have indicated that at higher doses, BoNT/A activity can spread to central neurons via a mechanism of retrograde transport within first-order neurons followed by transfer (transcytosis) of the active toxin to second-order neurons where it can potentially cleave SNAP25

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Abbreviations: 2D, 2 dimensional; BoNT/A, botulinum neurotoxin type A; ChAT, choline acetyltransferase; CNS, central nervous system; CTB AF-488, cholera toxin B subunit Alexa Fluor 488 conjugate; DAS, digit abduction score; GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; Hc/A, heavy chain type A; Lc/A, light chain type A; MoA, mechanism of action; MN, motor neuron; MNT, motor nerve terminals; NMJ, neuromuscular junction; OCT, optimal cutting temperature; pAb, polyclonal antibody; PBS, phosphate-buffered saline; SC, spinal cord; SNAP25, synaptosomal associated protein 25 kD; $SNAP25_{197}$, synaptosomal associated protein cleaved at position 197; $SNAP25_{206}$, synaptosomal associated protein uncleaved; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; TA, tibialis anterior; TeNT, tetanus toxin; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter; vGlut1, vesicular glutamate transporter 1; vGlut2, vesicular glutamate transporter 2; VLSC, ventrolateral spinal cord.

http://dx.doi.org/10.1016/j.neuroscience.2017.03.049

and inhibit transmitter release (Matak and Lackovic, 2014; Mazzocchio and Caleo, 2014). While it was reported that only a fraction of the total internalized BoNT/A actually undergoes retrograde transport and transcytosis (Restani et al., 2012a), it was proposed that this fraction utilizes similar mechanisms to those exploited by tetanus neurotoxin (TeNT) to gain access to central neurons (Lalli et al., 2003; Bercsenyi et al., 2013; Bomba-Warczak et al., 2016). Consequently, it was hypothesized that peripheral applications of BoNT/A can directly transcytose to neurons within the central nervous system (CNS; Mazzocchio and Caleo, 2014).

Nonetheless, questions have been raised regarding the tools, models and BoNT/A dosing levels utilized in these studies (Lawrence et al., 2012). For example, these studies relied on a rabbit polyclonal antibody (pAb) to detect $SNAP25_{197}$ in BoNT/A-treated samples using both Western Blot and immunohistochemical (IHC) assays (Antonucci et al., 2008; Matak et al., 2011, 2012, 2014; Restani et al., 2011, 2012). The $SNAP25_{197}$-positive staining observed in these studies was purported as indicative of the presence of active BoNT/A in both first-order and second-order neurons.

We recently developed site-specific, recombinant monoclonal antibodies (rMAb) against the BoNT/A-cleaved SNAP25 epitope and demonstrated their superior specificity over pAbs and other commercial monoclonal antibodies at recognizing $SNAP25_{197}$, especially with regards to IHC (Rheaume et al., 2015). Since many of the conclusions regarding BoNT/A retrograde transport and transcytosis were based on IHC analysis of the cleaved product, we resolved to explore this possibility utilizing one of our rMAbs (Ab632) in combination with high-resolution three-dimensional (3D) imaging and quantitative analysis to characterize $SNAP25_{197}$ expression in a rat motor neuron (MN) pathway, following intramuscular (i.m.) BoNT/A injections at various doses. We performed a systematic evaluation to determine whether $SNAP25_{197}$ is confined to primary MNs or is also found in neighboring cells and/or nerve fibers within the spinal cord (SC). We selected the motor pathway leading from the rat hindlimb tibialis anterior (TA) muscle through the sciatic nerve and into the L4–L5 segment of the ventrolateral SC (VLSC). This anatomical pathway represents a relevant, *in vivo* system to study the effects of BoNT/A, as motor nerve terminals (MNT) are the target of numerous therapeutic indications.

To accurately carry out this analysis, it was necessary to understand and appreciate the complex motor circuitry within the VLSC (Fig. 1). MNs have extensive dendritic arbors that extend radially from the soma in all axes (i.e., mediolateral, dorsoventral and rostrocaudal), often reaching the dorsal SC (Cook and Woolf, 1985; Kurz et al., 1990). Furthermore, MN dendritic branches form a multitude of synaptic contacts with neighboring neurons and nerve fibers, including Renshaw cells (Alvarez and Fyffe, 2007), excitatory and inhibitory interneurons (Kiehn, 2006; Nishimaru and Kakizaki, 2009), sensory afferents (Snider et al., 1992) and descending catecholamine fibers (Han et al., 2007; Sharples et al., 2014), as well as resident glial cells (Blackburn et al., 2009) that collectively serve to modulate sensorimotor signals to the muscle (Hall, 2004). Under the present experimental conditions, our results suggest that BoNT/A is confined to MNs and any evidence of distal activity is due to limited systemic spread of the toxin at higher doses and not through transcytosis within the SC.

EXPERIMENTAL PROCEDURES

Materials

Primary antibodies used: recombinant human anti-$SNAP25_{197}$ (Ab632) rMAb (Allergan plc, Irvine, CA, USA); rabbit anti-SNAP25 monoclonal antibody (Epitomics-Abcam, Cambridge, MA,, USA); rabbit anti-vesicular acetylcholine transporter (VAChT) pAb (Sigma–Aldrich, Saint Louis, MI, USA); mouse anti-syntaxin 1 (sc-12736) was from Santa Cruz Biotechnology (Dallas, TX, USA); mouse anti-choline acetyltransferase (ChAT) monoclonal antibody, rabbit anti-calbindin polyclonal antibody, mouse anti-vGlut1 monoclonal antibody, mouse anti-vGlut2 monoclonal antibody, mouse anti-GFAP monoclonal antibody, rabbit anti-GAD65 + GAD67 polyclonal antibody (Abcam, Cambridge, MA, USA); rabbit anti-tyrosine hydroxylase (TH) polyclonal antibody (EMD Millipore, Billerica, MA, USA). Cholera toxin B subunit Alexa Fluor 488 conjugate (CTB AF-488) and α-bugarotoxin Alexa Fluor-488 conjugate (α-Bgt AF-488) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

Animals

Male Sprague–Dawley rats (225–250 g; Charles River, Wilmington, MA, USA) were group housed on a 12-h light–dark cycle with food and water available *ad libitum*. A total of ~60 Sprague–Dawley rats were used for this study. Animal protocols and procedures were approved by the Allergan Institutional Animal Care and Use Committee and followed National Institutes of Health guidelines for the care and use of laboratory animals.

BoNT/A preparation and injection procedures

Working solutions of BoNT/A (onabotulinumtoxinA, 900-kDa complex, Allergan, plc., Irvine, CA, USA) were prepared in 0.9% saline. For single-injection studies, 20 μl of BoNT/A (3, 10, 30 U/kg) in 0.9% saline or AF-488 conjugated CTB (10 μg/kg) was injected intramuscularly (i.m.) into the mid-belly of the TA muscle of the right hindlimb. Doses of BoNT/A utilized were equivalent to ~22, 75 and 225 pg/kg, respectively (based on 150 kDa BoNT/A neurotoxin protein). Rats were sacrificed at 1, 3 or 6 days post-BoNT/A injection. For multi-injection studies, CTB AF-488 was first injected into five equidistant spots spanning the right TA rat muscle (10 μg/kg, 10 μl each). The following day, BoNT/A (10 U/kg) was injected into the mid-belly of the muscle. All animals were sacrificed 6 days post-BoNT/A injection. Separate control animals received saline injections into the right TA muscle.