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**TRANSLOCATION AND DISSEMINATION TO TARGET NEURONS
OF BOTULINUM NEUROTOXIN TYPE B IN THE MOUSE
INTESTINAL WALL**

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ABSTRACT

Botulinum neurotoxins (BoNTs) are responsible for severe flaccid paralysis (botulism) which in most cases enter the organism via the digestive tract and then disseminate into the blood or lymph circulation to target autonomic and motor nerve endings. The passage way of BoNTs alone or in complex forms with associated non-toxic proteins through the epithelial barrier of the digestive tract still remains unclear. Here we show using an *in vivo* model of mouse ligated intestinal loop that BoNT/B alone or the BoNT/B C-terminal domain of the heavy chain (HCcB), which interacts with cell surface receptors, translocate across the intestinal barrier. The BoNT/B or HCcB translocation through the intestinal barrier occurred via an endocytosis-dependent mechanism within 10-20 min, since Dynasore, a potent endocytosis inhibitor, significantly prevented BoNT/B as well as HCcB translocation. We also show that HCcB or BoNT/B specifically target neuronal cells and neuronal extensions in the intestinal submucosa and musculosa expressing synaptotagmin, preferentially cholinergic neurons and to a lower extent other neuronal cell types, notably serotonergic neurons. Interestingly, rare intestinal epithelial cells accumulated HCcB suggesting that distinct cell types of the intestinal epithelium, still undefined, might mediate efficient translocation of BoNT/B.

INTRODUCTION

Clostridium botulinum produces potent neurotoxins (botulinum neurotoxins, BoNTs) which are responsible for severe neuroparalytic illness (botulism) in man and animals resulting from inhibition of spontaneous and nerve-evoked acetylcholine (ACh) release at cholinergic nerve endings. BoNTs are synthesized as an inactive single protein (~150 kDa) that is proteolytically cleaved into a ~100 kDa heavy chain (HC) and a ~50 kDa light chain (LC). Both chains remain linked by a disulfide bridge. The di-chain molecule constitutes the active neurotoxin. The half C-terminal part of HC (HCc) recognizes specific receptors on the surface of target neuronal cells and is involved in driving the toxin entry pathway into cells, whereas the N-terminal part permits the translocation of the L chain into the cytosol. LC catalyzes a zinc-dependent proteolysis of one or two of the three proteins of the SNARE complex, which play an essential role in neurotransmitter exocytosis (Meunier *et al.*, 2002, Poulain *et al.*, 2008, Bercsenyi *et al.*, 2013, Simpson, 2013, Rossetto *et al.*, 2014).

BoNTs are divided into 7 toxinotypes (A to G) according to their immunological properties based on neutralization with polyclonal antibodies. Each toxinotype is specifically neutralized only with the corresponding antibodies (Hill *et al.*, 2013). A new toxinotype called H or rather a new hybrid F/A type has been recently reported but still needs further characterization (Dover *et al.*, 2014, Gonzalez-Escalona *et al.*, 2014). *C. botulinum* strains show genetic variations and are classified into 4 groups (I to IV) based on *16s rRNA* gene sequences and biochemical characteristics. Most of the strains produce only one BoNT type. Genetic diversity is also observed in *bont* genes, and multiple subtypes (or genetic variants) have been identified in each BoNT toxinotype (reviewed in (Hill *et al.*, 2013)).

Three forms of botulism are recognized in humans in natural conditions: foodborne botulism, infant botulism or botulism by intestinal colonization, and wound botulism. Foodborne botulism is due to the ingestion of preformed BoNT in contaminated food and it is the main form of botulism in adults. In contrast, in infant botulism and some adult cases, ingested *C. botulinum* spores develop in the intestinal content and produce the toxin *in situ* (Tacket *et al.*, 1989, Sobel, 2005). In both forms, foodborne botulism and botulism by intestinal colonization, BoNT escapes the gastro-intestinal tract to reach the target cholinergic nerve endings, possibly through the blood and lymph circulation (Maksymowych *et al.*, 1999). Previous observations using experimental models of animal intoxication have shown that following oral administration, BoNT enters the blood stream and lymph circulation (Maksymowych *et al.*, 1999). The upper small intestine was found to be the primary site of toxin absorption (Kitamura *et al.*, 1969, Sugii *et al.*, 1977, Bonventre, 1979, Fujinaga *et al.*, 1997). However, BoNT can also be absorbed from the other parts of the digestive tract including the buccal cavity, stomach and colon, but to a lower extent than in the upper small intestine (Bonventre, 1979, Sakaguchi, 1983, Maksymowych *et al.*, 1999). Therefore, BoNT translocation through the intestinal barrier and trafficking to the cholinergic nerve ending target represent the initial and critical steps of botulinum intoxication.

The mechanism of BoNT passage through the intestinal epithelial barrier remains partially unknown. Botulinum complexes formed by BoNT and associated non-toxic proteins (ANTPs) including hemagglutinin (HA) components have reported to pass across the epithelial barrier upon HA-mediated opening of intercellular junctions. Indeed, HA subunits have been shown to bind to epithelial cadherin (E-cadherin) and to disrupt the intercellular junctional complexes between epithelial cells (Jin *et al.*, 2009, Sugawara *et al.*, 2010, Sugawara *et al.*, 2011, Lee *et al.*, 2014, Lam *et al.*, 2015). However, purified BoNT has also been shown to undergo transcytosis through intestinal epithelial cells without the help of HAs

(Fujinaga *et al.*, 1997, Maksymowych *et al.*, 1998, Park *et al.*, 2003, Maksymowych *et al.*, 2004, Nishikawa *et al.*, 2004, Ahsan *et al.*, 2005, Couesnon *et al.*, 2008). BoNT/A seems to preferentially use specific subsets of intestinal cells such as crypt enteroendocrine cells to enter the intestinal mucosa (Couesnon *et al.*, 2012). In this study, we investigated the passage of BoNT/B in the mouse intestinal mucosa since it is a major cause of foodborne botulism and infant botulism in various countries (Fox *et al.*, 2005, Peck, 2009, Malaska, 2014, Mazuet *et al.*, 2014).

RESULTS

Entry of HCc/B into the mouse intestinal mucosa

Recombinant HCc from BoNT/B (HCc/B) labeled with Cy3 has been used to monitor BoNT/B entry into mouse intestinal mucosa. HCc domain of clostridial neurotoxins (BoNTs and tetanus neurotoxin), which specifically interacts with cell receptors, has already been extensively used to investigate internalization and intracellular trafficking of the neurotoxins in neuronal cells, as well as toxin translocation through intestinal epithelial cells (Lalli *et al.*, 2003, Maksymowych *et al.*, 2004, Bohnert *et al.*, 2005, Roux *et al.*, 2006, Harper *et al.*, 2011, Restani *et al.*, 2012, Lam *et al.*, 2015). Similar strategy was performed in the analysis of BoNT/A passage through the mouse intestinal mucosa (Couesnon *et al.*, 2012). Therefore, fluorescent HCcB was injected into ligated ileum loops of anesthetized mice. The animals were then killed at various time intervals and the intestinal loops were washed, fixed and prepared for confocal microscopy observations. As shown in Fig. 1A (upper panel) limited HCcB fluorescence was observed in the intestinal lumen between the villi after 5 min incubation. Strikingly, rare epithelial cells of the villi accumulated fluorescent HCcB (Fig. 1A, middle panel). A few filamentous structures labeled with HCcB were also detected in some intestinal villi and in some submucosa areas (Fig. 1A, lower panel). After 10 and 20 min incubation, labeled HCcB was visualized in the inter-villi spaces, the lumen of intestinal crypts, and on the surface of some intestinal villi, but no HCcB staining was evidenced in crypt epithelial cells (Fig. 1B, upper and middle panels, Fig. 1C, upper panel). Again, only very few cells of the villus epithelium were stained with HCcB (Fig. 1B and 1C upper panels). Filamentous elements stained with HCcB were observed in the bottom of intestinal villi surrounding intestinal crypts (Fig. 1B, middle panel and Fig. 1C, upper and middle panels). A dense network of filamentous structures in the musculosa was stained with

fluorescent HCcB (Fig. 1B lower panel, Fig. 1C, upper and lower panels). In addition, fluorescent filament structures were also evidenced inside the core of the villi (Fig. 1A middle panel, Fig. 1B upper panel). The most marked observations were the abundant filament labeling in the submucosa and to a larger extent in the musculosa after 10 and 20 min incubation of the ileal loops with fluorescent HCcB (Fig. 1B lower panel, Fig. 1C, upper and lower panels). Albeit the *in vivo* experiments at different incubation time periods did not allow to determine a precise kinetics of the toxin passage through the intestinal barrier, they permitted to evidence the progressive translocation of HCcB lasting 5 to 20 min from the intestinal lumen to target structures in the submucosa and musculosa. This finding was further supported by the quantification of the HCcB fluorescence intensity showing HCcB accumulation in villi overtime, and entry into the submucosa/musculosa at 10-20 min (Fig. 1C).

Dynasore impairs the translocation of HCcB into the intestinal mucosa

BoNT/A has been evidenced to cross the epithelial barrier through a transcytotic mechanism in the absence of ANTPs (Maksymowych *et al.*, 1998, Maksymowych *et al.*, 2004, Ahsan *et al.*, 2005, Couesnon *et al.*, 2008). Previous work also suggests that BoNT/B is able to use a similar transport (Maksymowych *et al.*, 1998). To investigate whether HCcB enters the mouse intestinal mucosa in *in vivo* conditions through a similar transcytotic mechanism, Dynasore, a specific inhibitor of endocytosis which blocks dynamin function (Harper *et al.*, 2013), was injected (10 μ g) into an ileal loop of anesthetized mice 20 min prior the injection of fluorescent HCcB. Mice were then sacrificed 10 min after the administration of fluorescent HCcB. As controls, we checked that administration of Dynasore and/or HCcB did not induced morphological alteration of the intestinal mucosa as monitored by E-cadherin staining of intestinal epithelial cells (Fig. 2A and B). In ileal loops, which only received fluorescent HCcB, an intense intracellular staining of rare intestinal epithelial cells and staining of filamentous elements in the mucosa and musculosa was observed (Fig. 2A). In contrast, in the ileal loops pretreated with Dynasore, fluorescent HCcB showed a different staining pattern. Thus, HCcB staining was mostly observed on the surface of intestinal villi and in the inter-villi spaces, whereas only a few filamentous structures were labeled in the submucosa and musculosa (Fig. 2B). No morphological alteration of the basolateral epithelial cell membranes was evidenced as monitored by E-cadherin immunostaining (Fig. 2A and B, middle panels). Quantification of fluorescence intensity in the submucosa and

musculosa indicated that Dynasore impaired by almost 90% the transcytosis of fluorescent HCcB across the intestinal barrier (Fig. 2C). The significant Dynasore-induced inhibition of HCcB staining of target cells in the submucosa and musculosa strongly supports a transcytotic passage of HCcB through the intestinal epithelial barrier.

Endocytosis-dependent entry of BoNT/B into the mouse intestinal mucosa

Experiments were performed with purified BoNT/B to further assess that the results obtained with HCcB were representative of the trafficking of the whole toxin. For this, ligated mouse intestinal loops were injected with purified BoNT/B (100 μ g) and were then processed for imaging studies as described above. After 10 min administration, BoNT/B was detected with immunopurified polyclonal antibodies directed against the HCc domain. BoNT/B was visualized on the surface of some villi after 10 min toxin administration as shown in Fig. 3A (upper panel), and decorated filamentous structures in the villi, submucosa and musculosa. BoNT/B was mostly localized in the submucosa and musculosa after a 20 min incubation time period (Fig. 3A, lower panels). Thereby, a similar pattern of staining was observed between HCcB and purified BoNT/B.

Next, we checked whether BoNT/B like HCcB enters the intestinal mucosa through a transcytotic mechanism. Pretreatment of intestinal loop with Dynasore (10 μ g) for 20 min significantly reduced the detection of BoNT/B in the submucosa and musculosa (Fig. 3C). No morphological alteration of the intestinal mucosa was observed in the intestinal loops treated with Dynasore and/or BoNT/B (not shown). Compared to intestinal loops treated with BoNT/B in the absence of Dynasore, only a few cells and filament structures were labeled in the submucosa and musculosa from Dynasore pretreated intestinal loops (Fig. 3C). Dynasore induced a 80% decrease in the BoNT/B staining in both submucosa and musculosa (Fig. 3D), further supporting that similarly to HCcB, BoNT/B uses a transcytotic pathway to pass through the intestinal epithelial barrier. In addition, these results support the view that HCcB and BoNT/B use a similar traffic pathway in the mouse intestinal mucosa.

HCcB and BoNT/B target neuronal cells in mouse intestinal submucosa and musculosa

Imaging analyses with antibodies against neurofilaments (NF) were performed to identify the cells and filamentous structures recognized by fluorescent HCcB in the submucosa and musculosa. Fluorescent HCcB was localized on long cell extensions co-labeled with anti-NF antibodies (Fig. 4A and B). However, not all but only certain cell

extensions were recognized by both HCcB and anti-NF antibodies (Fig. 4A and B). Partial colocalization between the two markers (Pearson coefficient 0.38 ± 0.06) suggested that HCcB did not bind directly to NF, but rather bound to cells and cell extensions containing NF.

We took advantage of Thy1-yellow fluorescent protein (YFP) transgenic mouse in the C57BL6 genetic background, which have been widely used to analyze the morphology and function of neurons in the mouse brain (Feng *et al.*, 2000, Vuksic *et al.*, 2008), to visualize neurons of the enteric nervous system (ENS). Thy1-YFP is mainly expressed in the Golgi apparatus of certain subpopulations of neurons (Feng *et al.*, 2000). In the intestinal submucosa and musculosa of Thy1-YFP mice, neuronal cells were discontinuously labeled, to some extent due to the accumulation of YFP around the nuclei (Fig. 5A). Some neuronal cells showed an intense YFP expression in the cytosol in cell bodies and extensions. Fluorescent HCcB injected into the intestinal lumen of Thy1-YFP mice co-stained YFP labeled cells and cell extensions after 10 min after injection (Fig. 5A). However, not all but only some subpopulations of YFP-expressing neurons were recognized by HCcB (Fig. 5A). The low Pearson coefficient (0.241 ± 0.11) between YFP and Cy3-HCcB, indicates that the two markers labelled distinct structures of the same cells.

Synaptotagmin (Syt) has been evidenced as the receptor protein part which in combination with gangliosides of the GD1b and GT1b series forms the high affinity receptor of BoNT/B. Indeed, BoNT/B binds to the intraluminal domain of synaptotagmin I (SytI) and (SytII) through the HCc domain (Nishiki *et al.*, 1996, Dong *et al.*, 2003, Rummel *et al.*, 2007, Rummel, 2013). Antibodies against SytII labeled cells and cell extensions in mouse intestinal submucosa and musculosa which were co-labelled with YFP in Thy1-YFP mice (Fig. 5A). Similar results were obtained with anti-SytI antibodies (not shown). Fluorescent HCcB specifically recognized cells and cell extensions expressing both Thy1 and SytII (Fig. 5A). This supports that HCcB targeted specific neurons expressing SytII in mouse intestine. The low Pearson's coefficient (0.24 ± 0.11) between HCcB staining and YFP expression also indicates that HCcB did not colocalize with YFP molecules but recognized cells expressing YFP-Thy1. In contrast, the high level of colocalization between HCcB and SytII (Pearson's coefficient: 0.51 ± 0.11) supports that SytII is a specific BoNT/B receptor in intestinal neuronal cells.

Glial cells are abundant in the nerve tissues as well as in the ENS where they show multiple extensions, mainly in the submucosal and myenteric plexus, and in close contact with neuronal cells (Yu *et al.*, 2014). Glial cells from the mouse intestinal mucosa were

specifically visualized with antibodies against the glial fibrillary acidic protein (GFAP) (Fig. 5B). Since glial cells, are wound around neuronal cells an apparent high Pearson's coefficient (0.35 ± 0.03) was observed between HCcB-Cy3 and GFAP stained cells (Fig. 5B). Indeed, glial cell extensions are in close contact with those of neuronal cells but they are shorter. HCcB labeling pattern of cell extensions correlated with that of NF staining but was distinct from that obtained with GFAP staining (Fig. 5B). These findings suggest that HCcB entered the intestinal mucosa and targeted specific neuronal cells and extensions in the submucosa and musculosa and did not interact with glial cells in the mouse intestine.

HCcB targets cholinergic neurons in the mouse intestine

BoNTs are known to interact with cholinergic neurons and to block spontaneous and evoked quantal ACh release (Schiavo *et al.*, 2000, Poulain *et al.*, 2008, Rossetto *et al.*, 2014). However, BoNTs are able to enter various neuronal cell types and to inhibit the release of a large range of other neurotransmitters such as ACh, glutamate, gamma-aminobutyric acid (GABA) (reviewed in (Dolly *et al.*, 2009, Popoff *et al.*, 2010).

First, imaging studies were carried out to test whether HCcB targets cholinergic neurons using antibodies directed against choline acetyltransferase (ChAT). Most of the ChAT immunoreactive neuronal cell extensions from the submucosa and musculosa were co-labelled with HCcB ($82.2 \% \pm 3.8$) 10 min after its administration in the intestinal loop (Fig. 6A, and Table 1). ChAT neuronal cells labeled with HCcB also contained SytII (Sup. Fig. 1A). Similar results were obtained with whole BoNT/B injected into the intestinal lumen and detected with specific immunopurified antibodies (Fig. 7A). After 10 min incubation, a large proportion of ChAT immunoreactive neuronal cells of the submucosa and musculosa were co-stained with anti BoNT/B antibodies ($87\% \pm 2.5$) (Fig. 7A). However, it is noteworthy that HCcB or BoNT/B also labelled other neuronal cell types than ChAT neuronal cells, but to a lower extent.

Distinct neuronal cells recognized by HCcB in the mouse intestine

Binding of BoNT/B to the diverse neuronal cell types in mouse intestine was investigated by colocalisation analysis of HCcB-Cy3 with specific neuronal cell markers. Only a low number of neuronal cells were stained with anti-serotonin (5-hydroxytryptamine, 5-HT) antibodies in the submucosa and myenteric plexuses of the mouse intestine. After 10 min administration of HCcB or BoNT/B into the ileum lumen, a significant proportion (25 –

35 %) of serotonin-immunoreactive cells was co-labeled with fluorescent HCcB or anti BoNT/B antibodies (Fig. 6B and 7B, and Table 1). It is noteworthy that the serotonin-immunoreactive cells labeled with HCcB also expressed SytII (Sup. Fig. 1B).

The other neuronal cell types are underrepresented in ENS and were variably recognized by HCcB-Cy3. Indeed, a low proportion estimated to be about 10% of vasoactive intestinal peptide (VIP)-immunoreactive cells in the submucosa was co-stained with HCcB (Fig. 6C and Table 1). Furthermore, rare glutamate- and gamma-aminobutyric acid GABA-immunoreactive cells stained with antibodies against vesicular glutamate (vGLUT) and GABA transporter (vGAT), respectively, were visualized in the intestinal submucosa and most of them were partially co-stained with fluorescent HCcB (Fig. 6D, 6E, and Table 1).

Interestingly, HCcB labeled filamentous structures inside the core of villi which were co-stained mostly with antibodies against ChAT ($52.5 \pm 2.9\%$) and to a lower extent with VIP antibodies ($5 \pm 2.5\%$) (Fig. 8). No serotonin-immunoreactive neuronal extensions were visualized in the intestinal villi. These structures recognized by HCcB are likely projections of intrinsic afferent neurons whose cell bodies are localized in the submucosa and which are mainly ChAT- and to a lower extent VIP-immunoreactive neurons (Furness *et al.*, 2004).

Intestinal cells involved in the passage of BoNT/B

Subsequently to administration of fluorescent HCcB in the intestinal lumen, certain cells of villous epithelium were intensely fluorescent. Albeit HCcB was observed inside the lumen of most intestinal crypts, no crypt cells retained HCcB fluorescence (Fig. 1). Similar findings were observed with BoNT/B injected into the intestinal lumen and detected with anti-HCcB antibodies (Fig. 2, Fig. 3C and D). The question arises as to whether the rare cells from the villous intestinal epithelium accumulating HCcB fluorescence reflect the transcellular translocation of HCcB and BoNT/B, or alternatively represent a site of toxin storage. Because these cells were mostly located in the upper part of intestinal villi, we first investigated whether they undergo apoptosis and thereby might non-specifically accumulate HCcB or BoNT/B. As shown in Sup. Fig. 2, no co-staining between fluorescent HCcB and activated Caspase3 antibodies was evidenced indicating that the fluorescent HCcB stained cells were not apoptotic.

The inhibitory effect of Dynasore on the transport of HCcB or BoNT/B across the intestinal epithelium (Fig. 2 and 3B) suggests an apical endocytic uptake and subsequent

transcytotic delivery of HCcB or BoNT/B through the basolateral side. Colchicine, which is a tubulin filament disruption agent, impairs transcytosis in epithelial cells such as CaCo-2 cells (Bose *et al.*, 2007), and has been used to inhibit the translocation of *Listeria monocytogenes* across the intestinal epithelium in an intestinal ligated loop model (Nikitas *et al.*, 2011). Pretreatment of mouse intestinal loops with colchicine in the same conditions than for the *L. monocytogenes* experiments (10 µg/ml, 20 min) (Nikitas *et al.*, 2011) prior to the injection of fluorescent HCcB, induced a significant increased number of villous epithelial cells accumulating HCcB (Fig. 9A and 9B). In addition, the HCcB fluorescence in intestinal loops pretreated with colchicine was mainly localized at the periphery of the epithelial cells which accumulated HCcB, whereas in the non-pretreated intestinal loops the epithelial cells which retained HCcB were uniformly stained, possibly resulting from a different traffic pathway than in colchicine-treated cells (Figs. 9A and 9B). However, colchicine pretreatment did not significantly prevent HCcB staining of neuronal cells in the submucosa and musculosa (Fig. 10), indicating a delayed passage of HCcB through certain villous cells, independently of the one inhibited by colchicine.

The cells which accumulated fluorescent HCcB, were not stained with phalloidin, a marker of actin filaments (Fig. 9A). This finding strongly suggests that HCcB accumulated in a subset of intestinal cells devoid of apical brush border, and raises the question whether BoNT/B uses specific cells for its transport through the intestinal barrier? To address this question, we investigated whether the HCcB labeled cells belong to a specific sub-population of the intestinal epithelium, using various markers of already characterized cell subsets of the intestinal epithelium: wheat germ agglutinin (WGA) which binds specifically to sialic acid and N-acetylglucosaminyl carbohydrate residues in mucous of goblet cells (Jang *et al.*, 2004), Lectin *Ulex europaeus* agglutinin type 1 (UEA1) which recognizes Paneth cells (Garabedian *et al.*, 1997), antibodies anti-CD3 and anti-CD11C two markers of lymphocytes and dendritic cells, respectively, which are localized in the intestinal lamina propria (Montufar-Solis *et al.*, 2007, Persson *et al.*, 2013), anti-villous M cells shown to be the principal site of gut luminal antigen uptake (Jang *et al.*, 2004), chromogranin A which represents a common marker of enteroendocrine cells (Portela-Gomes *et al.*, 2000), and the double cortin kinase 1 protein (DCLK1) specific of tuft cells, a class of secretory intestinal cells distinct from enteroendocrine cells, Paneth, cells and goblet cells (Gerbe *et al.*, 2011). Additional markers of goblet cells were used like antibodies specific of the intestinal trefoil factor (ITF) or cytokeratin18 (Poulsom *et al.*, 1993, Hesse *et al.*, 2007). Double immunofluorescence studies

using the various cell-specific antibodies or markers and fluorescent HCcB clearly showed that none of the markers tested colocalized or co-stained with HCcB labelled cells in the intestinal epithelium (Sup. Fig. 3 and 4). Furthermore, the rare cells of the villous epithelium exhibiting ChAT immunoreactivity were not targeted by HCcB (Sup. Fig. 3).

Transcytosis through intestinal cells indicates that HCcB or BoNT/B recognizes specific cell surface receptor(s). Are BoNT/B receptors on intestinal cells the same than those on neuronal cells (gangliosides of the GD1b/GT1b series in combination with SytII)? An excess of GT1b has been found to prevent BoNT/B binding to synaptosomes (Atassi *et al.*, 2014). A competition experiment was performed between HCcB and GT1b for binding and entry of toxin into the intestinal mucosa. As shown in Fig. 11, in contrast to control intestinal loop treated with only labeled HCcB, preincubation of HCcB with GT1b significantly prevented HCcB staining of intestinal villi, submucosa and musculosa. Only a faint HCcB staining was observed in certain villi, whereas no staining was visualized in the submucosa and musculosa. As control, GM1 was found to not impair HCcB entry into the intestinal mucosa (Sup. Fig. 5). Moreover, no labeling of intestinal epithelial cells with anti-SytII antibodies was evidenced, suggesting that BoNT/B uses GT1b but a distinct protein receptor from that of neuronal cells to enter intestinal cells.

DISCUSSION

Translocation of BoNT through the intestinal barrier is a critical initial step in botulism resulting from the ingestion of toxin preformed in food or from intestinal colonization by *C. botulinum*. In *in vitro* cultures, food or intestinal content, BoNT is produced in complex forms by non-covalent association with ANTPs. Whether whole botulinum complexes or only BoNT pass through the intestinal barrier and the mode of its transport remain under debate. ANTPs play an essential role in the protection of BoNT against the stomach acidic pH and digestive proteases. The non-toxic non-hemagglutinin (NTNH) component of *C. botulinum* type A, is structurally related to BoNT/A and associates with BoNT/A in a pH-dependent manner to form a medium size botulinum complex highly resistant to acidic pH and protease degradation (Gu *et al.*, 2012, Gu *et al.*, 2013). It is noteworthy that NTNH is synthesized by all *C. botulinum* types and likely retains the same function in the various corresponding botulinum complexes. HAs, which are found in various BoNT complex types, have been evidenced to mediate the absorption of botulinum complex

from the gut. HAs interact with oligosaccharides on the intestinal epithelial cells and possibly facilitate the toxin uptake into intestinal cells (reviewed in (Fujinaga *et al.*, 2013, Gu *et al.*, 2013)). The HA-mediated transport of BoNT/A complex through the intestinal barrier seems to preferentially occur via microfold (M) cells (Matsumura *et al.*, 2015). In addition, HAs bind to and disrupt E-cadherin-mediated intercellular junctions, thus allowing the passage of BoNT through the intestinal barrier via the paracellular route (reviewed in (Fujinaga *et al.*, 2013, Gu *et al.*, 2013)). However, HAs are not produced by *C. botulinum* E, F and certain type A strains which are also involved in human botulism by the oral route (Simpson, 2013, Singh *et al.*, 2014). These *C. botulinum* strains form BoNT complexes containing OrfX proteins instead of HAs (Hill *et al.*, 2013, Popoff *et al.*, 2013). However, OrfX proteins have not been found to interact with intestinal cells indicating that BoNT can itself cross the intestinal barrier without the help of additional protein. Therefore, when administrated into the small intestine, BoNT free of HAs and BoNT complexes are absorbed through the intestinal barrier in an equally efficient manner (Maksymowych *et al.*, 1999). Moreover, purified BoNT was demonstrated to bind to intestinal cells, and to undergo receptor-mediated endocytosis, transcytosis, and subsequent release from the baso-lateral side (Maksymowych *et al.*, 1998, Maksymowych *et al.*, 2004, Ahsan *et al.*, 2005, Couesnon *et al.*, 2009). Here, we investigated the entry of BoNT/B into the intestinal mucosa in an *in vivo* mouse model using purified BoNT/B or the recombinant HCcB fragment. The corresponding C-terminal domain of BoNT/A has already been used to monitor the holotoxin trafficking in cultured cells or intact intestinal mucosa (Maksymowych *et al.*, 2004, Ahsan *et al.*, 2005, Couesnon *et al.*, 2012).

When administrated into a mouse jejuno-ileal ligated loop, a progressive passage of the fluorescent HCcB or BoNT/B from the intestinal epithelium to the submucosa and musculosa was visualized. Within 10-20 min, HCcB or BoNT/B targeted neuronal structures in the submucosa and musculosa. This rapid passage contrasts with the longer time (30-60 min) observed for the internalization of HCcA in the mouse intestinal mucosa (Couesnon *et al.*, 2012). However, the experimental conditions used for HCcA investigation were slightly different. Mouse intestinal loops were excised, transferred into oxygenated culture medium, and incubated at room temperature after intraluminally injection of HCcA (Couesnon *et al.*, 2012). In the present work, the vascularization of the intestinal loops and the physiological mouse temperature were maintained. Thereby, in the more physiological conditions, a more rapid trafficking was observed indicating that BoNT/B translocation into the mouse intestinal mucosa is a rapid process. However, the difference in the experimental procedures does not

fully exclude that BoNT types A and B may have a different way and kinetics of entry into the intestinal mucosa.

Interestingly, Dynasore which is an inhibitor of dynamin-dependent endocytosis process (Macia *et al.*, 2006, Harper *et al.*, 2013), significantly prevented the entry of HCcB or BoNT/B into the mouse intestinal mucosa (Fig. 2 and 3). The *in vivo* mouse model of ligated intestinal loop clearly supports that BoNT/B free of HAs or the receptor binding domain, HCcB, were able to pass through the intestinal barrier *via* an endocytic mechanism to target specific cells in the intestinal submucosa and musculosa. It should be noticed that the toxin amounts used in this study were higher than those expected in natural acquired botulism. Minimum amounts of fluorescent HCcB yielding detectable signal in intestinal tissues were around 10 μg per intestinal loop (Sup Fig. 6). We selected to use higher amounts (100 μg /intestinal loop) to more efficiently visualize the cells mediating toxin trafficking. Since high HCcB or BoNT/B doses were efficiently inhibited by endocytosis inhibitor (Fig. 2 and 3) or in competition experiments with specific receptor such as GT1b (Fig. 10), and since only a restricted number of cell types were visualized (Fig. 1 and 3), the physiological toxin trafficking was likely preserved in these experimental conditions. Although lower amounts of HCcA (0.5 μg / intestinal loop) (Couesnon *et al.*, 2012) or BoNT/A (0.6 μg orally per mouse) (Lam *et al.*, 2015) were used. However we were unable to reproduce the results with 0.6 μg BoNT/A per mouse. Moreover, it has to be taken in account that BoNT/B is less active than BoNT/A, about 10-fold less (Foran *et al.*, 2003, Rasetti-Escargueil *et al.*, 2009), and that only a low toxin fraction passes through the intestinal barrier based on *in vivo* experiments (reviewed in (Popoff *et al.*, 2014)) therefore justifying the amounts used in this study. However, one cannot fully exclude that in the present work, HCcB or BoNT/B uses different entry pathways than in the *in vivo* situation.

HCcB or BoNT/B enters the mouse intestinal mucosa and specifically targets neuronal cell bodies and neuronal cell extensions in the submucosa and musculosa as visualized by co-staining of the filamentous structures with anti-NF antibodies and HCcB or BoNT/B (Fig. 4). Glial cells which are abundant in the intestinal mucosa were not recognized by HCcB (Fig. 5). These observations were further supported by the use of the transgenic Thy1-YFP mouse which exhibits a nice labeling of the ENS, and co-staining of neuronal filaments expressing Thy1-YFP with HCcB (Fig. 5). It is noteworthy that HCcB or BoNT/B only recognizes certain neuronal cell extensions. Neuronal cell filaments identified by anti-NF antibodies or by Thy1-YFP expression were not all co-stained with HCcB (Fig. 5). Interestingly, HCcB and

BoNT/B colocalized with SytII antibodies on intestinal neuronal extensions (Fig. 5 and Sup Fig. 1) in agreement with previous characterization of SytII as the BoNT/B receptor (Nishiki *et al.*, 1996, Dong *et al.*, 2003, Chai *et al.*, 2006). ChAT and serotonin neurons labeled with HCcB also expressed SytII (Sup. Fig. 1), and likely the other neuronal cell types targeted by BoNT/B. Collectively the present findings support that BoNT/B uses SytII as specific receptor on neuronal cell extensions of the ENS.

Neuronal cells of the ENS are organized in the submucosa and myenteric plexuses, which project on the different layers of the intestinal mucosa. ENS neurons can synthesize a wide variety of neurotransmitters. More than 20 types of neurotransmitters have been identified in ENS and most enteric neurons may produce and secrete several of them (Goyal *et al.*, 1996, Grundy *et al.*, 2006, Furness, 2012). Cholinergic neurons are the greater population of enteric neurons. In the submucosal plexus, cholinergic neurons (about 55% of the enteric neurons) regulate ion secretion and are involved in local sensory pathways in response to the luminal content composition. In addition, they interact with Peyer's patch follicles (Kulkarni-Narta *et al.*, 1999, Furness *et al.*, 2004, Harrington *et al.*, 2010). Cholinergic neurons are preponderant in the myenteric plexus (about 80%) and have a main function in mediating muscle activity and controlling intestinal motility (Furness *et al.*, 2004, Harrington *et al.*, 2010). Serotonin is a major mediator of gastrointestinal function. Notably serotonin controls motility, secretion, and sensory responses. Serotonin is mainly produced by enteroendocrine cells from the mucosal epithelium of the intestine and 2 to 20% of enteric neurons are serotonin immunoreactive (McLean *et al.*, 2007, Mawe *et al.*, 2013). VIP-immunoreactive neurons are the most preponderant non-cholinergic neurons of the ENS and constitute the main class of inhibitory interneurons. VIP-immunoreactive neurons are estimated to represent about 45% of neurons of the submucosal plexus in the ileum and jejunum. VIP modulates several functions including vasodilatation of intestinal vessels, intestinal smooth muscle relaxation, and stimulation of electrolyte secretion and water (Brookes, 2001, Furness *et al.*, 2004, Hernandez *et al.*, 2004, Igarashi *et al.*, 2011, Furness, 2012). Glutamate and GABA are neurotransmitters produced by a few number of ENS neuronal cells (Furness, 2000, Furness *et al.*, 2004).

In *in vivo* experiments HCcB or BoNT/B targeted certain but not all neuronal cells at the Syt binding sites. Cholinergic neurons of ENS were the main target of HCcB or BoNT/B, since more than 80% of the ChAT immunoreactive neuronal cells were co-stained with HCcB or BoNT/B (Table 1). This is in agreement with the fact that BoNT/B like the other BoNT types, mainly target peripheral cholinergic motor neuron endings (Simpson, 2013, Rossetto *et*

al., 2014). HCcA was also found to preferentially localize to cholinergic neurons of the submucosa and musculosa of the mouse intestine (Black *et al.*, 1987, Couesnon *et al.*, 2012). HCcB also targeted cholinergic extensions in the villi and around the intestinal crypts. Constipation is a frequent symptom (about 70%) in food-borne botulism and is a major and early event in botulism resulting from intestinal colonization (Arnon *et al.*, 2001, Sobel, 2005, Brook, 2007, Mitchell *et al.*, 2008). Since cholinergic neurons are the main players of the control of intestinal motility and secretion (Furness, 2012), ChAT-immunoreactive targeting by BoNT likely account for the reduced intestinal peristalsis and secretion observed in botulism. During botulism by intestinal colonization, BoNT produced in the intestinal lumen might be absorbed in a higher local concentration able to enter the intestinal mucosa and inhibit the underlying cholinergic neurons compared to orally ingested toxin which disseminates more broadly through the digestive tract.

HCcB or BoNT/B targeted other neuronal cell types than cholinergic neurons in the mouse intestinal mucosa but to a lower extent. Serotonin-immunoreactive neuronal cells were significantly co-stained with HCcB or BoNT/B (25 to 35%) (Table 1), whereas a lower proportion (about 10%) (Table 1) of VIP-immunoreactive neurons was stained with HCcB. In the present work, only rare V-GLUT- and V-GAT-immunoreactive cells were visualized in the intestinal mucosa and most of them co-stained with HCcB. However, the low number of identified cells did not allow estimating precisely the proportion of these sub-cell types targeted by HCcB.

The pathophysiological effects of BoNT on the non-cholinergic neurons are still a matter of debate. Serotonin is an important neurotransmitter in the gastrointestinal tract which is involved in multiple functions including intestinal motility and secretion. Although most part of serotonin is secreted by enteroendocrine cells, serotonergic neurons have an essential role in the gastrointestinal motility (Li *et al.*, 2011, Gershon, 2013). BoNT/B effects on serotonergic neurons of the intestinal mucosa might contribute to the inhibition of intestinal motility in synergy with the blockade of ACh release at cholinergic nerve endings. The incidence of BoNT/B on the small part of VIP-immunoreactive neurons targeted by HCcB, remains speculative. Since one of the main roles of VIP is to control gastrointestinal secretion (Igarashi *et al.*, 2011, Furness *et al.*, 2014), BoNT/B may exert its anti-secretory effect partially through inhibition of VIP release. BoNT/B might use non-cholinergic neurons not only to contribute to the local paralytic effects, but also to disseminate to other target neurons locally or at distance from the intestine. Indeed, BoNT/A has been shown to use a retrograde transport and transcytosis to migrate from the peripheral neurons to the central nervous

system (Antonucci *et al.*, 2008, Restani *et al.*, 2011, Restani *et al.*, 2012). Since the neurons of ENS are highly interconnected between them and with neurons of the central nervous system (Goyal *et al.*, 1996, Grundy *et al.*, 2006, Furness *et al.*, 2014), one can speculate that BoNT/B uses non-cholinergic intestinal neurons for its transport to remote target neurons.

The present findings also raises an intriguing question: does BoNT/B use preferential cells to cross the intestinal barrier? In contrast to BoNT/A, which has been found to enter the intestinal mucosa preferentially via crypt enteroendocrine cells (Coesnon *et al.*, 2012), no HCcB or BoNT/B labeling was detected in epithelial crypt cells, or enteroendocrine cells using anti-chromogranin A antibodies. Accumulation of HCcB or BoNT/B was observed in a few villous epithelial cells lacking apical actin staining (e.g. devoid of apical brush border). The fact that cells accumulating HCcB were not apoptotic cells rule out the possibility of non-specifically fluorescent protein accumulation. Using a wide panel of markers of intestinal epithelial and lymphocytic cells, the villous cells labeled with HCcB or BoNT/B could not be assigned to any already known subset of cell type. Whether these cells represent the toxin passage through the intestinal barrier remains questionable? A transcytotic passage supposes a continuous trans-cellular toxin passage over time. However, a transient accumulation in some intracellular compartment is not fully excluded. Time lapse monitoring the passage of fluorescent HCcB could be more informative. The *in vivo* experiments used in this study allowed collection of intestinal samples only at different time points. Pretreatment with colchicine, an inhibitor of microtubule-dependent transcytosis, evidenced an increased number of villous epithelial cells accumulating HCcB, but with a distinct pattern of fluorescent labeling than in colchicine-untreated intestinal loops (Fig. 9). HCcB was mainly observed on the basolateral peripheries of epithelial cells from pretreated intestinal loops with colchicine, whereas HCcB accumulated broadly into the intracellular space in non-pretreated loops. However, colchicine did not prevent significantly the HCcB passage to the underlying neuronal cells. Thus, it cannot be excluded that colchicine delayed, but not completely blocked, the transcytotic passage of HcCB in these subsets of villous cells. The absorption of BoNT from the digestive tract is a very low efficient process. *In vivo* experiments showed that less than 0.01 to 0.1% of intraduodenally administrated BoNT can be recovered in lymph of blood circulation (reviewed in (Popoff *et al.*, 2014)). Thus, a low toxin passage through the enterocytes or at least some of them may support the weak intestinal absorption of toxin which has been observed *in vivo*.

In conclusion, the present study using an *in vivo* model of mouse ligated intestinal loop demonstrates that HCcB or BoNT/B free of HAs can pass through the intestinal barrier

via an endocytosis-dependent mechanism since Dynasore, a specific inhibitor of endocytosis, significantly prevented their translocation. After 10-20 min incubation time, HCcB or BoNT/B specifically targeted neuronal cells and neuronal extensions in the submucosa and musculosa. Binding of HCcB or BoNT/B to neurons correlated with Syt expression, in agreement with the identification of Syt as the specific BoNT/B receptor (Nishiki *et al.*, 1996, Dong *et al.*, 2003, Rummel *et al.*, 2007, Rummel, 2013). Cholinergic intestinal neurons were the main targets of HCcB or BoNT/B together with other neuronal cell types of the intestinal mucosa, notably serotonergic neurons and to a lower extent VIP, V-GLUT and V-GAT-immunoreactive neurons. Although HCcB or BoNT/B accumulated in some intestinal cells, those which mediate the passage of BoNT/B remain to be identified.

EXPERIMENTAL PROCEDURES

Ethic statements

All experiments were performed in accordance with French and European Community guidelines for laboratory animal handling. The protocols of experiments were approved by Pasteur Institute CETEA (Comité d'Ethique en Expérimentation Animale) with the agreement of laboratory animal use (n° 2013-0118).

Animals

Adult Swiss mice (Charles River) and Thy-1 yellow fluorescent protein protein (YFP) transgenic mice (C57BL6 background, Jackson Research) were used.

Reagents

Tissues were stained with Hoechst (Dako, 1:1000 dilution) for the nuclei, FITC-phalloidin (Sigma, 0.4mg/ml), FITC-Urexa europaea agglutinin type 1 (UEA1) (Sigma, 1 mg/ml), wheat germ agglutinin alexa fluor 488 conjugate (Invitrogen, 1:500 dilution). The primary antibodies used recognized E-cadherin (Invitrogen, rat, 1:250 dilution), neurofilament (Sigma, rabbit, 1:250 dilution), Choline Acetyltransferase (ChAT) (Millipore, goat, 1:100 dilution), serotonin (Abcam, goat, 1:1000 dilution), chromogranin A (Abcam; rabbit, diluted 1:250), synaptotagmin II (SytII) (Abcam, rabbit, 1:250 dilution), Glial Fibrillary Acidic Protein (GFAP) (Sigma; rabbit, 1:200 dilution), VIP (Abcam; rabbit, diluted 1:200), vesicular glutamate transporter 1, BNPI, SLC17A7 (VGLUT 1) (Synaptic System, rabbit, 1:250 dilution), vesicular GABA transporter (VGAT) (Synaptic System, rabbit, 1:250 dilution),

activated caspase-3 (Cell Signaling 9661, rabbit, 1:200), DCLK1 (Abcam, rabbit, 1:200 dilution), M cell villi (MACS Miltenyi Biotec, rat, 1:200 dilution), Anti CD11c clone HL3 (BD Bioscience, armenian hamster, 1:250 dilution), integrin α E (CD3) (BD Pharmingen, syrian hamster, 1:200 dilution), cytokeratin 18 (Abcam, biotinylated mouse monoclonal antibody, 1:100 dilution), intestinal trefoil peptide (Santa Cruz, rat, 1:100 dilution). The secondary antibodies were Alexa fluor 488 or 647 donkey anti goat, alexa fluor 488 or 647 goat anti rat, Alexa fluor 488 donkey anti rabbit, Alexa fluor 594 goat anti rabbit (Invitrogen, 1:500 dilution), Alexa fluor 647 anti Syrian hamster (Jackson Immunoresearch, 1/500 dilution), Alexa fluor 647 goat anti Armenian hamster (Jackson Immunoresearch, 1/500 dilution). Endocytosis assay were perform with Dynasore Hydrate (Sigma, 10 μ g/intestinal loop). Other reagents: Colchicine (Sigma, 10 μ g/ml), trisialoganglioside GT1b (Sigma G3767), ganglioside GM1 (Sigma 345724).

Botulinum neurotoxin and recombinant HCcB protein production

BoNT/B was produced and purified as previously described (Shone *et al.*, 1995). Recombinant His-tag HCc fragment of BoNT/B was produced and purified from pET-28a-c(+) vector containing DNA encoding for HCcB cloned into BamHI and Sall sites, as previously described (Tavallaie *et al.*, 2004). HCcB His-tag was labeled with Amersham Cy3 Mono-Reactive Dye Pack (Ge Healthcare) according to the manufacturer's recommendations. Free dye is removed from labelled protein using the de Zeba Spin Desalting Columns according to the manufacturer's recommendations (Thermo Scientific).

***In vivo* intestinal ligated loop experiment**

Swiss mice (between 20 and 22 g) or Thy-1 YFP transgenic mice were fasted for 16 h before surgery. Mice were deeply anesthetized with a mixture of ketamine (50 mg/kg body weight; Imalgene 1000; Merial) and medetomidine (0.5 mg/kg body weight; Domitor; Orion Corporation). A mouse laparotomy was performed and a jejuno-ileal loop (approximately 4cm long) was isolated. Three hundred μ l of labeled HCcB or purified BoNT/B containing 100 μ g protein were injected into the intestinal lumen. After incubation times (5 to 20 min) mice were euthanatized, the intestinal loops were harvested, opened longitudinally, washed repeatedly in 37°C Dulbecco's modified Eagle's medium (Invitrogen), and fixed flat (luminal side up) for 2 h in 4% paraformaldehyde (PFA) at room temperature.

In competition assay, Cy3-HCcB (70 μg) was preincubated with a 10-fold excess of ganglioside GT1b from bovine brain (Sigma G3767) for 20 min at room temperature and the mixture was injected into an intestinal loop. After 15 min incubation, the intestinal loops were processed for fluorescence microscopy.

Intestinal tissue preparations and indirect immunofluorescence

Fixed intestinal loops were embedded in 4% agarose and sections (150 μm) were cut with a vibratome (Polyscience – Bangs Laboratories). Sections were permeabilized with 0.4% triton X-100 for 1 h at room temperature, washed in PBS, and then incubated for 1 h in PBS-BSA (3 %). Samples were then incubated or not with specific antibodies for 2 h at room temperature for primary antibodies and 1 h at room temperature for secondary antibodies in PBS-BSA (0.5 %) - Triton (0.2%). After 3 washes in PBS, sections were mounted in Fluoromount (FluorProbes) and samples were imaged using a Zeiss LSM700 confocal laser scanning microscope.

Image analysis and statistics

Image analysis was performed using ImageJ (National Institutes of Health). Colocalization test were performed using JacOp plugin from ImageJ. Values throughout the text are expressed as means \pm standard deviations (SD). Differences were assessed using unpaired Student's t-test. Statistical significance was assumed for $P < 0.0001$ (***) on at least 3 independent experiments for integrated density measurements and a 100 counted cells for the percentage of co-labeled structures.

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LEGENDS TO FIGURES

Figure 1. Visualization of fluorescent HCcB in the mouse intestine following its injection into the intestinal lumen. HCcB-Cy3 (100 μ g) in Dulbecco's modified Eagle's medium was injected into ligated jejuno-ileal loop of anesthetized mice. The intestinal loop was then introduced again into the mouse abdomen. After 5 (**A**), 10 (**B**), and 20 min (**C**), the intestinal loop was washed, fixed, sliced, and prepared for fluorescent confocal microscopy. The preparations were co-stained with Hoechst (blue) to visualize nuclei. (**A**) At 5 min incubation, HCcB was visualized (red labeling) on the surface and/or inside some villous epithelial cells, as well as in the inter-villous spaces. No or weak HCcB labeling was observed in the submucosa. (**B**) HCcB was observed inside intestinal crypts, in a few villous epithelial cells, and in filamentous structures in the villi and around intestinal crypts. HCcB markedly stained filamentous structures in the submucosa and musculosa. (**C**) At 20 min the HCcB staining of filamentous structures in the submucosa and musculosa was prominent. Scale bars = 10 μ m. (**D**) Quantification of HCcB fluorescence in the villi and submucosa/musculosa of intestinal loops. Values are mean \pm SD from 3 independent experiments (3 to 5 quantifications in each experiment). *** $P < 0.001$.

Figure 2. Inhibition of HCcB entry into the intestinal mucosa by Dynasore. HCcB-Cy3 (100 μ g) was injected into a control ligated jejuno-ileal loop (**A**) or into a ligated intestinal loop pretreated with Dynasore (10 μ g, 20 min) (**B**). After 10 min HCcB incubation, the intestinal segments were prepared as described in Fig. 1. The HCcB staining (red) of filamentous structures in the submucosa and musculosa (**A**) was prevented by pretreatment with Dynasore (**B**). E-cadherin staining (A and B middle panels, white) showed no morphological alteration of the intestinal epithelium. The preparations were co-stained with Hoechst (blue) and ECDD2 (white). (**C**) Quantification of HCcB fluorescence in the submucosa and musculosa of intestinal loops pretreated or not with Dynasore and injected intraluminally with fluorescent HCcB. Fluorescence from intestinal loops treated with HCcB only was set to 100%. Values are mean \pm SD from 3 independent experiments (3 to 5 quantifications in each experiment).. Scale bars = 10 μ m. *** $P < 0.001$

Figure 3. BoNT/B enters the mouse intestinal mucosa and its entry is prevented by Dynasore. BoNT/B (100 μ g) was injected into ligated jejuno-ileal loop of anesthetized mice. After the indicated times, the intestinal segments were prepared as described in Fig. 1 and BoNT/B was detected with rabbit immunopurified immunoglobulins against HCcB and Alexa594-anti-rabbit immunoglobulins. **(A)** After 10 min incubation, BoNT/B was visualized in a few villous epithelial cells, as well as in filament structures inside the villi and to a lower extent in the submucosa. **(B)** At 20 min incubation, a marked BoNT/B staining of filamentous structures in the submucosa and musculosa was evidenced. Epithelium brush border was visualized by actin staining with phalloidin (green) and nuclei with Hoechst (blue). **(C)** Pretreatment with Dynasore (10 μ g, 20 min) followed by 10 min incubation with BoNT/B significantly prevented BoNT/B staining in the submucosa and musculosa. **(D)** Quantification of BoNT/B fluorescence in the submucosa and musculosa of intestinal loops pretreated or not with Dynasore and injected intraluminally with BoNT/B. Data are mean values \pm SD, from 3 independent experiments (3 to 5 quantifications in each experiment).. Scale bars = 10 μ m. *** $P < 0.001$.

Figure 4. HCcB stains neuronal cell extensions in mouse submucosa and musculosa. HCcB-Cy3 (100 μ g) was injected into ligated jejuno-ileal loop and after 10 min incubation the intestinal samples were prepared as described in Fig. 1. Neuronal cell extensions were labeled with anti neuro-filament (NF) antibodies, and nuclei with Hoechst (blue). Scale bars = 20 μ m and 10 μ m for the magnification panels.

Figure 5. HCcB recognizes neuronal cell extensions expressing synaptotagmin II but not glial cells in mouse intestinal submucosa and musculosa. A, HCcB-Cy3 (100 μ g) was injected into ligated jejuno-ileal loop of transgenic Thy1-YFP mice. After 10 min incubation the intestinal samples were prepared as described in Fig. 1. **A**, HCcB stained neuronal cells and cell extensions expressing Thy1-YFP (green) and co-stained with anti-synpatotagmin II antibodies (white). **B**, No co-staining of glial cells monitored with anti-GFAP antibodies and fluorescent HCcB was evidenced. Nuclei were stained with Hoechst (blue). Scale bars = 20 μ m and 10 μ m for magnification panels.

Figure 6. Neuronal cells specifically targeted by HCcB in mouse intestinal submucosa and musculosa. HCcB-Cy3 (100 μ g) was injected into ligated jejuno-ileal loop and after 10

min incubation the intestinal samples were prepared as described in Fig. 1. The preparations were co-stained with anti-ChAT (marker of cholinergic neurons), anti-serotonin, anti-VIP, anti V-GLUT (marker of glutamatergic neurons), anti-V-GAT (marker of GABAergic neurons) (green). Enlarged views of the drawn squares are shown on the right side. Hoechst (blue), ECCD2 (white). Scale bars = 20 μm and 10 μm for magnification panels.

Figure 7. BoNT/B targeted cholinergic and serotonin neurons in the intestinal submucosa and musculosa. BoNT/B (100 μg) was injected into mouse ligated jejuno-ileal loop and after 10 min incubation the intestinal samples were prepared as described in Fig. 1. BoNT/B was detected with rabbit immunopurified immunoglobulins against HCcB and Alexa594-anti-rabbit immunoglobulins. The preparations were co-stained with anti-ChAT (**A**) and anti-serotonin (**B**). Enlarged views of the drawn squares are shown on the right side. Hoechst (blue). Scale bars = 20 μm and 10 μm for magnification panels.

Figure 8. HCcB targeted cholinergic and VIP neurons in intestinal villi. HCcB-Cy3 (100 μg) was injected into ligated jejuno-ileal loop and after 10 min incubation the intestinal samples were prepared as described in Fig. 1. Preparations were co-stained with antibodies anti-ChAT (cholinergic cells) or anti-VIP (green), and anti E cadherin (ECCD2) (white) and Hoechst (blue). Scale bars =20 μm and 10 μm in the magnification panels.

Figure 9. HCcB accumulation in some cells of the intestinal epithelium. HCcB-Cy3 (100 μg) was injected into ligated jejuno-ileal loop and after 10 min incubation the intestinal samples were prepared as described in Fig. 1. (**A**) HCcB accumulated in a few cells of the intestinal epithelium which showed no apical actin staining. (**B**) Colchicine pretreatment (10 $\mu\text{g}/\text{ml}$, 30 min) increased the number of cells accumulating HCcB. HCcB is preferentially distributed on the apical or basolateral sides of the cells from colchicine pretreated intestinal loop (**B**), in contrast to a broad HCcB distribution through cells of untreated intestinal loops (**A**). Note that the cells accumulating HCcB in the intestinal epithelium did not show Phalloidin staining of their apical side. Hoechst (blue). Scale bar = 10 μm .

Figure 10. Colchicine does not totally block the intestinal passage of HCcB-Cy3. HCcB-Cy3 (100 μg) was injected into ligated jejuno-ileal loop with or without pretreatment with colchicine (10 $\mu\text{g}/\text{ml}$, 30 min). Albeit colchicine induced HCcB-Cy3 accumulation into

certain intestinal epithelial cells, it did not completely impaired HCcB-Cy3 dissemination to neuronal cells in the submucosa and musculosa. Hoechst (blue). Scale bar = 10 μ m.

Figure 11. HCcB uses ganglioside GT1b as receptor on intestinal cells. HCcB-Cy3 (70 μ g) was injected into ligated jejuno-ileal loop (A) or incubated with a 10-fold excess of GT1b for 20 min at room temperature prior to injection into intestinal loop (B). After 15 min incubation the intestinal samples were prepared as described in Fig. 1. Prior incubation with GT1b significantly prevented HCcB staining of intestinal villi, submucosa, and musculosa. (C) Quantification of HCcB staining of the intestinal wall. Scale bars = 20 μ m. *** $P < 0.001$. (3 to 5 quantifications in each experiment).

Supplementary Figure 1. HCcB recognizes neuronal cell extensions expressing synaptotagmin II in mouse intestinal submucosa and musculosa. HCcB-Cy3 (100 μ g) was injected into mouse ligated jejuno-ileal loop. After 10 min incubation the intestinal samples were prepared as described in Fig. 1. HCcB stained neuronal cells and cell extensions expressing serotonin (A) (green) or ChAT (B) (green) and co-stained with anti-synaptotagmin1 (SytII) antibodies (white), and Hoechst (blue). Scale bars = 20 μ m and 10 μ m for magnification panels.

Supplementary Figure 2. Cells of the intestinal epithelium which accumulated HCcB, were not immunoreactive with antibodies against caspase 3. HCcB-Cy3 (100 μ g) was injected into ligated jejuno-ileal loop and after 10 min incubation the intestinal samples were prepared as described in Fig. 1. The preparations were co-stained with antibodies against cleaved caspase 3. Scale bars = 10 μ m.

Supplementary Figure 3. Investigation of HCcB accumulating cells of the intestinal epithelium. HCcB-Cy3 (100 μ g) was injected into ligated jejuno-ileal loop and after 10 min incubation the intestinal samples were prepared as described in Fig. 1. The preparations were co-stained with antibodies anti-ChAT (cholinergic cells), anti-chromogranin A (enteroendocrine cells), anti-M villous cell, anti-serotonin, anti-DCLK1 (tuft cells), anti-WGA (goblet cells), anti-CD3 (T lymphocytes), anti-CD11C (dendritic cells) (green). Hoechst (blue). Scale bars = 20 μ m.

Supplementary Figure 4. Cells of the intestinal epithelium which accumulated HCcB, were not immunoreactive with antibodies ITF and cytokeratin18 specific of goblet cells. HCcB-Cy3 (100 μ g) was injected into mouse ligated jejunum-ileal loop. After 10 min incubation the intestinal samples were prepared as described in Fig. 1. The preparations were co-stained with antibodies anti-ITF (green) or anti-cytokeratin18 (green), as well as anti E cadherin (ECCD2) (white) and Hoechst (blue). Scale bars = 10 μ m.

Supplementary Figure 5. The ganglioside GM1 does not impair HCcB entry into the intestinal mucosa. HCcB-Cy3 (70 μ g) was injected into ligated jejunum-ileal loop (A) or incubated with a 10-fold excess of GM1 for 20 min at room temperature prior to injection into intestinal loop (B). After 15 min incubation the intestinal samples were prepared as described in Fig. 1.

Supplementary Figure 6. Visualization of fluorescent HCcB (10 μ g) in the mouse intestine following its injection into the intestinal lumen. HCcB-Cy3 (10 μ g) in Dulbecco's modified Eagle's medium was injected into ligated jejunum-ileal loop of anesthetized mice. After 20 min incubation the intestinal samples were prepared as described in Fig. 1. Preparations were co-stained with antibodies anti E-cadherin (ECCD2) and Hoechst (blue). Scale bars = 10 μ m.

Protein injected intraluminally	Neuronal marker	Colocalization (Pearson's coefficient)	Co-stained neuronal structures with HCcB or BoNT/B (%)
Cy3-HCcB	ChAT	0.310 ± 0.07	82.2 ± 3.8
BoNT/B	ChAT	0.314 ± 0.08	87 ± 2.5
Cy3-HCcB	serotonin	0.25 ± 0.09	25.8 ± 5.4
BoNT/B	serotonin	0.34 ± 0.08	34.7 ± 6.9
Cy3-HCcB	VIP	0.294 ± 0.13	10.0 ± 1.6
Cy3-HCcB	V-GLUT	0.58 ± 0.09	56.2 ± 7.4*
Cy3-NCcB	V-GAT	0.24 ± 0.07	100 ± 14*

Table 1. Quantification of colocalization between Cy3-HCcB or BoNT/B and neuronal markers in mouse submucosa and muscosa. Colocalization index was determined as Pearson's coefficient. The distribution of neuronal cells recognized by HCcB or BoNT/B was expressed as the ratio of the number of neuronal structures co-stained with HCcB or BoNT/B and with one neuronal marker to the total number of neuronal structures labeled with the corresponding neuronal marker. Results represent means ± SD accounting for at least 100 cells from five different experiments except for V-GLUT and V-GAT (*) the results of which represent only a few cells (< 10).