Distinct Kinetic Changes in Neurotransmitter Release After SNARE Protein Cleavage

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Science **309**, 491 (2005);
DOI: 10.1126/science.1112645

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26. This work was supported by Ministero dell'Istruzione, dell'Università e della Ricerca Prin grants to A.B., G.V., and E.P, and by a Bracco spa grant to G.B. and E.P.

Supporting Online Material
www.sciencemag.org/cgi/content/full/309/5733/488/DC1
Materials and Methods
References

3 February 2005; accepted 17 May 2005
10.1126/science.1110625

Distinct Kinetic Changes in Neurotransmitter Release After SNARE Protein Cleavage

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Neurotransmitter release is triggered by calcium ions and depends critically on the correct function of three types of SNARE [soluble *N*-ethylmaleimide–sensitive factor attachment protein (SNAP) receptor] proteins. With use of the large calyx of Held presynaptic terminal from rats, we found that cleavage of different SNARE proteins by clostridial neurotoxins caused distinct kinetic changes in neurotransmitter release. When elevating calcium ion concentration directly at the presynaptic terminal with the use of caged calcium, cleavage of SNAP-25 by botulinum toxin A (BoNT/A) produced a strong reduction in the calcium sensitivity for release, whereas cleavage of syntaxin using BoNT/C1 and synaptobrevin using tetanus toxin (TeNT) produced an all-or-nothing block without changing the kinetics of remaining vesicles. When stimulating release by calcium influx through channels, a difference between BoNT/C1 and TeNT emerged, which suggests that cleavage of synaptobrevin modifies the coupling between channels and release-competent vesicles.

Clostridial neurotoxins, which cleave SNARE [soluble *N*-ethylmaleimide–sensitive factor (NSF) attachment protein (SNAP) receptor] proteins (*1*), block Ca^{2+}-dependent neurotransmitter release (*2*). Distinct kinetic differences in their action (*3*, *4*) indicate that it matters which of the SNAREs is cleaved and at what particular site (Fig. 1A). However, studies disagree in their mechanistic interpretations regarding toxin action (*3*–*9*). Conventional synapses allow only limited manipulations at the presynaptic terminal, rendering it difficult to discern which steps between Ca^{2+} entry and transmitter release are impaired by a given toxin. The calyx of Held, a large glutamatergic nerve terminal in the auditory pathway, can be voltage-clamped (*10*, *11*); the intracellular Ca^{2+} concentration ($[Ca^{2+}]$) can be manipulated by caged Ca^{2+} as well as by controlled Ca^{2+} influx (*12*, *13*); and recombinantly produced light chains of the toxins can be introduced directly into the terminal. This allows for testing toxin action acutely, applying stimuli of graded strength, and monitoring Ca^{2+} influx. By using these possibilities, we uncovered remarkable differences in the action of toxins.

Presynaptic terminals were stimulated by voltage-clamp depolarization, and two toxins cleaving either syntaxin (BoNT/C1) (Fig. 1B) or SNAP-25 (BoNT/A) (Fig. 1C) were infused by a patch pipette (*14*). In each case, a pulse protocol consisting of 10 action potential–like (AP-like) depolarizations followed by a 50-ms depolarization was repeatedly applied to the presynaptic terminal. The presynaptic Ca^{2+} current did not change appreciably during the 10-min recording period (89 ± 7% and 91 ± 5%) (top traces, Fig. 1, B and C). The excitatory postsynaptic current (EPSC), however, changed strongly in this time interval (middle traces).

The earliest records (blue) were taken at a time when toxin action was still modest. Similar to control, the EPSCs evoked by AP-like pulses displayed facilitation during the first two to three stimuli, followed by depression. Subsequent long-lasting depolarizations elicited large EPSCs, which were sufficient to release all remaining vesicles of the releasable pool (RP) (*15*, *16*). The pattern of change during toxin action was simplest for the action of BoNT/C1 (Fig. 1B). About 10 min after the start of toxin infusion, only small postsynaptic currents were observed during both the initial 10 AP-like pulses and the long-lasting depolarization. At an intermediate time (red trace, Fig. 1B), EPSCs were substantially reduced both for AP-like and long depolarizations. The sequence of facilitation and depression was preserved throughout the time course of the experiment (Fig. 1B right). The pattern of change was strikingly different for BoNT/A (Fig. 1C): At about 10 min of toxin action, the responses to the AP-like pulses were completely blocked, whereas cumulative release elicited by the long depolarization was still almost intact (88 ± 9.0%, $n = 5$). Furthermore, at an intermediate time (7 min) responses during the AP-like pulses facilitated more strongly (Fig. 1C right).

The gradual and uniform decrease observed under BoNT/C1 is compatible with an all-or-nothing block of release sites, whereas the distinct kinetic changes induced by BoNT/A call for other mechanisms of action. Further characterization of the mechanisms is difficult to achieve by voltage-clamp experiments alone, because elevation of intracellular $[Ca^{2+}]$ through Ca^{2+} channels is spatially not homogenous, and different vesicles may be exposed to different $[Ca^{2+}]$ signals (*17*, *18*). Ca^{2+} uncaging circumvents this problem by elevating $[Ca^{2+}]$ uniformly within the presynaptic terminal. We infused a mixture of the caged-Ca^{2+} compound DM-Nitrophen (Calbiochem, Bad Soden, Germany) and the Ca^{2+} indicator dye Fura 2FF (TEFLABS, Austin, TX) into the cell together with toxins and rapidly elevated $[Ca^{2+}]$ by an ultraviolet flash to around 10 μM (Fig. 2). This $[Ca^{2+}]$ is within the range postulated to occur during nerve-evoked action potentials (*12*, *13*). Comparing control (Fig. 2A) with BoNT/C1 (Fig. 2B), BoNT/A (Fig. 2C), and a third toxin, tetanus toxin (TeNT) (Fig. 2D), which cleaves synaptobrevin. The absolute magnitudes of the EPSCs were found to be smaller under the influence of toxins. In all cases, the flash was followed after 60 ms by a long-lasting depolarization. At 8 min of toxin infusion, the total number of vesicles released shortly after the flash was 3120 ± 348 vesicles under control conditions and 1347 ± 258 vesicles and 995 ± 161 vesicles under BoNT/C1 and TeNT, respectively. However, the vesicles that escaped toxin action were released with a time course similar to that of control for both BoNT/C1 and TeNT (*14*). Subsequent depolarization evoked little further release. In contrast, the step-like elevation of $[Ca^{2+}]$ to 10 μM elicited only a

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trickle of release under BoNT/A (Fig. 2C right), whereas the depolarization, which further elevated [Ca^{2+}], led to a substantial EPSC. The amplitudes of [Ca^{2+}] and peak release rates (shown as rates per vesicle, i.e., measured peak rates during the flash response divided by the total number of vesicles released by flash and depolarization combined) indicate that the release rate of vesicles was reduced by almost two orders of magnitude under BoNT/A, whereas the remaining release-competent vesicles under BoNT/C1 and TeNT responded with about the same rate as that under control (Fig. 2E).

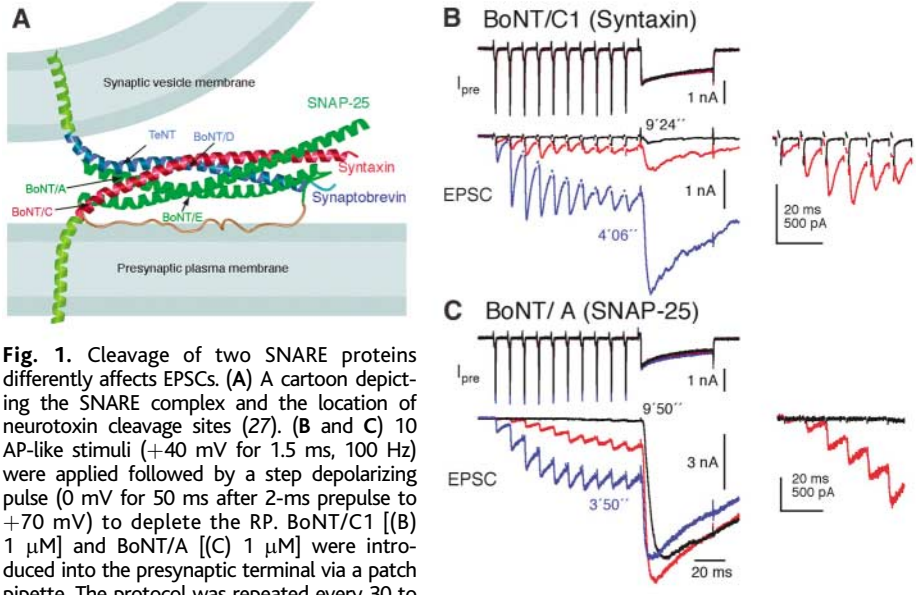

Fig. 1. Cleavage of two SNARE proteins differently affects EPSCs. (**A**) A cartoon depicting the SNARE complex and the location of neurotoxin cleavage sites (*27*). (**B** and **C**) 10 AP-like stimuli (+40 mV for 1.5 ms, 100 Hz) were applied followed by a step depolarizing pulse (0 mV for 50 ms after 2-ms prepulse to +70 mV) to deplete the RP. BoNT/C1 [(B) 1 μM] and BoNT/A [(C) 1 μM] were introduced into the presynaptic terminal via a patch pipette. The protocol was repeated every 30 to 45 s, and representative traces (presynaptic Ca^{2+} currents and EPSCs) at times early (blue), intermediate (red), and late (black) during dialysis of toxins were superimposed. Time of recording (after break-in) is indicated in minutes (′) and seconds (″). The first six EPSCs at intermediate and late during dialysis are shown on the right. In all experiments cyclthoazide and kynurenic acid were present (*14*).

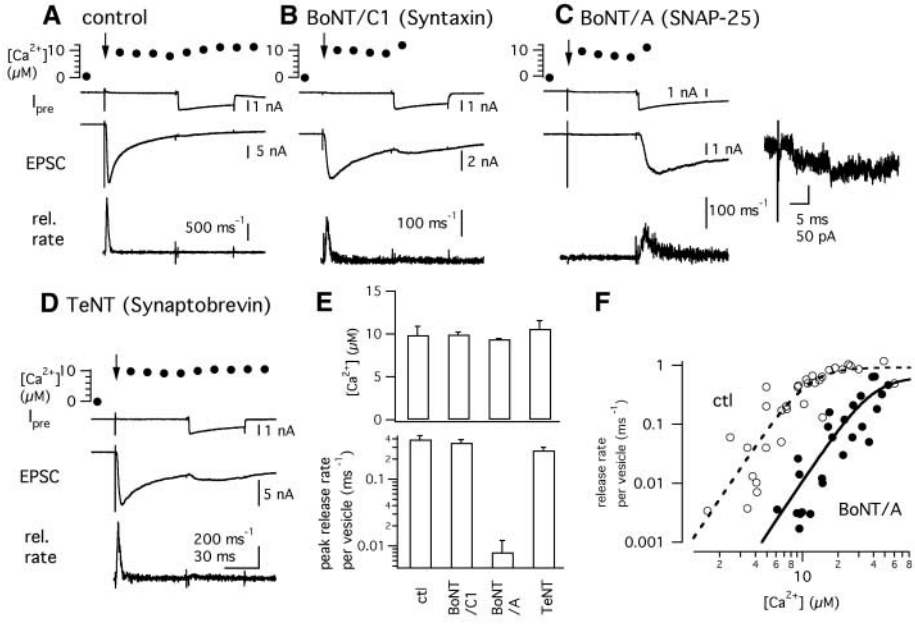

Fig. 2. The time course of transmitter release evoked by flash photolysis is altered only by BoNT/A. (**A** to **D**) A step-like [Ca^{2+}] increase from the basal concentration to 10 μM was obtained by flash photolysis of DM-Nitrophen (top). A depolarizing pulse was applied 60 ms after the flash to deplete the RP. Presynaptic Ca^{2+} currents, EPSCs, and release (rel.) rates are also shown. (A) to (D) are from control and in the presence of BoNT/C1 (2 μM), BoNT/A (1 μM), and TeNT (5 μM), respectively. Expanded EPSC trace under BoNT/A is shown on the right (C). (**E**) Average amplitudes of [Ca^{2+}] steps (top) and peak release rates per vesicle (bottom) during flash responses in control (ctl, six cells), BoNT/C1 (six cells), BoNT/A (six cells), and TeNT (five cells). (**F**) The relationship between [Ca^{2+}] and peak release rates per vesicle was plotted under control conditions (open circles, n = 14 cells) and in the presence of BoNT/A (solid circles, n = 13 cells). The data were fitted with a Hill coefficient of 3.

We further characterized the BoNT/A effect by performing Ca^{2+} uncaging with flashes of varying strengths, causing step-like Ca^{2+} increases to between 1 and 60 μM. Comparing peak release rates (per vesicle) for control cells and cells with BoNT/A indicated that BoNT/A decreased the Ca^{2+} sensitivity of vesicles about fourfold without a change in the slope of the Ca^{2+} dose-response curve (Fig. 2F). Release rates under BoNT/A reached values almost as high as those of control in the [Ca^{2+}] range of 50 to 60 μM (Fig. 2F). Further experiments using the Ca^{2+} ramp method (fig. S1) confirmed that BoNT/A strongly reduced the apparent Ca^{2+} sensitivity, whereas BoNT/C1 and TeNT only led to minimal kinetic changes (at most a factor of 2 in release rate) in the [Ca^{2+}] range of <10 μM. The results exclude the possibility that BoNT/A reduces maximal rate of secretion (*4*, *19*) or changes cooperativity for fusion (*20*, *21*). Rather, the shift of apparent Ca^{2+} affinity is best explained by an allosteric model of Ca^{2+} release coupling, as recently suggested to explain phorbol ester effects (*22*).

None of our Ca^{2+} uncaging experiments revealed any difference between cleavage of syntaxin and synaptobrevin, with all findings being readily interpretable as all-or-nothing blocks. However, when we studied TeNT (and BoNT/D, both cleaving synptobrevin) with the use of voltage-clamp depolarization, subtle but important differences in the action of two toxins became apparent. First, we observed that, in a pulse protocol as in Fig. 1, EPSCs after partial TeNT block showed different degrees of block for the responses to short and long depolarizations. At times when the cumulative amount of release during AP-like pulses was reduced to 37 ± 5% of control values, the release during a long depolarization was still 83 ± 13% (n = 5, fig. S2). Differences between TeNT and BoNT/C1 were most pronounced during long-lasting depolarizations. Thus, we analyzed release by using the deconvolution method (Fig. 3) (*14*, *23*) and applying 50-ms depolarizing pulses every 30 to 45 s with 0.5 mM EGTA in the presynaptic pipette. Such pulses deplete the RP almost completely, and two kinetic components of release can be separated under these conditions (time constants of 2 to 3 ms and 20 to 30 ms) (*16*). The amplitude of the fast component corresponds roughly to the cumulative amount of release by high-frequency trains (*15*). For control traces (Fig. 3A), cumulative release was fitted by a double exponential with an average RP size of 2901 ± 381 vesicles (n = 5), which remained constant at 98 ± 5.6% over 548 ± 17 s after the start of whole-cell recording (see Table 1 for kinetic

details). The amount of presynaptic Ca^{2+} influx during depolarizations stayed constant under control and also in the toxin experiments. Comparing the effect of BoNT/C1 to that of TeNT, in both cases EPSCs decreased strongly during 8 to 12 min of toxin action (Fig. 3, B and C). In the case of BoNT/C1, the decrease was uniform along the whole trace. Cumulative release decreased during toxin action without major changes in kinetics. This is best seen when traces were normalized to their values at 50 ms (Fig. 3B right). In contrast, under TeNT, cumulative release preferentially lost its fast component (Fig. 3 and Table 1). We then asked whether TeNT might also affect the rate at which new vesicles are recruited after depletion of the RP. Indeed, replenishment rates of the fast component under TeNT were slower than those of controls (fig. S4). In contrast, BoNT/C had no effects on the time course of recovery, showing once more that the two toxins act differently (fig. S4).

We postulate that the additional kinetic effects of TeNT most likely result from changes in the efficiency by which Ca^{2+} currents increase Ca^{2+} concentration at the Ca^{2+} sensor of the release apparatus (*17*, *18*), because differences between TeNT and BoNT/C1 were not observed when Ca^{2+} was directly applied to presynaptic terminals by caged Ca^{2+}. Furthermore, vesicles responded slowly to depolarizing stimuli after TeNT action, as if they were exposed to a reduced calcium concentration, in spite of the fact that Ca^{2+} currents were not reduced by any of the toxins (Figs. 1 and 3).

This effect of TeNT has interesting implications with respect to the general problem of Ca^{2+} channel–release coupling and the heterogeneity of release readiness of vesicles in the calyx of Held. The pool of release-ready vesicles can be divided into one subpool, which upon prolonged depolarization releases rapidly, and another one, which releases about 10-fold slower (*16*). The rapidly releasing pool recovers slowly upon depletion by strong stimulation, whereas the slowly releasing one recovers rapidly (*15*, *16*). The simplest explanation for these findings assumes that vesicles, after docking at the membrane, quickly reach a state in which their release apparatus is fully fusion-competent and only later become linked to regions of high Ca^{2+} channel density, which renders them more sensitive to Ca^{2+} influx. Such a sequence of events was suggested to explain the properties of different populations of vesicles visible by total internal reflection microscopy at terminals of retinal bipolar cells (*24*). In order to explain the special kinetic effect of TeNT, we only have to assume that after toxin action this maturation step cannot happen or happens slower (fig. S4), either because of the loss of the postulated interaction between vesicles and special release sites or because of a block of such sites by vesicles with compromised release machineries or with defective endocytosis (*25*). Asynchronous release during high-frequency stimulation persists under TeNT (*3*, *8*), and slowly releasing vesicles, which are less sensitive to TeNT (Fig. 2), contribute to asynchronous release during high-frequency stimulation at the calyx of Held (*26*). Asynchronous release, therefore, is likely to represent vesicles that have not yet reached their optimal position within the active zone.

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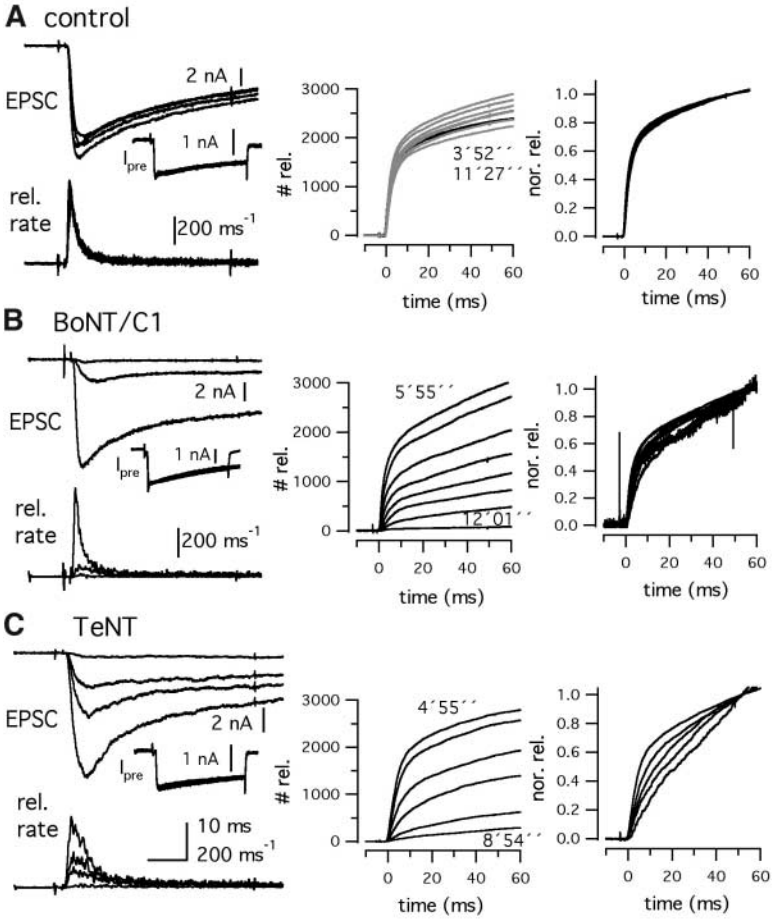

Fig. 3. Kinetic analysis of transmitter release using long-duration depolarizing steps. (**A** to **C**) Depolarizing pulses (to 0 mV for 50 ms after pre-depolarization to +70 mV for 2 ms) were applied every 30 to 45 s. This interstimulus interval is long enough such that responses recovered completely. Presynaptic patch pipettes contained 0.5 mM EGTA. Traces obtained at different times of whole-cell recording are superimposed. The left graphs show EPSCs, release rates, and presynaptic I_{Ca} as inset. The center and right graphs show cumulative release (number of vesicles) during depolarization and after normalization to the value at 50 ms, respectively.

Table 1. Effects of toxins on the time course of release during a depolarizing pulse. Dashed entries indicate not determined.

Condition	*N*	Early period (for control, initial 10 min)		Intermediate period		
		τ_1 (fraction)	τ_2	τ_1 (fraction)	τ_2	Inhibition of total release
Control	5	3.0 ± 0.4 ms (58 ± 4%)	41 ± 8 ms	—	—	—
BoNT/C1	5	3.8 ± 0.6 ms (53 ± 3%)	35 ± 5 ms	5.8 ± 0.7 ms (50 ± 7%)	55 ± 8 ms	34 ± 8%
TeNT	5	3.4 ± 0.7 ms (60 ± 2%)	34 ± 5 ms	21 ± 0.7 ms (100%)	—	32 ± 5%

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28. This study was supported by Deutsche Forschungsgeminschaft Center for Molecular Physiology of Brain. We thank R. Schneggenburger and S. Young for comments on the study.

Supporting Online Material
www.sciencemag.org/cgi/content/full/309/5733/491/DC1
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Figs. S1 to S4
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23 March 2005; accepted 1 June 2005
10.1126/science.1112645