Seven types (A–G) of botulinum neurotoxin (BoNT) target peripheral cholinergic neurons where they selectively proteolyze SNAP-25 (BoNT/A, BoNT/C1, and BoNT/E), syntaxin1 (BoNT/C1), and synaptobrevin (BoNT/B, BoNT/D, BoNT/F, and BoNT/G). SNARE proteins responsible for transmitter release, to cause neuromuscular paralysis but of different durations. BoNT/A paralysis lasts longest (4–6 months) in humans, hence its widespread clinical use for the treatment of dystonias. Molecular mechanisms underlying these distinct inhibitory patterns were deciphered in rat cerebellar neurons by quantifying the half-life of the effect of each toxin, the speed of replenishment of their substrates, and the degradation of the cleaved products, experiments not readily feasible at motor nerve endings. Correlation of target cleavage with blockade of transmitter release yielded half-lives of inhibition for BoNT/A, BoNT/C1, BoNT/B, BoNT/F, and BoNT/E ($\gg 31$, $>25$, $>10$, $<2$, and $<0.8$ days, respectively), equivalent to the neuromuscular paralysis times found in mice, with recovery of release coinciding with reappearance of the intact SNAREs. A limiting factor for the short neuroparalytic durations of BoNT/F and BoNT/E is the replenishment of synaptobrevin or SNAP-25, whereas pulse labeling revealed that extended inhibition by BoNT/A, BoNT/B, or BoNT/C1 results from longevity of each protease. These novel findings could aid development of new toxin therapies for patients resistant to BoNT/A and effective treatments for human botulism.

* This work was supported in part by Allergan Inc., United States Army Medical Research and Materiel Command under Contract DAMD17-01-C-6062, and a Biological and Biotechnological Research Council studentship (to G.O.L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed. Tel.: 44-208-393-4438; Fax: 44-208-224-7629; E-mail: dr.oliver@dolly.fsworld.co.uk.

The abbreviations used are: BoNT, botulinum neurotoxin; DIV, days in vitro; KHR, Krebs-Ringer-HEPES; LC, light chain; Sbr, synaptobrevin; SNAP-25, 25-kDa synaptosomal-associated protein; SNARE, soluble Y-ethylmaleimide-sensitive factor attachment protein receptor; STx1, syntaxin1; TeTx, tetanus toxin; CHAPS, 3-[3-cholamidopropyl]-1-propanesulfonic acid.

2 Meunier, F. A., Lisk, G., Sesardic, D., and Dolly, J. O. (2003) Mol. Cell. Neurobiol., in press.
protease for many weeks contributes to the extended inhibition of secretion; also, SNAP-25A has been shown to be inhibitory to BoNT/A and BoNT/E results in a more rapid recovery of neuromuscular function, equivalent to that of BoNT/E alone, prompting many scientists (16, 22, 24) to suggest that both proteases have equivalent lifetimes in the motor nerve ending and the prolonged paralysis by BoNT/A arises from slow replacement of SNAP-25A. Accordingly, Meunier et al. (5) observed that type E hastens the removal of inhibitory SNAP-25β from BoNT/A-treated mouse neuromuscular synapses by converting it to SNAP-25βp, which is replaced rapidly; thus, resumption of synaptic transmission is accelerated.

In this study, biochemical analyses (not practical with motor nerve endings or isolated motoneurons; see “Discussion”) were performed on cultured cerebellar neurons to quantify the half-lives of toxin inhibition and the rates of turnover of SNAREs and their toxin-cleaved products. Although noncholinerigic, these neurons provide a useful model for studying the intracellular fate of BoNTs, because we observed the same relative durations of neurotoxic paralysis of BoNT/A, BoNT/B, BoNT/C1, BoNT/E, and BoNT/F as measured in motor nerves in vitro (see above). In addition, these homogeneous cerebellar neurons are very susceptible to BoNTs and could be obtained in sufficient numbers for these quantitative measurements. In this way, we have extended earlier findings (22, 25) and explained how exocytosis can be blocked for dissimilar periods by the different BoNT serotypes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media and general reagents were supplied by Sigma-Aldrich. N2 supplement and serum (extensively dialyzed before use) were from Invitrogen. [14C]Glutamine was from Amersham Biosciences, and [35S]methionine was from ICN. Monoclonal antibodies selectively reactive with STx1, Sbr–1, and Sbr–2; termed HV62, synaptotagmin1 (its last 20 amino acids), and SNAP-23 (11 residues at the C terminus) were produced in rabbits and affinity-purified as before (26, 27). Anti-glial fibrillary acidic protein-reactive astrocytes but an abundance of STx1, Sbr–1, and 2 (a 62-mer peptide precursor) (31); all of the steps were performed at 37 °C. After 48 h, the neurones were washed twice and harvested immediately or “chased” in standard medium supplemented with 0.25 mM unlabeled t-methionine. Washed neurones were detergent solubilized for 30 min (0.6 ml) using 2% (v/v) CHAPS and 2% (v/v) n-octyl-β-D-glucopyranoside in 20 mM HEPES-NaOH, pH 7.5, containing 10 mM EDTA, 150 mM NaCl, 1% (v/v) glycerol, and 2% (v/v) of a protease inhibitor mixture (P8340). Sigma). All of the steps were performed at 0–4 °C.

In solubilized material was removed by centrifugation at 15,000 × g for 40 min, and the extracts were incubated for 3–4 h in an end-over agitator with the relevant anti-SNARE Ig-protein A-agarose complex (10 μg of IgG/50 μl of resin). Resin was collected by centrifugation (5 s at 100 × g) and washed eight times over 30 min (1 ml each wash) before dissolving the protease mixture and containing only 0.1% (v/v) each of CHAPS and n-octyl-β-D-glucopyranoside. Nonreducing SDS-PAGE sample buffer was added to the agarose slurry and heated at 80 °C for 20 min. The radioactive immunoprecipitated SNAP-25 was subjected to SDS-PAGE, fixed, treated with Amplify™ (Amersham Biosciences), dried for fluorography, and detected using Hyperfilm MT™. Control experiments found that BoNT/A, BoNT/B, BoNT/E, or BoNT/F pretreatments had no effect on protein synthesis, by measuring the amounts of radioactivity incorporated into precipitable protein relative to toxin-free controls (measured by scintillation counting; data not shown).

**Immunoblotting and Quantitation of Antigen Levels**—For analyzing the transmitter release assay, the cells were solubilized in 1% (w/v) SDS in 20 mM HEPES-NaOH, pH 8.5, containing 20 mM EDTA plus 150 mM NaCl; the total protein was quantitatively isolated using chloroform-methanol precipitation (outlined in Ref. 27). For optimal resolution of intact SNAP-25 from its toxin-truncated products, the samples were subjected to SDS-PAGE using NOVEX™ 12% Bis-Tris gels and a MOPS-based buffer system (Invitrogen). The proteins were electrotransferred and immunoblotted, as detailed previously (27), with detection by anti-species-specific IgGs conjugated to horseradish peroxidase and visualized by enhanced chemiluminescence. The blots were densitometrically scanned, and the bands were quantified using image analysis software (Scion Image for Windows); the standard curves of the amounts of SNAPRE plotted against band intensity were constructed for the linear range of SNAPRE levels and were used to calculate the amount of SNAPRE released into the medium.
much more prominent in neurons than glia (Fig. 1B). In contrast, SNAP-23, a BoNT/A-insensitive but BoNT/E-cleavable non-neuronal SNAP-25 homologue (27, 36, 37) was apparently absent from the granule cells but present in glia (Fig. 1C). By microscopy (28) and immunoblotted using SMI-81 Ig (SNAP-25) and anti-HV62 Ig (Sbr2) one could see a partial blockade, with minimal inhibition of exocytosis in the absence of toxin after BoNT/E treatment or 3 (28) or 7 (29) days after toxin was washed away, no significant recovery being noted at 7 days (28, 29). In both cases, exocytosis appeared to resume more rapidly from a partial blockade, with minimal inhibition of exocytosis after replenishment of intact SNAREs coincident with resumption of exocytosis. From the dose dependence, a half-life of the duration of inhibition \( t_{1/2} \) by each toxin was determined by monitoring reduction in the extent of blockade of exocytosis at different times after initial exposure to a given concentration of toxin. In the case of BoNT/E, the concentrations required to yield a 40% inhibition of exocytosis at 0, 2, and 4 days after the removal of toxin were 0.021, 0.22, and 0.80 nM, respectively (Fig. 2A). When these values were subjected to first order decay analysis, a \( t_{1/2} \) of 0.70 ± 0.15 days (mean ± S.D.; n = 6) was calculated; a second series of experiments yielded a comparable value. The mean of both experiments was 0.73 ± 0.11 days (mean ± S.D.; n = 12; Table I). Similarly, analyses of data from two recovery experiments with BoNT/F yielded a mean \( t_{1/2} \) of 1.76 ± 0.28 days (Table I). It is assumed that these \( t_{1/2} \) values represent a combination of the times required for cellular removal of the toxin protease activity and synthesis of functional intact SNAREs.
from inhibition of release or replenishment of intact SNAP-25 was detected at any of the BoNT/A concentrations employed (Fig. 3, A and B), upon weekly monitoring up to 31 days. Additional experiments also demonstrated a lack of significant recovery from blockade of exocytosis 1 month after toxin exposure (data not shown and detailed later). Therefore, the \( t_{1/2} \) of BoNT/A exceeds 31 days (Table I).

In contrast, BoNT/B induced a concentration-dependent inhibition of transmitter release achieving \( \approx 80\% \) blockade (Fig. 3C; 100 pM gave \( 98.4 \pm 2.12\% \) inhibition; Table I), together with nearly complete cleavage of Sbr2 (Fig. 3D), precluding SbrI that is resistant (38). Notably, the truncated Sbr2 fragments produced by BoNT/B or BoNT/F, potential contributors to the poisoning, were not visible on Western blots (see below). Upon removing BoNT/B by washing, recovery from inhibition occurred in a time-dependent manner and was accompanied by equivalent partial replacement of intact Sbr2 (Fig. 3, C and D). Exponential decay analysis of data from two BoNT/B dose dependence recovery studies yielded a mean \( t_{1/2} \) of 9.84 ± 2.12 days (Table I). Therefore, compared with the long lasting BoNT/A and short acting BoNT/E and/F, type B exhibits an intermediate duration of inhibition.

**BoNT/C1 Exerts a Long Lasting Inhibition of Exocytosis and Is Neurotoxic**—Two days after a 24-h exposure to BoNT/C1, concentration-dependent cleavage of STx1 and SNAP-25 was observed (Fig. 4B). A concomitant inhibition of \( K^+ \)-evoked exocytosis occurred (Fig. 4A; 13 pM gave 50% inhibition; Table I) that correlates well with the proteolysis of SNAP-25 but not STx1 (Fig. 4B). Whereas there was extensive formation of a SNAP-25 product with a size corresponding to the known N-terminal fragment (residues 1–198) (Fig. 4D; see the Introduction), only a residual content of the toxin-truncated N-terminal STx1 product (residues 1–253) (reviewed in Ref. 3) could be detected (Fig. 4D). Short exposure to a higher concentration of BoNT/C1 (2 h) resulted in \( \approx 80\% \) cleavage of STx1 and SNAP-25; however, the STx11 product was not more abundant and only clearly visible if immunoblots were overdeveloped (Fig. 4D, asterisk); thus, this fragment is rapidly degraded. The possibility that this potential competitor of the SNARE complex contributes to the inhibition of exocytosis is therefore unlikely.

Closer examination of neuron abundance in control and BoNT/C1-treated cultures revealed a dose-dependent effect on survival with 0.33 nM yielding \( \approx 50\% \) lethality within 2 days (Fig. 4C and Table I). Indeed, C1 lethality was much more apparent after 18 days exposure as indicated by the diminution of BoNT/C1-resistant markers, Sbr2 and synaptotagmin1 (data not shown); also, direct neuron counting (Fig. 4C) revealed that \( \approx 60\% \) of the neurons treated with 33 pM had died. It is apparent that neurons exposed to 10 pM BoNT/C1, showing 47.3 ± 5.3 and 7.7 ± 9.6% cleavage of SNAP-25 and STx1 at 2 days, survived well over the additional 16 days (Fig. 4, B and C), but those that experienced more extensive initial proteolysis of SNAREs fared poorly. For instance, only \( \approx 10\% \) of neurons survived 18 days if their initial intact STx1 content had been diminished by 48.0 ± 8.0% (Fig. 4, B and C). Despite the difficulties experienced with neuron survival, it was still possible to demonstrate that neither significant recovery from the dose-dependent inhibition of exocytosis (Fig. 4A) nor increased contents of intact SNAP-25 and STx1 occurred (Fig. 4B). Additional separate experiments lasting either 18 or 25 days post-intoxication (Table I) also demonstrated a lack of significant recovery from BoNT/C1-induced blockade of neuroexocytosis.

**[35S]Metabine Pulse Labeling Demonstrates That BoNT/A Protease Has a Long Lifetime in Central Neurons:** SNAP-25 was Turned Over as Rapidly as the Intact Polypeptide—Failure to recover exocytosis from BoNT/A-intoxicated neurons and persistence of SNAP-25 was suggested that the prolonged inhibition arose from an extended lifetime of SNAP-25 (known to block exocytosis) (22, 23) and/or the continued activity of the toxin. To address the former possibility, the \( t_{1/2} \) of SNAP-25 in BoNT/A-pretreated neurons was assessed relative to the intact protein (Fig. 5). The cells were treated for 24 h with BoNT/A and subjected to a 4-h pulse labeling before being harvested (i.e. 0 h chase) or chased for the specified times in label-free medium (Fig. 5). After immunoprecipitation of SNAP-25, fluorography revealed time-dependent decreases in [35S]Met-SNAP-25 and -SNAP-25A (Fig. 5, A and B). Additionally, immunoblotting of the precipitates with an anti-SNAP-25 antiserum indicated that equivalent amounts of SNAP-25 were analyzed (Fig. 5, C and D) and that the toxin had proteolysed a substantial fraction in advance of pulse labeling (Fig. 5D). Less than 50% of the newly synthesized [35S]Met-SNAP-25 was proteolyzed by BoNT/A during the 4-h pulse labeling period (Fig. 5B; i.e. 0 h chase); this contrasts with the >85% cleavage of total SNAP-25.

---

**TABLE I**

Potencies and durations of inhibition of exocytosis by BoNTs in cerebellar granule neurons

| Purified nicked toxin | Concentration causing 50% blockade of transmitter release \( \mu M \) | \( t_{1/2} \) of inhibition \( \text{days} \pm \text{S.D.} \) |
|-----------------------|----------------------------------|---------------------|
| BoNT/A               | 10                               | \( >31^{*} \)       |
| BoNT/B               | 100                              | 9.84 ± 2.12         |
| BoNT/C1              | 13                               | \( >25 \)           |
| BoNT/E               | 43                               | 0.73 ± 0.11         |
| BoTx/F               | 1350                             | 1.76 ± 0.28         |
| TeTx                  | 6.5                              | Not studied         |

---

\( ^* \) These values were determined from detailed concentration dependence studies plotted in Figs. 2–4.

\( ^{*} \) The \( t_{1/2} \) is calculated by subjecting the time-dependent decreases of inhibition to first order decay analysis.

\( ^{*} \) No significant diminution in the extent of inhibition was noted at the listed times, in several experiments.
Durations of Transmitter Release Block by Botulinum Toxins

**Experimental Procedures**

**Durations of Transmitter Release Block by Botulinum Toxins**

![Fig. 4. BoNT/C1 potently blocks transmitter release for many weeks because of a corresponding persistence of SNAP-25A, and a reduced STx1 content; this serotype can cause cell death. Neurons (7-9 DIV) were incubated for 20 h, in the specified medium without the specified concentrations of BoNT/C1. After washing away the toxin, the medium was replaced, and the neurons were assayed 2 (filled symbols) or 18 (open symbols) days later. A, the extents of blockade of evoked transmitter release were measured (as in Fig. 1 legend) and expressed as the means ± S.D. (n = 3 or 4), B, neuronal protein was immunoblotted using IgG specified in the Fig. 2 legend. The data (means ± S.D.; n = 3) from densitometric scanning of blots were used to determine the extents of cleavage of SNAP-25 (2 days, •; 18 days, ○) or STx1 (2 days, ▼; 18 days, ▽). C, neuron survival at 2 (△) and 18 (□) days was assessed microscopically by counting viable cells. D, following a 2-h exposure in the absence or presence of BoNT/C1, equal amounts of protein were immunoblotted using the specified antibodies (an asterisk indicates the toxin-truncated product, STx1 1–23; this was only visible after a prolonged development time).**

![Fig. 5. [35S]Met-methionine pulse labeling and immunoprecipitation determine the t1/2 values of intact and SNAP-25, in developing neurons and demonstrate that BoNT/A protease persists for at least 3 weeks. Neurons cultured for 7 DIV were exposed for 16 h in the absence (A and C) or presence (B and D) of 100 pM BoNT/A prior to [35S]Met-methionine pulse labeling (see "Experimental Procedures"). After the specified chase, the neurons were washed, detergent solubilized, and immunoprecipitated. [35S]Met-labeled SNAP-25 was subjected to SDS-PAGE and fluorography (A and B) and Western blotting (C and D) using an anti-SNAP-25 Ig of an alternate species to that used for immunoprecipitation to ensure equal contents in each sample. E, the SNAP-25 bands were excised, and their radioactive contents were measured by scintillation counting; the data (± S.D.) are plotted from three experiments each performed in duplicate or triplicate; ○, SNAP-25; □, SNAP-25A. F, neurons cultured for 7 DIV were exposed for 24 h in the absence or presence of 10 pM BoNT/A and then maintained in culture without toxin for the specified period, prior to pulse labeling (with or without chase) and immunoprecipitation of SNAP-25. Immunoassayed SNAP-25 was fractionated by SDS-PAGE, and the newly synthesized radiolabeled protein was analyzed by fluorography and Western blotting (see "Experimental Procedures").

detected immunologically (Fig. 5D); therefore, newly synthesized SNAP-25 can only represent a minor portion of total SNAP-25. Accurate measurement of radioactivity remaining in the SNAP-25 bands from multiple experiments by scintillation counting (Fig. 5E) revealed time-dependent decreases in SNAP-25A in BoNT/A-treated cells with decay kinetics comparable with the intact protein in toxin-free cells. Extending the chase period beyond 4–8 days revealed a diminution of almost all of the residual [35S]Met-SNAP-25A detected (Fig. 5, B and E); the t1/2 values of SNAP-25 and SNAP-25A, extrapolated were 0.89 ± 0.28 and 0.95 ± 0.20 days, respectively (Fig. 5E). Therefore, the t1/2 of SNAP-25A does not account for the longevity of BoNT/A-induced inhibition (N.B. t1/2SNH > 31 days), at least, in these cultured central neurons.

Next, the persistence of the BoNT/A protease was assessed by examining whether the newly synthesized SNAP-25 was still being proteolyzed at various periods after toxin exposure, visualized using pulse labeling and immunoprecipitation (Fig. 5F). Thus, neurons incubated for 24 h in the absence or presence of 10 pM BoNT/A (a concentration sufficient to yield a nearly maximal SNAP-25 cleavage) were cultured in the absence of toxin for the specified period prior to pulse labeling and isolation of SNAP-25, as outlined above (Fig. 5F). Additionally, toxin-treated neurons were chased for 14 h to allow sufficient time for the toxin to proteolyze new [35S]Met-SNAP-25. Immunoblotting revealed ~90% cleavage of SNAP-25 in precipitates from type A-treated neurons at all periods examined (Fig. 5F). Importantly, 7, 15, and 20 days after intoxication, newly synthesized [35S]Met-SNAP-25 was still efficiently proteolyzed, particularly following the additional 14-h chase (Fig. 5F). Reduced neuron survival precluded assessments longer than 3 weeks. Therefore, the notable longevity of BoNT/A-induced inhibition in these cultured central neurons results from persistence of its protease.

**Co-exposure of BoNT/A-treated Neurons to Type E Failed to Shorten the Inhibition of Exocytosis: Removal of up to 26 Residues from SNAP-25 Did Not Alter Its Turnover—In view of the observed ability of BoNT/E to foreshorten the paralysis time induced by type A at human and murine neuromuscular junctions (16),2 neurons were pre-exposed for 24 h in the absence or presence of 10 pM type A (Fig. 6A, hatched bars) or the latter plus 2 nM BoNT/E (Fig. 6A, cross-hatched bars) prior to assessment of blockade of transmitter release and SNAP-25 cleavage (Fig. 6B). The BoNT/A and E concentrations employed yielded nearly maximal inhibition (Fig. 6A) and cleavage of intact SNAP-25 (Fig. 6B); SNAP-25E predominated in doubly treated cells, consistent with the ability of type E to proteolyze SNAP-25A as efficiently as intact substrate (39). Following a 7-day recovery period, sufficient for nearly complete recovery from the 2 nM BoNT/E used (Fig. 2A), evoked release from the...
Toxin(s) by washing, the blockade of evoked release was measured at the specified times (means ± S.E.; n = 4; see Fig. 1); the values in brackets represent percentages of inhibition of transmitter release relative to non-toxin-treated controls. The samples were blotted using anti-SNAP-25 A; anti-HV62 Igs (A); SNAP-25 A, or SNAP-25 C1-truncated SNAP-25, Sbr2, or STx1 in fully differentiated neurons. Cerebellar granule cells cultured for 16 DIV were exposed for 16 h in the absence or presence of the specified BoNT prior to [35S]methionine pulse labeling (see “Experimental Procedures”). After the specified chase, the neurons were washed and detergent-solubilized, and their [35S]-labeled SNAPERs were isolated by immunoprecipitation, using either SMI-81 Ig A; SNAP-25), anti-HV62 Igs (B; Sbr2), or HPC-1 Ig C; STx1). The samples were subjected to SDS-PAGE and fluorography (F) or Western blotting (W) to ensure equal SNAPER contents in each sample, using anti-SNARE antibodies generated in species different to those used for immunoprecipitation. Following fluorography, the SNAPER bands were excised, and their radioactive contents were measured by scintillation counting; the values for SNAP-25 (1), SNAP-25 A, SNAP-25 C1 (3), SNAP-25 C1 (1), Sbr2 (1), or STx1 (1) were expressed relative to their appropriate chase-free controls (means ± S.E.; n = 3 or 4).

Measurement of the $t_{1/2}$ of Sbr2 and STx1 would also indirectly indicate the rate of SNAPER synthesis. Multiple assessments of equivalent immunoprecipitated SNAPER samples from different chase periods revealed time-dependent decreases in radiolabeled Sbr2 and STx1, using fluorography (Fig. 7, B and C) and scintillation counting (Fig. 7D). A $t_{1/2}$ of 4–5 days was recorded for Sbr2, because the longest times employed (5 days) failed to yield a 50% reduction of radiolabeled STX1, the $t_{1/2}$ can only be estimated as ~6 days.

The Rates of Replacement of Truncated Sbr2 and STx1 in Cerebellar Neurons Are Not Primarily Responsible for the Intermediate or Long Inhibition Exhibited by BoNT/B or BoNT/C—Because prolonged inhibition by BoNT/B or BoNT/C1 may have arisen from slow rates of replacement of cleaved Sbr2 or STx1, this possibility was examined. Because mature neurons exhibit maximal SNAPER contents from ~13 DIV onwards (only diminished by gradual loss of cell numbers; Fig. 1C and data not shown), the rates of SNAPER synthesis and degradation must be equivalent in mature neurons (see “Discussion”). Therefore, measurement of the $t_{1/2}$ of Sbr2 and STx1 would also indirectly indicate the rate of SNAPER synthesis. Multiple assessments of equivalent immunoprecipitated SNAPER samples from different chase periods revealed time-dependent decreases in radiolabeled Sbr2 and STx1, using fluorography (Fig. 7, B and C) and scintillation counting (Fig. 7D). A $t_{1/2}$ of 4–5 days was recorded for Sbr2, because the longest times employed (5 days) failed to yield a 50% reduction of radiolabeled STX1, the $t_{1/2}$ can only be estimated as ~6 days.

The Rates of Replacement of Truncated Sbr2 and STx1 in Cerebellar Neurons Are Not Primarily Responsible for the Intermediate or Long Inhibition Exhibited by BoNT/B or BoNT/C—Because prolonged inhibition by BoNT/B or BoNT/C1 may have arisen from slow rates of replacement of cleaved Sbr2 or STx1, this possibility was examined. Because mature neurons exhibit maximal SNAPER contents from ~13 DIV onwards (only diminished by gradual loss of cell numbers; Fig. 1C and data not shown), the rates of SNAPER synthesis and degradation must be equivalent in mature neurons (see “Discussion”). Therefore,
its protease was directly examined using pulse labeling and immunoprecipitation. Neurons exposed for 24 h in the absence or presence of 100 µM BoNT/B were pulse labeled immediately (0 days) or cultured for 18 days prior to pulse labeling and Sbr2 isolation. This treatment caused 76.2 ± 3.9% (mean ± S.D.; n = 4) and 76.2 ± 5.0% (mean ± S.D.; n = 4) proteolysis of Sbr2, immunolabeled or radiolabeled; the values for intact Sbr remain intact. 

Therefore, persistence of the toxin protease activity is the primary determinant of the longevity of BoNT/B-induced inhibition of exocytosis. 

**DISCUSSION**

The detailed pulse-chase study of native and BoNT-cleaved SNAREs reported herein provides the first unambiguous and direct demonstration of a persistence of BoNT/A protease in central neurons, together with convincing evidence that it is the major factor responsible for prolonged inhibition of neuroexocytosis. Unexpectedly, SNAP-25A exhibited the same turnover rate as the full-sized protein in cerebellar neurons, in contrast with its reported persistence (2, 24) in peripheral motor nerve endings. Apparently, an exceptional situation must exist in motor nerve terminals in vivo (discussed in Refs. 2 and 22), allowing SNAP-25A to squat at the presynaptic membrane because co-treatment of human or murine endplates with BoNT/A and BoNT/E causes a rapid recovery, equivalent to that of BoNT/E alone (16). The latter would seem to exclude an adequate level of toxin protease persisting—but another study did not detect such a rescue although different conditions (e.g., higher toxin dose) were used (41)—though perturbation of the otherwise persistent BoNT/A protease activity or localization following treatment with BoNT/E cannot be precluded. Notably, BoNT/A protease persisted unabated for longer than 1 month in cerebellar neurons, thereby precluding BoNT/E-mediated rescue of exocytosis or depletion of SNAP-25A, the apparent lack of replacement of the latter has been observed previously for spinal cord neurons in culture, although SNAP-25 turnover or protease longevity were not directly measured (25). Similarly, a study performed on cultured neuroendocrine cells observed negligible recovery of catecholamine release or replacement of SNAP-25A over 2 months following BoNT/A treatment, apparently resulting from protease persistence (22). Therefore, SNAP-25A, but not the E-truncated protein, is retained in motor nerve terminals in vivo at the synaptic vesicle release sites; this intriguing dissimilarity with peripheral and central neurons in vitro warrants further investigation.

Despite the obvious differences that exist between central cerebellar neurons and motor nerves, many similar neuronal characteristics are conserved; these include common exocytic mechanisms and proteins, neurite extension, and synapse development. Also, our data reveal that picomolar concentrations of several BoNT serotypes block exocytosis when directly applied to central neurons in culture with potencies matching that observed for motor nerve terminals. In vivo, this has not been observed because toxin access to central and nonmotor spinal neurons is largely prevented by anatomical barriers (e.g., the blood brain barrier). Moreover, BoNTs do not exhibit detectable levels of retrograde transport, characteristic of TeTx. Preliminary unpublished studies comparing BoNT potency in cultured central neurons and motoneurons have indicated that BoNTs poison cholinergic nerves more rapidly. However, if toxin exposures are performed overnight (i.e. when the rate of toxin internalization is not the limiting factor), comparable potencies were observed in both cell types. Most importantly, however, for the purpose of this study concerned with the bases for the different longevities of BoNT serotypes, their relative lifetimes in these neurons are remarkably similar to the distinct durations of neuromuscular paralysis observed in vivo for rodents (see the Introduction).

Generation of an avid antibody specific for the LC protease of BoNT/E has allowed tracking of the minute quantities that remain after exposure to nanomolar concentrations. Immunoblotting of cell extracts, after a 2-h treatment with BoNT/E, for several chase periods up to 3 days later revealed that the majority of BoNT/E LC remained as a covalently linked dimer, inconsistent with its delivery to the cytosol (where it would have been reduced). Therefore, there are at least two pools of toxin in these neurons: endosomal and cytosolic. Although it was necessary to use concentrations of toxins supermaximal to those needed to inhibit exocytosis, nevertheless, the t½ values reported herein correspond to a t½ of ~16 h obtained for cell-associated BoNT/E LC immunoreactivity (data not shown).

The different degradation rates found herein for SNAP-25 in developing and mature cerebellar granule neurons (~1 and 2 days, respectively) accord with data from earlier studies (42), which showed that the accumulation of SNAP-25 during development of neurons results from both increased expression and...
reduced rates of degradation, processes that stabilize by 14 DIV. The \( t_{1/2} \) values of Sbr2 and STX1 in mature neurons (4–5 and 6–6 days) are reported for the first time. These collective findings allowed consideration of the contribution that toxin-truncated SNARE replacement makes to the different durations of transmitter release inhibition by BoNT serotypes. Indeed, the results suggest that the rate of SNAP-25 synthesis governs the length of BoNT/E-induced inhibition. Interestingly, removal of up to 26 C-terminal residues from SNAP-25 does not alter its degradation rate, implicating other signals for regulation of its turnover. The rates of synthesis and degradation of Sbr2 must be more rapid in developing neurons relative to the much longer \( t_{1/2} \) of 4–5 days observed for the fully mature protein (i.e. analogous to SNAP-25), because a \( t_{1/2} \) of 2 days was found for BoNT/F in developing neurons. Because another Sbr2-cleaving toxin, BoNT/B, persists for much longer (\( t_{1/2} \) \( \approx \) 10 days) than the periods required for SNARE synthesis or degradation of the truncated N-terminal fragment, persistence of its protease must account for the prolonged inhibition of exocytosis.

Recent work (43) highlighted the potential risks associated with the clinical use of large quantities of BoNT/B for achieving paralysis of medium length, because of a much reduced safety margin relative to BoNT/A. Although the \( t_{1/2} \) values determined herein are dependent upon both the times required for removal of the BoNT protease and replacement of cleaved SNARE with intact, protease persistence primarily dictates the larger \( t_{1/2} \) values measured in neurons treated with BoNT/A, BoNT/C1, or BoNT/B. Attempts by others to examine the \( t_{1/2} \) of the LC of the closely related Clostridial neurotoxin, TeTx, in cultured spinal neurons, found that a highly radio labeled toxin disappeared long before even an initial onset of recovery from blockade of neurotransmission (44); the authors correctly suggest that degradation of TeTx LC (\( t_{1/2} \) = 6 days) may underlie the slow recovery from neuroinhibition. Indeed, it has been estimated that only 10–100 intracellular toxin molecules are required to inhibit exocytosis (45), precluding straightforward radiolabeled detection; furthermore, this approach does not distinguish between relevant functional toxin protease in the cytosol and that which may reside in other cellular locations (i.e. endosomes). Therefore, the methodology used herein for measuring the kinetics of recovery from inhibition offers obvious advantages.

Detailed BoNT dose dependence studies revealed good correlations between losses of intact SNAREs and inhibition of evoked transmitter release, providing a direct demonstration of their involvement in up to 90% of the Ca\(^{2+}\)-dependent evoked glutamate exocytosis measured. Note that microanatomical features of motor neurons in vitro are not reproduced by neurons in culture (including motoneurons), and they could play important roles in determining the duration, localization, and molecular basis of paralysis (2). However, an imperfect relationship was observed regarding SNAP-25 content and inhibition of evoked release in BoNT/A-treated cells; this component of release (~30% of the total) is apparently mediated by SNAP-25A, because it was reduced by sequential BoNT/E administration. A similar situation has been found in permeabilized neuroendocrine cells (39, 46) and synaptosomes (47).

A small number of patients are primary nonresponders to BoNT/A therapy; also, multiple administrations may gradually elicit immunity in a tiny minority of responders and limit the efficacy of treatment (reviewed in Ref. 14). Therefore, an alternative serotype with the potency and duration of type A is required. In this context, these studies have demonstrated that BoNT/C1 may possess such therapeutic potential (17), except that it has been reported to impair neurite/axonal growth and cause cell death, an effect not ascribable to contamination (Ref. 20 and this work). From the present investigation, it seems that such BoNT/C1 toxicity may result from its proteolysis of STX1 because the dose dependence study revealed that only minimal cleavage of STX1 coincides with the lethal effects, whereas extensive SNAP-25 cleavage was not lethal; also, the SNAP-25/1 minus fragment is known to be nonlethal (22). Additional proteolysis of one or more of the other five syntaxin isoforms reported (9) has not been excluded; only STX4 and STX5 are known to be resistant to BoNT/C1 (reviewed in Ref. 3). An essential nonsynaptic vesicle docking fusion role for STX1 in developing neurons is suggested by its notable abundance in immature cerebral neurons, which are almost devoid of the other SNAREs and lack the functional Ca\(^{2+}\)-dependent exocytotic machinery (Fig. 1C). In conclusion, this first detailed examination of the molecular basis for the extended action of BoNT/A relative to shorter acting serotypes in neurons has provided novel information that should aid the extension of therapies as well as the development of countermeasures for botulism.

Acknowledgments—We thank M. C. Goodnough, W. H. Tepp, and C. J. Molizio for purifying BoNT/B and/E in the laboratory of E. A. Johnson.

REFERENCES
1. Cherington, M. (1998) *Mucosal Res.* 21, 701–710
2. Dolly, J. O., Liak, G., Foran, P. G., Meunier, F., Mohammed, N., O’Sullivan, G., and dePauw, A. (2002) in *Scientific and Therapeutic Aspects of Botulinum Toxins* (Brin, M., Jankovic, J., and Hallet, M., eds.) pp. 91–102, Lippincott Williams and Wilkins, Philadelphia, PA
3. Schiavo, G., Matteoli, M., and Montecucco, C. (2000) *Physiol. Rev.* 80, 717–766
4. Dolly, J. O., Black, J., Williams, R. S., and Melling, J. (1984) *Nature* 347, 457–460
5. Daniels-Holgate, P. U., and Dolly, J. O. (1996) *J. Neurosci. Res.* 44, 263–271
6. Black, J. D., and Dolly, J. O. (1996) *J. Cell Biol.* 130, 534–544
7. Sollier, T., Bennett, M. K., Newlands, S. A., Scheller, R. H., and Rothman, J. E. (1993) *Cell* 73, 409–418
8. Oyler, G. A., Higgins, G. A., Hart, R. A., Battenberg, E., Billingsley, M., Bloom, F. E., and Wilson, M. C. (1989) *J. Cell Biol.* 109, 3039–3052
9. Bennett, M. K., Garcia-Arraras, J. E., Elferink, L. A., Peterson, K., Fleming, A. M., Hanaka, C. D., and Scheller, R. H. (1993) *Cell* 74, 863–873
10. Trimble, W. S., Cowan, D. M., and Scheller, R. H. (1988) *Proc. Natl. Acad. Sci. U. S. A.* 85, 4538–4542
11. Baumert, M., Maycox, P. R., Navone, F., De Camilli, P., and Jahn, R. (1989) *EMBO J.* 8, 379–384
12. Hayashi, T., McMahon, H., Yamasaki, S., Bine, T., Hata, Y., Sudhof, T. C., and Niemann, H. (1994) *EMBO J.* 13, 5051–5061
13. Pellegrini, L. L., O’Connor, V., Lottspeich, F., and Betz, H. (1995) *EMBO J.* 14, 4705–4713
14. Brin, M. F. (1997) *Muscle Nerve* 26, (suppl.) 146–168
15. Sloop, R. R., Cole, B. A., and Escutin, R. O. (1997) *Neurology* 40, 189–194
16. Eleopra, R., Tugnoli, V., Rossotto, O., De Grandis, D., and Montecucco, C. (1998) *Neurosci. Lett.* 256, 135–138
17. Eleopra, R., Tugnoli, V., Rossotto, O., Montecucco, C., and De Grandis, D. (1997) *Neurosci. Lett.* 224, 91–94
18. de Paiva, A., Meunier, F. A., Molgo, J., Aoki, K. R., and Dolly, J. O. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 3200–3205
19. Juraskins, C. V., Lieth, E., Dang Do, A. N., and Schengrund, C. L. (2001) *Toxicon* 39, 1309–1315
20. Williamson, L. C., and Neale, E. A. (1998) *J. Neurosci. Res.* 56, 569–583
21. Huang, X. H., Wheeler, M. B., Kang, Y. H., Xu, L., Lukaas, G. L., Trimble, W. S., and Gaisano, H. Y. (1998) *Neurosci. Lett.* 256, 135–138
22. Racibraska, D. A., and Charlton, M. P. (1999) *Can. J. Physiol. Pharmacol.* 77, 679–688
23. Keller, J. E., Neale, E. A., Oyler, G., and Adler, M. (1999) *FEBS Letts* 456, 137–142
24. Lawrence, G. W., Foran, P., and Dolly, J. O. (1996) *Eur. J. Biochem.* 236, 877–886
25. Chen, F. S., Foran, P., Shone, C. C., Foster, R. A., Melling, J., and Dolly, J. O. (1999) *Biochemistry* 38, 5719–5728
26. Cambray-Dean, M. A. (1995) in *Neural Cell Culture: A Practical Approach* (Cohen, J., and Wilkin, G. P., eds.) pp. 3–13, IRL Press, Oxford, UK
27. Thangnipon, W., Kingsbury, A., Webb, M., and Balazs, R. (1993) *Brain Res.* 582, 189–191
28. Thangnipon, W., Kingsbury, A., Webb, M., and Balazs, R. (1998) *Brain Res.* 834, 17–25
29. Gallo, V., Ciotti, M. T., Coletti, A., Aloisi, F., and Levi, G. (1982) *Proc. Natl. Acad. Sci. U. S. A.* 79, 7919–7923
Durations of Transmitter Release Block by Botulinum Toxins

32. Schramm, M., Eimerl, S., and Costa, E. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1193–1197
33. Gallo, V., Kingsbury, A., Balazs, R., and Jorgensen, O. S. (1987) J. Neurosci. 7, 2203–2213
34. Van Vliet, B. J., Sebben, M., Dumuis, A., Gabrion, J., Bockaert, J., and Pin, J. P. (1989) J. Neurochem. 52, 1229–1239
35. Cousin, M. A., and Nicholls, D. G. (1997) J. Neurochem. 69, 1927–1935
36. Ravichandran, V., Chawla, A., and Roche, P. A. (1996) J. Biol. Chem. 271, 13300–13303
37. Foran, P. G. P., Fletcher, L. M., Oatey, P. B., Mohammed, N., Dolly, J. O., and Tavare, J. M. (1999) J. Biol. Chem. 274, 28087–28095
38. Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Delaureto, P. P., DasGupta, B. R., and Montecucco, C. (1992) Nature 359, 832–835
39. Lawrence, G. W., Foran, P., Mohammed, N., DasGupta, B. R., and Dolly, J. O. (1997) Biochemistry 36, 3961–3967
40. Foran, P., Lawrence, G., and Dolly, J. O. (1995) Biochemistry 34, 5494–5503
41. Adler, M., Keller, J. E., Sheridan, R. E., and Deshpande, S. S. (2001) Toxicon 39, 253–243
42. Sanders, J. D., Yang, Y., and Liu, Y. (1998) J. Neurosci. Res. 53, 670–676
43. Aoki, K. R. (2002) Toxicon 40, 925–928
44. Habig, W. H., Bigalke, H., Bergey, G. K., Neale, E. A., Hardegree, M. C., and Nelson, P. G. (1986) J. Neurochem. 47, 930–937
45. Erdal, E., Bartels, F., Binscheck, T., Erdmann, G., Frevert, J., Kistner, A., Weller, U., Wever, J., and Bigalke, H. (1995) Naunyn-Schmiedeberg's Arch. Pharmacol. 351, 67–78
46. Dolly, J. O., Lawrence, G. W., and Foran, P. (1999) in Proceedings of Biomedical Aspects of Clostridial Neurotoxins, International Conference, Oxford (Tranter, H. S., ed) pp. 97–102, Center for Applied Microbial Research, Salisbury, UK
47. Ashton, A. C., and Dolly, J. O. (2000) J. Neurochem. 74, 1979–1988
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

Patrick G. Foran, Nadiem Mohammed, Godfrey O. Lisk, Sharuna Nagwaney, Gary W. Lawrence, Eric Johnson, Leonard Smith, K. Roger Aoki and J. Oliver Dolly

J. Biol. Chem. 2003, 278:1363-1371.
doi: 10.1074/jbc.M209821200 originally published online October 14, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M209821200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 11 of which can be accessed free at http://www.jbc.org/content/278/2/1363.full.html#ref-list-1