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ORIGINAL PAPER

Neuronal Functions Associated with Endo- and Exocytotic Events-cum-Molecular Trafficking may be Cell Maturation-Dependent: Lessons Learned from Studies on Botulism

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Abstract The passion in the scientific endeavors of Marshall Warren Nirenberg had been his quest for knowledge regarding the storage, retrieval, and processing of information in the cell. After deciphering the genetic code for which he shared the Nobel Prize in Physiology and Medicine in 1968, Nirenberg devoted his attention to unraveling the mysteries in the most complex cellular organization in the body, i.e., the nervous system, especially those governing neuronal development, plasticity, and synaptogenesis. During the tenure of the primary author (RR) as a postdoctoral Staff Fellow in the Nirenberg laboratory in the late seventies to early eighties, he had the opportunity of working on projects related to what Nirenberg used to broadly define as the "synaptic code." The major aspects of these projects dealt with the functional macromolecules relevant to neuronal growth, organization, lineage, selectivity, stabilization, synaptogenesis, and functions such as neuroexocytosis. This author's emphasis was particularly on voltage-gated calcium channels that regulate stimulus-induced neurotransmitter release. One central as well as crucial theme in these studies was the fact that the neurons had to be mature and differentiated in order to study these entities (Science 222: 794–799, 1983; Cold Spring Harb Symp Quant Biol 48: 707–715, 1983). In this communication, we illustrate how did this basic knowledge, i.e., cell maturation-dependent properties being essential for neuronal functions, led to a successful experimental design and demonstration of the validity of the targeted neurologic therapeutic delivery approach based on recombinant botulinum toxin serotype A (BoNT/A) heavy chain (rHC) serving as a neuron-specific targeting molecule (BMC Pharmacol 9: 12, 2009).

Keywords Neurons · Functions · Endocytosis · Exocytosis · Molecular trafficking · Cell maturation · Botulism · Targeted therapeutic

Background

Botulinum neurotoxins (BoNTs) are produced by the anaerobic *Clostridium botulinum* species of bacteria and are the cause of botulism, a life-threatening neuroparalytic disease. BoNTs are di-chain peptides consisting of a 50 kDa light chain (LC) and a 100 kDa heavy chain (HC) linked through a disulfide bond (Inoue et al. 1996). The HC binds specifically to receptors on target nerve cells (through its C-terminus) and facilitates uptake of the holotoxin via endosomes. Inside the endosomes, the toxin dissociates into the HC and the LC and the HC forms a transmembrane channel; the HC channel serves as a conduit for the translocation of the LC into the cytosol (Singh 2000; Li and Singh 1999). In the cytosol, the LC acts as a Zn^{2+}-endopeptidase to degrade specific intracellular protein targets present either on the plasma membrane or on

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, Publication no. 85-23, 1996), and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

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the synaptic vesicle, and inhibits neurotransmitter release by disabling the exocytotic docking/fusion machinery (Singh 2000; Li and Singh 1999).

Current therapy for botulism involves respiratory supportive care and the administration of antitoxin. However, only a few effective antitoxins, which must be administered before toxins reach the nerve cells, are available. Thus, the therapeutic window for using an antitoxin is short. Once the syndrome is developed, the antitoxin is less effective since it cannot penetrate the nerve cell to neutralize the toxin. This highlights the importance of developing a pharmacological therapeutic approach for botulism. At present, some examples of the proposed pharmacological antidotes for BoNT poisoning are a protease inhibitor, a phospholipase A_2 activator or a modulator of intracellular free Ca^{2+} concentration. Since all of these parameters are involved in normal body functions, a systemic therapeutic approach is inadvisable due to potential toxicity concerns. Therefore, therapeutic targeting offers two important advantages: (a) delivering an effective high concentration of the therapeutic compound to the site of toxicity, i.e., nerve terminals for botulism, and (b) minimizing systemic toxicity, if any, due to treatment compounds.

For therapeutic targeting, we attempted to develop a model drug delivery vehicle (DDV) comprising the Cy3-labeled (red fluorescence) non-toxic recombinant heavy chain (rHC) of BoNT/A coupled to an Oregon green 488-labeled (green fluorescence) 10-kDa amino dextran via the heterobifunctional linker 3-(2-pyridylthio)-propionyl hydrazide (Fig. 1). The rHC would serve to target BoNT/A-sensitive cells and promote internalization of the DDV complex, while the dextran molecule would serve as a drug carrier to deliver multiple therapeutic molecules to the targeted neurons. In this DDV construct, the dextran moiety was conjugated to the rHC via a disulfide linkage in such a manner as to allow its dissociation from the rHC exactly the way the LC dissociates from the HC inside endosomes. In other words, for its functioning, the DDV molecules are supposed to undergo the sequential steps of (a) receptor binding, (b) endosomal uptake, and (c) molecular trafficking in the same fashion as BoNT/A, i.e., to include the release of labeled dextran through the transmembrane channel formed by the HC. The success of this DDV approach would be demonstrated if the DDV would be taken up by neurons via BoNT/A receptor mediated endocytosis and subsequently processed to translocate the green fluorescent dextran into the neuronal cytosol. We conducted the studies in a cultured mouse (c57BL/6NCR strain, Frederick Cancer Research and Development Center, Frederick, MD) spinal cord neuronal model and used confocal microscopy to monitor DDV trafficking and its processing inside neurons as previously described (Zhang et al. 2009). The results showed that like many other neuronal functions as mentioned above, a proof of the DDV concept could be demonstrated only in mature, but not in immature neurons. The factors attributable to this

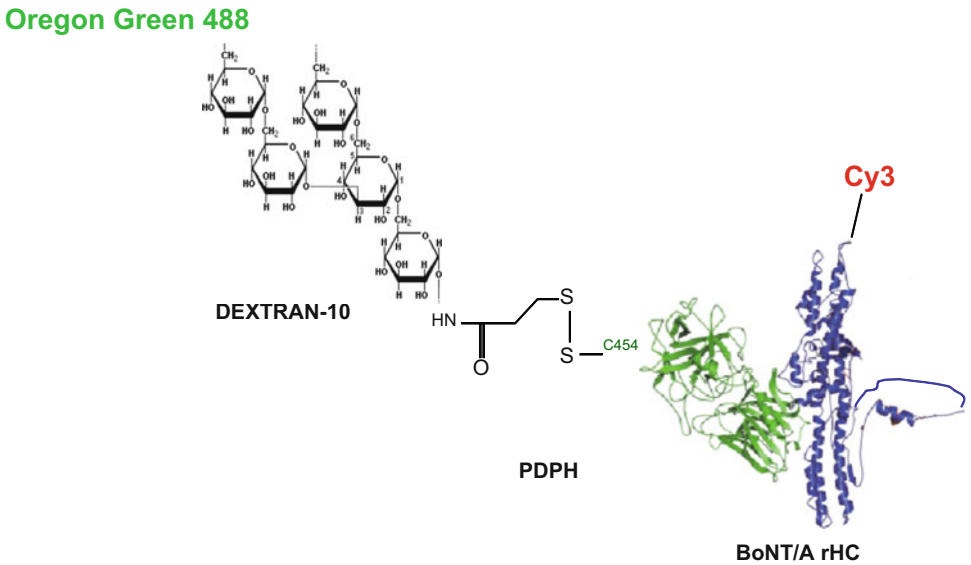

Fig. 1 Schematic representation of the DDV for transport of BoNT/A antagonists. The DDV construct consisted of a drug targeting molecule, i.e., Cy3-labeled rHC, which was linked by a disulfide bond to a drug carrier simulant, Oregon green 488-labeled 10 kDa dextran. The PDPH (3-(2-pyridylthio)-propionyl hydrazide) linker was bound to one of four possible cysteine (C) sulfhydryl groups on the BoNT/A rHC. It was attached to C454, which normally participates in the disulfide linkage between the HC and the LC in the native BoNT/A. Cy3 and Oregon green 488 were bound to O-amino groups of lysine in the rHC and dextran, respectively. The dextran was conjugated to the rHC by a C–N bond in one of the glucose residues. In a functional DDV, multiple drug molecules may be attached to the dextran carrier

difference between mature versus immature neurons constitute the subject of future research. However, the main reasons appear to be related to the levels of neuronal development and differentiation properties such as membrane composition and functions, protein expression, receptors, endocytosis, exocytosis, etc.

Methods

Construct a Model Drug Conjugated Drug Delivery Vehicle

The DDV was constructed with a targeting molecule, Cy3-labeled BoNT/A heavy chain (rHC) linked by a disulfide bond at Cys454 of rHC to a drug simulant, which was 10 kDa dextran labeled with Oregon green 488 (OG488) (Zhang et al. 2009) (Fig. 1).

Spinal Cord Cultures

Spinal cords were removed from fetus of timed pregnant C57BL/6NCR mice at gestation day 13. Cells were dissociated with trypsin and plated in collagen-coated 4-well coverslips or 35 mm diameter 6-well culture plates at a density of 10^5 cells/cm^2. Cells were grown in Eagle's Minimum Essential Medium with 5% horse serum and a nutrient supplement (N3) at 37°C in 90% air/10% CO_2. Cell cultures were treated with 54 mM 5-fluoro-2-deoxyuridine and 140 mM uridine from day 5–9 after plating to inhibit glial cell proliferation. Cultures were used for experiments at 1–3 weeks after plating.

[^{3}H]Glycine Release Assay

[^{3}H]glycine release was determined by a method described by Zhang et al. (2009). Spinal cord cells (35 mm wells) were incubated at 37°C for 30 min in HEPES-buffered saline (HBS) containing 2 mCi/ml [^{3}H]glycine. The cells were washed briefly with Ca^{2+}-free HBS and incubated sequentially for 7 min in each of the following modified HBS solutions: 5 mM KCl/0 mM Ca^{2+}, 80 mM KCl/2 mM Ca^{2+}, and 5 mM KCl/0 mM Ca^{2+}. Each incubation solution was collected, and the radioactivity was determined by scintillation counting.

Uptake of DDV by Spinal Cord Neurons and Release of Dextran into the Cytosol

Cells were exposed to DDV in growth medium for 16 h at a concentration of 200 nM at 37°C. Cells were subsequently washed three times with growth medium and fixed overnight using 2% paraformaldehyde. The coverslips containing fixed cells were mounted between a glass slide and glass coverslip and viewed on a Bio-Rad 2000 laser confocal microscope. Oregon green 488 was excited at 488 nm and read through a 515-nm cutoff filter. Cy3 was excited at 543 nm and read through a 565-nm cutoff filter. Micrographs were obtained using a Bio-Rad laser confocal microscope with a 100× oil immersion objective. Images were collected with Bio-Rad software. Co-localization of rHC with dextran and separation of dextran from DDV were then calculated by utilizing the Bio-Rad AutoDeblur and AutoVisualize software to quantitate fluorescence intensity. The extent of separation/release of OG488-dextran was expressed as 100% total (in images) minus % co-localization.

Results and Discussion

We have previously shown that (a) the rHC is a safe DDV component for delivery of prospective BoNT/A antidotes, (b) DDV entry into neurons is via BoNT/A receptor mediated endocytosis, and (c) inside endosomes, the model drug carrier dextran moiety dissociates from the DDV in a fashion similar to BoNT/A LC dissociates from the HC (Zhang et al. 2009). Here we show evidence to emphasize that three important neuronal functions, i.e., endocytosis, molecular trafficking associated with DDV function, as well as neuroexocytosis are cell maturation-dependent. Different stages of spinal cord cell culture growth were used to evaluate the efficiency of separation of drug carrier from DDV. Confocal image analysis revealed that in 1-week-old culture, both Cy3-labeled rHC and OG488-labeled dextran were mostly colocalized inside neurons (Fig. 2, top right panel); there was a visible, but only a small separation of the drug carrier from rHC. On the contrary, in 2- and 3-weeks-old cultures, which represented a relatively more enhanced maturation status of the neurons, the dextran moieties separated from the rHC molecules in a progressively increasing fashion (Fig. 2, middle and bottom right panels). Using the images shown in the right column in Fig. 2, we calculated the extent of separation of OG488-dextran from the DDV and its cytosolic localization. The results are shown in Fig. 3b. About 20, 32, and 40% of the drug carrier component (OG488-dextran) separated from DDV and diffused into the cytosol from endosomes in 1-, 2-, and 3-weeks-old cultures, respectively. These results indicated that the separation of the drug carrier from DDV was neuronal maturation-dependent. A question may be asked whether the higher levels of separation and cytosolic localization of the OG488-dextran molecules seen in more mature neurons could be because these neurons have a greater uptake of

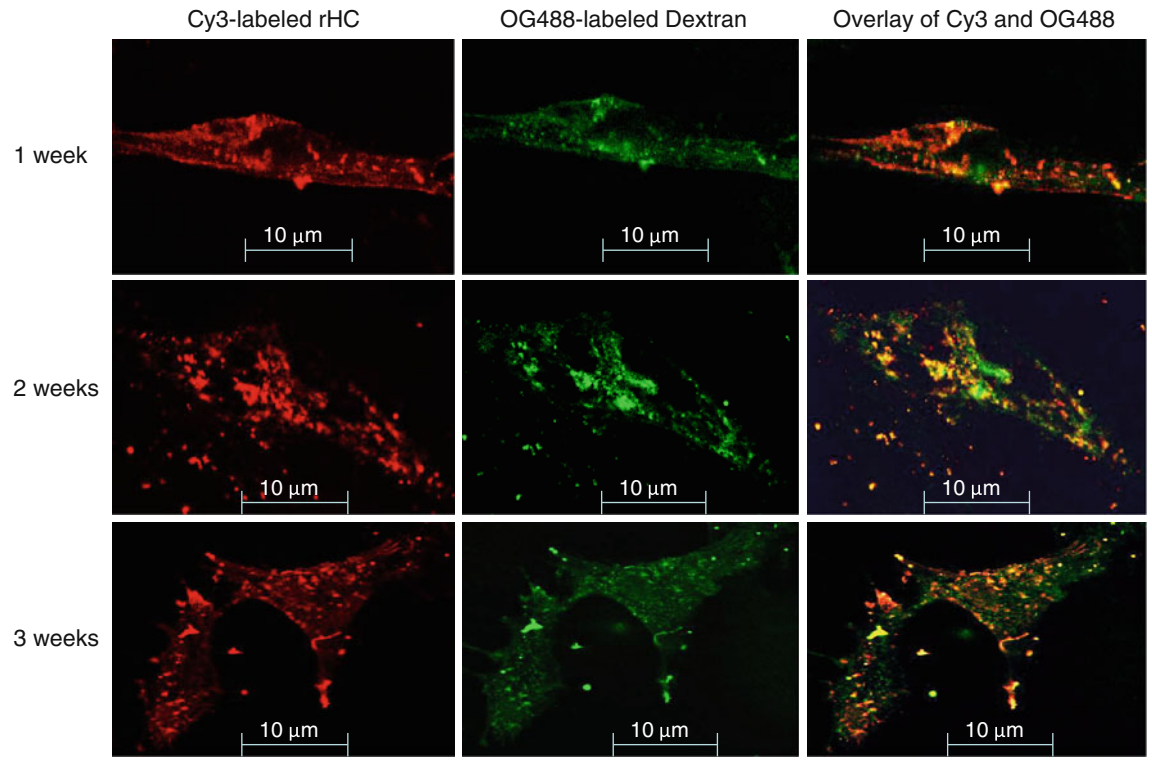

Fig. 2 Dissociation of the drug carrier molecule in DDV and its translocation into the cytosol in 1-, 2-, and 3-weeks-old spinal cord neuronal cultures. The figure shows fluorescent images of mouse spinal cord neuronal cultures grown for different times and incubated with DDV. 1-, 2-, and 3-weeks-old cultures are shown in *top*, *middle*, and *bottom rows*, respectively. Fluorescent images from Cy3-labeled (*red*) rHC, oregon green 488 (OG488)-labeled (*green*) dextran and superimposed *red* and *green* are shown in the *left*, *middle* and *right columns*, respectively. Overlays of *red* and *green* show either co-localization (*orange*) or separation of rHC (*red*) and dextran (*green*). Cells at different stages of culture growth were incubated for 16 h with 200 nM solutions of fluorescently labeled DDV. The results clearly demonstrated that the rHC component of the DDV remained localized in the endosomes (punctate distribution, *left column*), while the OG488-dextran separated from the DDV and migrated into the cytosol (*right column*). This phenomenon, i.e., dextran separating from DDV and then being released into the cytosol progressively improved with the age of the neuronal cultures indicating that DDV processing and cytosolic translocation of the drug carrier were neuronal maturation-dependent. Reproduced with permission from Zhang et al. 2009

DDV due to increased rHC receptors compared to less mature neurons or due to some other processes that are comparatively more active in mature neurons. The answer seems to be the latter, i.e., due to processes which are characteristic of more mature neurons. This conclusion was derived from the previous confocal microscopic studies on 1-, 2-, and 3-weeks-old neuronal cultures that had been labeled first with DDV (Cy3-rHC and OG488-dextran) and then stained for a fluorescent (blue) endosomal probe (Zhang et al. 2009). From visual observations, these cultures did not exhibit any remarkable age-dependent differences in their abilities for endosomal uptake of DDV molecules; however, the capacity for cytosolic translocation of the OG488-dextran molecules progressively increased with the age of the cultures (data not shown).

In the same growth phases of spinal cord neuronal cultures, i.e., in 1-, 2-, and 3-weeks-old cultures, we also tested the capability of evoked neuronal transmitter release by high K^+-stimulated [^{3}H]glycine release assay. As cultures matured over a three-week period, there were progressive increases in the amount of [^{3}H]glycine release due to high K^+ (80 mM) stimulation in the presence of normal extracellular Ca^{2+} (2 mM). These results suggested that the function of evoked neurotransmitter release was also neuronal maturation-dependent (Fig. 3a). In Fig. 3, we have also shown a comparison of the developmental aspects of the two neuronal properties: (1) DDV function as demonstrated by the extent of cytosolic translocation of the drug carrier molecules, and (2) stimulated neurotransmitter ([^{3}H]glycine) release. It is interesting to note that in the mouse spinal cord neuronal model, both properties improve with the age of the cultures; however, there seems to be a difference in the time-course of their development.

In conclusion, evidence presented here clearly demonstrate that in the cultured mouse spinal cord neuronal model, nerve functions such as endocytosis, molecular

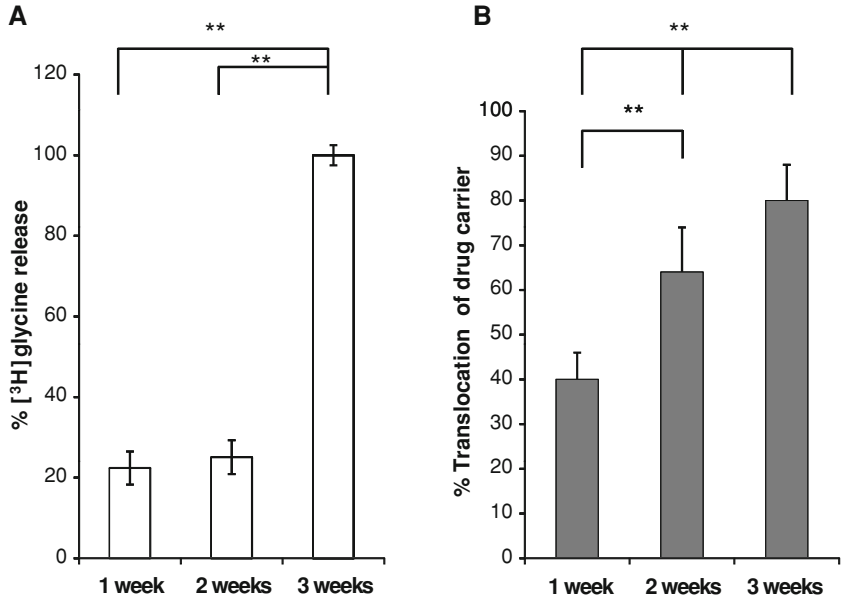

Fig. 3 The evoked neurotransmitter release (neuroexocytosis) property in primary mouse spinal cord cultures also seems to be cell maturation-dependent like molecular trafficking. **a** Replicate cell cultures (35 mm wells, $N = 3$) at different stages of growth were assayed for depolarization (80 mM K^+)-induced and Ca^{2+} (2 mM)-dependent release of [^{3}H]glycine. Data shown as release in 3-weeks-old culture as 100%. **b** Replicate cell cultures (coverslips, $N = 3$) at different stages of growth were exposed to DDV for 16 h. The translocation of drug carrier into neuronal cytosol was determined by confocal microscopy. Co-localization of rHC with dextran and separation/cytosolic release of dextran from DDV were then calculated by utilizing the Bio-Rad AutoDeblur and AutoVisualize software to quantitate fluorescence intensity. Results shown are means ± SD. Statistical analyses were done using one way ANOVA (**$P < 0.01$). The results indicated that the properties of neuroexocytosis and DDV processing, which involves endocytosis combined with molecular trafficking, appear to be developmentally regulated

processing-cum-trafficking and stimulated neurotransmitter release (exocytosis) are neuronal maturation-dependent. Immature neurons are incapable of executing a mature neuronal function such as DDV processing or neuroexocytosis. These observations exemplify the importance of considering such factors in design of experiments to test hypotheses on and also to study mature nerve functions. Moreover, the findings reported here lead to a new direction in developmental neurobiology research by indicating the importance of a coupling between cellular structural and functional components in nerve functions.

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