Interaction of 125I-Labeled Botulinum Neurotoxins with Nerve Terminals.  
I. Ultrastructural Autoradiographic Localization and Quantitation of Distinct Membrane Acceptors for Types A and B on Motor Nerves  

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Abstract. The labeling patterns produced by radioiodinated botulinum neurotoxin (125I-BoNT) types A and B at the vertebrate neuromuscular junction were investigated using electron microscopic autoradiography. The data obtained allow the following conclusions to be made. (a) 125I-BoNT type A, applied in vivo or in vitro to mouse diaphragm or frog cutaneous pectoris muscle, interacts saturably with the motor nerve terminal only; silver grains occur on the plasma membrane, within the synaptic bouton, and in the axoplasm of the nerve trunk, suggesting internalization and retrograde intra-axonal transport of toxin or fragments thereof. (b) 125I-BoNT type B, applied in vitro to the murine neuromuscular junction, interacts likewise with the motor nerve terminal except that a lower proportion of internalized radioactivity is seen. This result is reconcilable with the similar, but not identical, pharmacological action of these toxin types. (c) The saturability of labeling in each case suggested the involvement of acceptors; on preventing the internalization step with metabolic inhibitors, their precise location became apparent. They were found on all unmyelinated areas of the nerve terminal membrane, including the preterminal axon and the synaptic bouton. (d) Although 125I-BoNT type A interacts specifically with developing terminals of newborn rats, the unmyelinated plasma membrane of the nerve trunk is not labeled, indicating that the acceptors are unique components restricted to the nerve terminal area. (e) BoNT types A and B have distinct acceptors on the terminal membrane. (f) Having optimized the conditions for saturation of these binding sites and calibrated the autoradiographic procedure, we found the densities of the acceptors for types A and B to be ~150 and 630/μm² of membrane, respectively. It is proposed that these membrane acceptors target BoNT to the nerve terminal and mediate its delivery to an intracellular site, thus contributing to the toxin's selective inhibitory action on neurotransmitter release.

BOTULINUM toxins, produced by the anaerobic bacterium Clostridium botulinum in several antigenically different but structurally related forms, are the most neurotoxic agents known, and are responsible for the neuroparalytic condition, botulism (reviewed in reference 39). Although many of these toxins occur in association with other protein(s) that exhibit haemagglutinin activity, the isolated neurotoxic protein has a molecular weight of ~150,000 and normally consists of two heterologous polypeptides (~100,000 and ~50,000). The unique toxicity of both the free botulinum neurotoxin (BoNT) (4, 37, 38, 42, 43) and its complex (5) is attributable to their ability to inhibit reversibly the spontaneous and evoked release of acetylcholine at peripheral synapses. The specificity of this action on the nerve terminal is highlighted by the absence of measurable effects on any other parameters (6, 18). Therefore, BoNT may prove useful for the identification of functional membrane components on motor nerves and in the elucidation of cellular processes concerned with transmitter release.

Light-microscopic autoradiographic studies have shown that 125I-labeled BoNT type A binds specifically (7, 22) and saturably (7) to components of the neuromuscular junction in mouse diaphragm. The degree of resolution at the level of the light microscope, however, did not permit precise localization of the label in the synaptic region. Ultrastructural studies were necessary to distinguish between pre- and postsynaptic labeling and to establish whether the neurotoxin was associated with the membrane and/or intracellular targets. Using a radioiodinated derivative of BoNT type A (125I-BoNT) that exhibits a high level of neurotoxicity (46), direct evidence for the saturable interaction of BoNT type A solely with the motor nerve terminal was presented for the first time (8). When nerve–muscle preparations were treated with this neurotoxin in vitro, under conditions known to block neurotransmission (2), resulting autoradiograms showed a dis-
tinct deposition of silver grains at motor nerve endings. The labeling was not restricted to the plasma membrane of the synaptic bouton, as silver grains were also present within the terminal cytoplasm. This finding was consistent with the suggestion (38) that the toxin's neuroparalytic action involves a target site within the nerve terminal.

It is important to note that the acceptors for the toxin were found (8) to be distributed uniformly on all areas of the synaptic bouton membrane; i.e., they were not restricted to the active zones where neurotransmitter release is thought to occur (21). However, it is not known whether these membrane acceptors are unique to the terminal membrane or if they are also present on the myelinated axon. Furthermore, in relation to the basis of BoNT-induced neuroparalysis, it is of interest to obtain ultrastructural evidence for the proposed transport of BoNT (15) along the nerve trunk. It needs to be established whether BoNT type A and type B, similar in structure and overall pharmacological action (cf. references 37, 39, and 41), share common neuronal acceptors at the neuromuscular junction.

In this study, distinct populations of acceptors for 125I-BoNT types A and B were localized on all areas of the unmyelinated motor nerve terminal using electron microscopic autoradiography. Moreover, the density of these acceptors was determined after conditions for their saturation had been established; their role in targeting BoNT to cholinergic nerve terminals and in mediating the subsequent internalization is discussed with respect to the molecular basis of botulism.

Materials and Methods

Radioiodination of Neurotoxins and Bovine Serum Albumin (BSA)

BoNT types A and B were purified and radiolabeled by Dr. R. Williams of this laboratory (43, 46). Radioiodination was carried out using the chloramine-T method of Greenwood et al. (13), with important modifications. The 125I-labeled preparations were of high specific radioactivity (450-1,700 Ci/mmol) and retained 60-85% of the neurotoxicity of native toxin preparations (representing 2 × 10^9 and 1.1 × 10^9 of the amount of toxin that killed 50% of injected mice in 4 d (LD50/mg for types A and B, respectively). BSA was radioiodinated in a similar manner; after accurate determination of its specific radioactivity, it was used as a standard for measuring the efficiency of the autoradiographic procedure.

Tissue Labeling

In vivo studies. Mice were injected intraperitoneally with 125I-BoNT type A (1 μg, equivalent to 8.8 × 10^8 mouse LD50 in 100 μl phosphate-buffered saline (PBS)/1% BSA; after death due to respiratory failure (~2 hr after injection), the paralyzed diaphragm was excised, washed, and processed for electron microscopic autoradiography as detailed below.

In vitro studies. Small pieces of mouse diaphragm tissue were incubated with 125I-BoNT type A or B in 0.5 ml Krebs-Ringer's solution (124 mM NaCl, 5 mM KCl, 1.175 mM CaCl2, 10 mM glucose, 1.3 mM MgSO4, 1.2 mM KH2PO4, and 20 mM Na2HPO4, previously gassed for at least 30 min with 95% O2-5% CO2 and adjusted to pH 7.4) for 90 min at 22°C before extensive washing in Krebs-Ringer's solution at 4°C (five times, 5 min each) and fixation with 2% glutaraldehyde in the latter solution. After brief washing, the endplate regions were dissected, cut into small sections (~1 X 0.5 mm), and treated with 1% OsO4 for 1 hr, before being dehydrated in ethanol and embedded in Spurr's resin. Cutaneous pectoris muscle from a frog was processed similarly except that amphibian Ringer's solution (116 mM NaCl, 2 mM KCl, 2 mM CaCl2, 0.5 mM Na2HPO4, 3 mM glucose, and 5 mM Hepes [pH 7.2]) was used for the incubations and washes. Control samples were treated as above except that a 50-100-fold excess of native BoNT type A or B was included in the incubation medium.

To examine the basis of toxin internalization, mouse diaphragm tissue was pretreated with 15 mM Na azide and labeling with 125I-BoNT types A and B was then examined. Concentration dependence of toxin binding was studied by incubating small pieces of the tissue with toxin concentrations of 125I-BoNT (1.5-150 nM) for 90 min at 22°C, while the time course was investigated by labeling with type A radiolabeled toxin for various times (20-150 min), followed by the standard processing for microscopy. The distribution of acceptors for 125I-BoNT type A along the motor nerve axon was investigated using the diaphragm of newborn rats, because the motor nerve is unmyelinated at this stage in development. The interaction of the different toxin types (A and B) at the motor nerve terminal was studied by pretreating the tissue with unlabeled toxin of one type for 1 h at 22°C followed by incubation with labeled toxin of the other type (for 90 min).

Electron-microscopic Autoradiography

Autoradiograms were prepared essentially by the method of Salpeter and Bachmann (33). Ultrastructural sections of pale gold interference color were cut using an ultramicrotome (LKB Instruments, Inc., Gaithersburg, MD) and transferred to formvar-coated slides with a platinum wire loop. At least six groups of three to five test sections from a minimum of two different blocks were placed along one side of the slide and at least three groups of two to three control sections were arranged on the other. This ensured that all sections were exposed under the same conditions. The slides were then dipped in Ilford L4 nuclear emulsion (8 g in 24 ml) at 32°C using a semiautomatic coating device; the speed of withdrawal was adjusted to give a monolayer of crystals with a purple interference color (~170 nm thick). Slides were exposed for 3-6 wk at 4°C in light-tight plastic slide boxes. The emulsion was then developed in Kodak D-19 developer, fixed in 25% sodium thiosulfate, and the formvar film was stripped off onto the surface of distilled water. Sections were picked up with paraffin and stained with 12% uranyl acetate for 20 min before being viewed in a Hitachi-600 electron microscope.

Quantitation

For comparison of the extent of labeling under different conditions, 25-35 endplates from each preparation were photographed at a magnification of 10,000 and prints were prepared at a final magnification of 20,000. The thickness of the sections was measured by Small's method (40), using appropriate folds in the sections; only those of similar thickness were used for quantitative studies. The membrane enclosing the synaptic boutons was traced onto acetate sheets and its length measured in micrometers using a digitizer (Hewlett-Packard Co., Palo Alto, CA). Grains were then counted and expressed per unit length of presynaptic membrane.

Absolute Quantitation of Acceptors for 125I-BoNT Types A and B

Acceptors were quantitated by the method of Furtuck and Salpeter (11). Nerve-muscle preparations were treated with saturating concentrations of type A or B 125I-BoNT for 90 min at 22°C to ensure total occupancy of specific sites at the motor nerve terminal. After small pieces of the endplate regions were processed for microscopy, at least nine groups of three to five pale gold tissue sections from different blocks containing large numbers of nerve endings were arranged on each slide. After exposure periods ranging from 1 to 6 wk, the emulsion was developed and the stained sections viewed in the electron microscope. Nerve terminals from sections of similar thickness only were photographed and prints prepared at a magnification of 28,300. The density of acceptors was determined using the following equation (11):

\[
sites/\mu m^2 = \frac{G \times D}{A_{125I,800} (1 - e^{-at})} \times \frac{S_C}{S_A}
\]

where G is the grain density (No. grains/μm^2 membrane), D is the decay corrected for one developed grain of the tissue with increasing concentration time in days, A is Avogadro's number (6.023 × 10^23 molecules/mmol), S_C is the specific activity of the radiolabeled toxin at the beginning of the exposure period (curies per millimole), and S_A is the disintegrations per minute or 2.22 × 10^7. Grain density was determined by measuring the length of the presynaptic membrane in micrometers by digitization and counting the silver grains along its length. The percent efficiency of the autoradiographic technique used was determined by processing sections of 125I-BSA of known radioactivity for electron microscopic autoradiography and developing the emulsion after different exposure periods (34). The number of grains produced per radioactive decay could then be calculated.
Figure 1. Interaction of $^{125}$I-BoNT type A with motor nerves after in vivo administration to mice. (a) Motor nerve terminals (nt) labeled with $^{125}$I-BoNT type A after intraperitoneal injection of $\sim$1 µg of radiiodinated toxin (in 100 µl PBS/1% BSA) into a mouse. Autoradiograms were developed after 3-wk exposure. Note the absence of silver grains on muscle fibers (mf), Schwann cells (S), blood vessels (bv), and collagen (c). (b) Transverse section through a myelinated axon from a mouse diaphragm treated as above. Grains are seen in the axoplasm (a) but no labeling of the protective myelin sheath (m) is observed.

**Results**

Labeling Profiles for $^{125}$I-BoNT Types A and B in Mouse Diaphragm and Frog Cutaneous Pectoris Muscle

Sections of mouse diaphragm tissue labeled with $^{125}$I-BoNT type A in vivo showed deposition of silver grains at the motor nerve terminal (Fig. 1 a). No labeling of muscle fibers, Schwann cells, blood vessels, collagen fibrils, or myelin was detected in any of the sections, emphasizing the specificity of the interaction of radiolabeled BoNT type A with the motor nerve terminal. At the labeled nerve terminals, grains were observed both on the presynaptic plasma membrane and within the cytoplasm, indicating the involvement of an internalization step (Fig. 1 a). Most of the developed grains (63% of the total) were on the membrane (Table I) and could be seen on all areas of the unmyelinated nerve terminal (see below). Moreover, silver grains were observed within the
axoplasm of myelinated axons, suggesting that retrograde intra-axonal transport had occurred (Fig. 1 b).

A similar pattern of labeling was observed in sections from samples treated with 15 nM [125I]-BoNT type A in vitro, thus confirming the validity of the in vitro model for localization studies (Fig. 2 a). Silver grains were observed only at the unmyelinated motor nerve terminals; these occurred both on the plasma membrane (62%) and within the terminal (38%) (Table I). The internalized radioactivity did not appear confined to any particular intraterminal compartment, although grains were sometimes seen associated with the membrane of vacuolar structures. Control sections, treated with [125I]-BoNT type A in the presence of excess native neurotoxin, were virtually devoid of silver grains (Fig. 2 b); native BoNT reduced the extent of labeling to 4% of that seen in test samples. Collectively, these results showed that the interaction of [125I]-labeled type A toxin with its target tissue was both selective and saturable, thus demonstrating the involvement of acceptors. Saturable labeling of the neuromuscular junction was also observed using light-microscopic autoradiography after incubation of the tissue with 0.2 nM [125I]-BoNT type A, a concentration in the range of that required for neuropaanalysis in vitro (38), but too low to allow detection in ultrathin sections. Where autoradiography was combined with histochemical localization of acetylcholinesterase (7, 44) (to label the neuromuscular junction), silver grain clusters coincided with areas reacting with acetylcholine (data not shown).

[125I]-BoNT type B also interacted specifically and saturably with the motor nerve terminal (Fig. 2 c); likewise, the presence of silver grains within myelinated axons suggested retrograde axonal transport of radioactivity had taken place (data not shown).

A similar pattern of labeling was also observed with frog cutaneous pectoris (Fig. 3); silver grains were observed both on the membrane and in the cytoplasm of test sections labeled with [125I]-BoNT type A, while controls were completely devoid of silver grains, indicating saturation of the binding (data not shown). The extent of labeling at the amphibian nerve terminal was, however, considerably less than in mammalian tissue; this difference is reconcilable with the apparent lower sensitivity of frog muscle to the action of the toxin (31).

### Interaction of Types A and B Botulinum Neurotoxins

Preincubation of a mouse nerve–hemidiaphragm with a 100-fold excess of type B neurotoxin did not prevent binding or internalization of [125I]-BoNT type A at the motor nerve terminal (Fig. 4 a). Quantitative analysis of resultant autoradiograms (see Materials and Methods) showed that the number of grains per micrometer of plasma membrane was the same in sections incubated with [125I]-BoNT type A in the presence and absence of unlabeled type B (Table II).

In the converse experiment, type A did not prevent completely the binding of [125I]-BoNT type B, as silver grains were still seen at terminals preincubated with unlabeled type A neurotoxin (Fig. 4 b). However, quantitation of the data obtained demonstrated that specimens exposed to [125I]-BoNT type B in the presence of type A showed a 24% reduction in the extent of labeling relative to those treated with radioiodinated type B alone (Table II). Thus, in contrast to the lack of antagonism seen when terminals were labeled with [125I]-BoNT type A in the presence of type B BoNT, this partial inhibition suggests that type A interacts indirectly with at least some of the acceptors for type B.

### Localization of Acceptors for [125I]-BoNT Types A and B at the Murine Motor Nerve Terminal

Although the saturability of the labeling of motor nerve terminals with [125I]-BoNT in vitro implicated membrane acceptors, their exact location was not apparent due to masking by grains resulting from internalized radioactivity. As detailed in the accompanying report (2), the internalization step can be prevented totally using inhibitors of energy production (such as Na azide and dinitrophenol) and by low temperature (e.g., 4°C); this allows the interaction of the radiolabeled toxins with their acceptors to be examined.

In the presence of 15 mM Na azide, radioactivity seemed to occur solely on the plasma membrane (Fig. 5, a and b; Table I); nonspecific labeling was minimal (data not shown). To ensure that the nerve terminal membrane was indeed the source of the silver grains (particularly those opposite the postsynaptic folds [29]), quantitative studies were carried out as follows (35): the distance from the midpoint of at least 500 silver grains to the plasma membrane was measured and used to construct a grain-density distribution histogram. As Fig. 6 a shows, the distribution was bell-shaped, indicating that the majority of grains were found on the terminal membrane or very close to it. Since the scatter is symmetrical on both sides of the "biological line source," the presence of acceptors on the postsynaptic plasma membrane can be-

| Labeling conditions | Na Azide (15 mM) | Inside |
|---------------------|-----------------|--------|
| In vivo             | 125I-BoNT type A (~1 µg) | - | 63 | 37 |
| In vitro            | 125I-BoNT type A (15 nM) | - | 62 | 38 |
|                     | 125I-BoNT type A (11 nM) | + | 100 | - |
|                     | 125I-BoNT type B (10 nM) | - | 75 | 25 |
|                     | 125I-BoNT type B (20 nM) | + | 100 | - |

*Table I. Distribution of Silver Grains at Motor Nerve Terminals Labeled under Different Conditions with [125I]-BoNT Types A and B*
The half-distance value, i.e., the distance from a radioactive line within which half of the developed grains are located, was determined from the integrated grain distribution histogram shown in Fig. 6b, and found to be ~56 nm. At a distance of only 100 nm, the number of grains was very low, indicating that the autoradiographic method used gives good resolution.

The saturable acceptors for both toxin types were not restricted to the active zones but extended uniformly to all the unmyelinated terminal arborizations of the nerve as far as the
terminal node of Ranvier (Fig. 7, a–c). No grains were detectable on the protective myelin sheath, but the possibility of specific acceptors also occurring on the plasma membrane of the myelinated axons (to which toxin molecules had no access) could not be excluded. As the motor neurons innervating the diaphragm of newborn rats are still unmyelinated at this stage of development (47) and the Schwann cells that loosely surround the axons do not appear to prevent toxin molecules from reaching the axonal plasma membrane, the possible distribution of acceptors on the axon could be evaluated in this preparation. Newborn rats are highly susceptible to intoxication by 125I-BoNT type A, dying within a few hours of an intraperitoneal injection of low (<1 LD50 for adult mice) doses of the toxin. Accordingly, saturable binding of 125I-BoNT type A was detected at the developing motor nerve terminals in test sections (Fig. 8, a and b); control autoradiograms were completely devoid of silver grains (Fig. 8 c). Although a few grains were present on the axonal areas nearest the developing terminal matrices, no labeling was apparent in the large bundles of unmyelinated axons more distant from the terminal regions (Fig. 8 d). These observations suggest that the acceptors are located only in those areas of the axonal plasma membrane not destined to become myelinated, i.e., the terminal areas. Most of the nerve terminals observed were too small to allow detection of internalization, but in those that were slightly larger uptake was apparent (Fig. 8 b).

Density of Acceptors for 125I-BoNT Types A and B on the Unmyelinated Nerve Terminal Membrane

Optimization of conditions for saturation of specific acceptors with 125I-BoNT. Inhibition of the internalization step of intoxication by 125I-BoNT types A and B using metabolic inhibitors permitted the absolute quantitation of their acceptors at the nerve terminal. To carry out such determinations, it was essential to establish the toxin concentration and incubation period required to saturate the sites for each toxin type. Saturation of the acceptors in the presence of azide was achieved using 15 nM 125I-BoNT type A for 90 min at 22°C (Table III). A significant amount of radioactivity was seen associated with motor nerve terminals after only 20 min (84% of maximum binding, see Table III); maximum binding was attained by 90 min with this toxin concentration, despite the restricted diffusion into this tissue (1).

In the case of 125I-BoNT type B (Table III), the labeling observed using 20- and 100-nM concentration (for 90 min at 22°C) did not differ appreciably; for quantitative studies, 100 nM was used to ensure saturation.

Calibration of the autoradiographic technique. To determine the percent efficiency (32) of the method used routinely, ultrathin sections containing a known amount of radioactivity (in the form of 125I-labeled BSA) were processed for autoradiography and developed after varying lengths of time. A linear increase was observed (Fig. 9 a) in the number of silver grains produced during the first 3-wk exposure; longer exposure times, however, resulted in a slight leveling of the curve. Percent efficiency (Fig. 9 b) decreased from ~63% (almost maximum; see reference 32) for the shortest exposure time (4 d) to 57% for the longest (42 d), the average being 60 ± 3.2% (n = 6). The background grain density, obtained by photographing random areas of the sections that did not contain 125I-labeled BSA, was negligible.

To ensure that 60% efficiency was applicable to test specimens incubated with 125I-BoNT, groups of sections from samples that had been previously saturated with 125I-BoNT type A and processed for electron microscopic autoradiography were developed each week for 6 wk. The number of grains per micrometer of plasma membrane was then determined for each exposure time and expressed as a function of time (Fig. 10). The plots obtained for 125I-BSA and 125I-BoNT type A were very similar and 60% was considered a valid estimate of the efficiency in this system.

Acceptor densities. The average number of acceptors for BoNT type A was 152(± 20%) pm2 of membrane when 150 nM 125I-BoNT and 7-42-d exposure were used (Table II).
Figure 4. Interaction of types A and B BoNT at the murine motor nerve terminal. (a) Cross-sectional view of four synaptic boutons labeled with $^{125}$I-BoNT type A in the presence of unlabeled type B BoNT. The tissue was preincubated with type B BoNT (1.5 μM) for 1 h at 22°C before incubation for 90 min with radiolabeled type A, at a final concentration of 15 nM. (b) Section of a motor endplate pretreated with unlabeled type A BoNT (1 μM) for 1 h at 22°C followed by addition of $^{125}$I-BoNT type B to a final concentration of 10 nM, with further incubation for 90 min. Silver grains are present at the nerve terminals, but labeling is somewhat less extensive than that observed in control specimens.
Figure 5. Localization of acceptors for 125I-BoNT types A and B at the murine motor nerve terminal. Nerve-muscle preparations were treated with Na azide (15 mM) for 30 min at 22°C before a 90-min incubation with 125I-BoNT type A or B, in the presence of the metabolic inhibitor. Autoradiograms of samples treated (a) with 125I-BoNT type A (150 nM) or (b) with type B (20 nM).

When quantitation of acceptors was performed using 15 and 35 nM 125I-BoNT type A, similar numbers were obtained, confirming that these concentrations do, in fact, saturate the binding sites (Table III). The density of 125I-BoNT type B sites was determined, likewise, by processing two different blocks of diaphragm tissue (treated with 100 nM 125I-BoNT type B, see above) for electron microscopic autoradiography. Using an efficiency value of 62% (more appropriate for the exposure used), the average site density was 627 ± 21% (the range of values being 495-759; Table IV). Hence, the density of sites for 125I-BoNT type B is approximately four times greater than that for A.

Discussion

The grain distribution in autoradiograms of mouse diaphragm labeled in vivo with 125I-BoNT type A using a concentration (extrapolated) of <3 nM was indistinguishable from that seen after in vitro labeling with 1.5 nM (or higher); this emphasizes the validity of the latter as a means for observing labeling patterns that are relevant to BoNT poisoning. Moreover, the presence of silver grains in the axoplasm of myelinated nerves demonstrates that the toxin, or a portion of the molecule, undergoes retrograde intra-axonal transport as has been shown for tetanus toxin (30) and nerve growth
factor (19). The pathway for retrograde axonal transport of exogenous proteins usually begins with uptake by endocytosis at the nerve ending or unmyelinated portions of the preterminal axon (12). Protein is carried in the axon inside various vesicular or tubular organelles to the cell body, where it may be transferred to lysosomes (25), to some other compartment in the cell, or by transsynaptic migration to presynaptic terminals that synapse on that cell body (e.g., various vesicular or tubular organelles to the cell body where it may be transferred to lysosomes (25), to some other compartment in the cell, or by transsynaptic migration to presynaptic terminals that synapse on that cell body (e.g., tetanus toxin). The purpose for the neural ascent of BoNT is unknown; however, studies carried out by Hagenah et al. (16), in which toxin injected into muscle and allowed to ascend the motor nerve was found not to affect central cholinergic neurotransmission suggest that, unlike tetanus toxin, a (modified) inactive form is involved. Transport of BoNT to the cell body may be necessary for its degradation because the neuronal cell body is richer in both primary and secondary lysosomes than the axon and nerve endings (24).

The pattern of labeling with type B is comparable to that seen with A, in accordance with the related pharmacological action of these toxin types. However, although both neurotoxins appear to be internalized, the extent of uptake of B is appreciably lower (~40% less) than that of type A. If, as has been suggested previously (38), internalization of toxin is required for expression of toxicity, the demonstrable reduction in the proportion of translocated radioactivity could account for the lower toxicity of BoNT type B in mice.

Using the inhibitor of oxidative phosphorylation Na azide to prevent internalization of radioactivity, the exact location of the specific acceptors for 125I-BoNT types A and B was established. Resolution studies confirm that these are indeed presynaptic and membrane-associated; this result is consistent with the lack of direct postsynaptic effects being induced by BoNT (36). In both cases, silver grains were uniformly distributed on all unmyelinated areas of the nerve terminal membrane; thus, the acceptors are not located solely in the synaptic region but also on preterminal axonal plasma membrane. Since the protective myelin sheath does not allow access of molecules to the axonal membrane it encloses, the presence of acceptors in areas beyond the terminal node of Ranvier was investigated in newborn rats. Myelination of motor neurons is incomplete at this stage of development (26), leaving the entire length of the nerve trunk exposed to the external environment. Saturable acceptors for 125I-BoNT type A were found only on the terminal membrane of these immature synapses; large bundles of nerves destined to become myelinated were unlabeled. It appears, therefore, that the sites are restricted to the unmyelinated terminal arborizations of the motor nerve; a similar distribution was recently reported for α-latrotoxin binding sites except that only the synaptic bouton membrane, and not the preterminal axon, was labeled (45). It is interesting that types A and B do not appear to share common membrane acceptors at the nerve terminal. Further evidence for the presence of a distinct population of sites for each toxin type is given by their different densities. The absolute number of sites for the two toxin types represents the first direct measurement of the density of any component on the unmyelinated mammalian motor nerve terminal membrane. The number of sites per

Figure 6. Histogram of experimental grain-density distribution around a radioactive line source: determination of the half-distance (HD) value (35). (A) The distance from a biological line source (i.e., the plasma membrane of motor nerve terminals treated with 125I-BoNT type A in the presence of Na azide as described in Fig. 5) to the midpoint of >500 silver grains was measured and a histogram of grain-density distribution about the line was constructed with the data. Because this histogram appeared symmetrical on both sides (indicating that the source was in fact a line), a one-sided histogram (shaded bins) was also plotted. A smooth curve was then drawn in by eye. Most of the grains were located on or very close to the membrane, as reflected by the bell-shaped curve. (B) Following the method of Salpeter et al. (35), the results shown in A were used to plot an integrated grain distribution histogram. Each column gives the total number of grains within that distance from the source. The number of grains changed very little after ~300 nm and counting was stopped at a cut-off distance of ~600 nm. The half-distance value was determined readily from this histogram: the total number of grains is 532; half the total (i.e., 266 grains) can thus be found within ~56 nm of the source.
square micrometer of membrane for $^{125}$I-BoNT types A and B is $153 \pm 31$ and $627 \pm 131$, respectively.

The acceptors studied autoradiographically appear to be involved in the action of the toxin because of the discrete and selective labeling patterns seen both in vivo and in vitro with BoNT concentrations in the range that blocks neurotransmission (38). Additional support for this deduction is provided by their demonstrated presence only at terminals (3) where BoNT effectively inhibits transmitter release (3, 8). Regarding the precise manner in which the acceptors participate in the neuroparalytic action of the neurotoxin, analogies are apparent with other bacterial and plant toxins in certain respects. As in the case of these other toxins (e.g., diphtheria toxin, tetanus toxin, abrin, ricin, and modeccin), BoNT acceptors may ensure that toxin is concentrated on the cell surface (for review, see reference 28) and directed to a particular intracellular vesicular compartment. In the light of the results presented here the theoretical “pipe and valve” model of Hanig and Lamanna (17), which suggests that the interaction of BoNT with surface acceptors is directly responsible for toxicity, does not seem a viable proposition. The acceptors for $^{125}$I-BoNT types A and B, which appear to be distinct, are not restricted solely to the active zone areas opposite the postsynaptic folds (although the presence of a small number of higher affinity sites within the active zones has not been excluded). Furthermore, the calculated density of acceptors far exceeds the estimated number of 500 active zones per nerve terminal (20). Finally, it is difficult to reconcile
Figure 8. Interaction of $^{125}\text{I}\text{-BoNT}$ type A with the developing motor nerves of newborn rats. The diaphragm of a newborn rat was incubated with $^{125}\text{I}\text{-BoNT}$ (30 nM) in 0.5 ml Krebs-Ringer's solution for 90 min at 22°C, followed by extensive washing at 4°C. Sections processed for autoradiography were exposed for 4 wk. (a) Developing motor nerve terminals labeled with $^{125}\text{I}\text{-BoNT}$. Note the postsynaptic folds (pf) in the muscle cell and the numerous small axon terminals (t) embedded in an overlying Schwann cell (S). (b) Larger nerve terminal from a newborn rat diaphragm labeled with $^{125}\text{I}\text{-BoNT}$ type A. Note the internalized silver grain (arrow) possibly associated with a vesicular structure (v), the overlying Schwann cell (S), and the postsynaptic folds in the muscle cell (pf). (c) Control nerve terminal (t) treated with radiolabeled BoNT in the presence of excess (3 μM) unlabeled toxin. (d) Unmyelinated axons (a) loosely wrapped by Schwann cells (S) in a newborn rat diaphragm preparation treated as described above. Although the nerve terminal (t) areas (left) are labeled with $^{125}\text{I}\text{-BoNT}$, no silver grains are detectable on the unmyelinated axonal membrane.

This theoretical proposal with the apparent requirement for toxin internalization before expression of neurotoxicity (5, 38) and with the action of other toxins of similar overall structure. It does not appear, therefore, that BoNT acts by blocking physically the neurotransmitter release sites; of course, the possibility that toxin molecules interact with the active zones intracellularly remains likely. Another proposed model suggests that the toxin acts as a Ca$^{++}$ channel blocker (23). The distribution of BoNT sites, however, does not correspond with that predicted for Ca$^{++}$ channels from electron microscopic freeze-fracture studies (21). Moreover, the absence of binding sites for BoNT at adrenergic and several other synapse types (3, 8) would exclude the Ca$^{++}$ channel as its possible acceptor, as this channel ought to be similar in, at least, some of the nerves studied. In accord with this, BoNT is known to be ineffective towards Ca$^{++}$ currents measurable in presynaptic membranes (9, 14).

Regardless of the physiological function of the acceptors, their density is far too high to be reconcilable with the number of neurotoxin molecules needed to induce paralysis in vivo. For example, the LD$_{50}$ for a mouse is $\sim1.2 \times 10^{-11}$ g or $8 \times 10^{-17}$ mol, equivalent to $5 \times 10^7$ molecules of BoNT type A. From this LD$_{50}$, it can be calculated that an absolute maximum of $10^7$-10$^8$ molecules are required to induce blockade of neurotransmission at each synapse (calculated as described in reference 39). As the observed densities of acceptors are $\sim150$ and 630/μm$^2$ of membrane for $^{125}\text{I}\text{-BoNT}$ types A and B, respectively, these greatly exceed the number of molecules needed to inhibit neurotransmitter release at a synapse. Thus, these autoradiographic data appear to dismiss totally a "one-hit" model for the mechanism of action of these BoNT types. This is further supported by the finding that, although types A and B have a similar pharmacological action at the neuromuscular junction, they do not appear to share acceptors on the neuronal membrane.

Collectively, our findings show that the acceptors are
Table III. Concentration Dependence and Time Course of the Binding of Types A and B ¹²⁵I-BoNT to Nerve Terminal Membranes

| Toxin concentration (90 min)* | Number of grains per micrometer of plasma membrane‡ |
|------------------------------|---------------------------------------------------|
| nM                           | Type A                | Type B                |
| 1.5                          | 0.23 ± 0.03           | -                     |
| 10.0                         | -                     | 0.53 ± 0.06           |
| 15.0                         | 0.55 ± 0.06           | -                     |
| 20.0                         | -                     | 0.81 ± 0.07           |
| 35.0                         | 0.56 ± 0.06           | -                     |
| 100.0                        | -                     | 0.95 ± 0.07           |

Incubation with 15 nM toxin*

| min | Type A                | Type B                |
|-----|-----------------------|-----------------------|
| 20  | 0.46 ± 0.08           | -                     |
| 90  | 0.55 ± 0.06           | -                     |
| 150 | 0.53 ± 0.09           | -                     |

* Mouse diaphragms were preincubated in Krebs-Ringer's solution containing 15 mM Na azide for 30 min at 22°C followed by labeling with ¹²⁵I-BoNT type A or B in the latter solution, under the conditions specified above.

‡ After the standard processing for electron microscopic autoradiography, average grain densities were determined at a minimum of 25 endplates from several sections (of the same thickness) taken from different blocks of the tissue; these (± SE) are expressed (27) per unit length of presynaptic membrane.

Responsible for targeting BoNT to motor nerve terminals where they mediate internalization; this could facilitate inhibition of acetylcholine release by direct or indirect interaction of the toxin (or a fragment) with an intraterminal component concerned with the process. The large number of acceptors detectable, relative to the number of BoNT molecules required to cause neuroparalysis, should ensure an efficient delivery of toxin to its pharmacological target; this could be one contributory factor to its unique potency. Apart from being utilized by BoNT, these naturally occurring membrane components undoubtedly must serve some as yet unidentified function in their own right. Apparently, the BoNT types recognize determinants unique to cholinergic nerve terminals, implicating them in some characteristic function. The toxins may thus prove to be powerful tools for investigations on the nature of these terminals; they may also provide insight into the differences between the unmyelinated...
nerve terminal membrane and the myelinated axonal membrane of motor neurons.

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