Effects of transgenic expression of botulinum toxins in *Drosophila*

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ABSTRACT

Clostridial neurotoxins (botulinum toxins and tetanus toxin) disrupt neurotransmitter release by cleaving neuronal SNARE proteins. We generated transgenic flies allowing for conditional expression of different botulinum toxins and evaluated their potential as tools for the analysis of synaptic and neuronal network function in *Drosophila melanogaster* by applying biochemical assays and behavioral analysis. On the biochemical level, cleavage assays in cultured *Drosophila* S2 cells were performed and the cleavage efficiency was assessed via western blot analysis. We found that each botulinum toxin cleaves its *Drosophila* SNARE substrate but with variable efficiency. To investigate the cleavage efficiency in vivo, we examined lethality, larval peristaltic movements and vision dependent motion behavior of adult *Drosophila* after tissue-specific conditional botulinum toxin expression. Our results show that botulinum toxin type B and botulinum toxin type C represent effective alternatives to established transgenic effectors, i.e. tetanus toxin, interfering with neuronal and non-neuronal cell function in *Drosophila* and constitute valuable tools for the analysis of synaptic and network function.

Introduction

Clostridial neurotoxins (CNT) as tetanus toxin (TNT) and the seven different botulinum toxin types (BoNT-A, -B, -C, -D, -E, -F and -G) disable neuronal function by cleaving components of the SNARE apparatus that is key for Ca$^{2+}$-triggered neurotransmitter release. The SNARE apparatus is composed of three components: the v-SNARE neuronal Synaptobrevin (n-Syb), which resides on the vesicular membrane and the t-SNAREs Syntaxin (Syx) and SNAP-25, which are associated with the presynaptic plasma membrane. Upon elevation of the presynaptic Ca$^{2+}$ concentration, these proteins form a dense complex that pulls the vesicle membrane to the plasma membrane initiating fusion of the two lipid bilayers (Chen and Scheller 2001, Bruns and Jahn 2002). CNTs have been used in various model organisms as pharmacological tools or expressible transgenes to study the physiology of neurotransmitter release or to functionally block specific neuronal subsets in order to study their involvement in network function (Sakaba et al. 2005, Neuser et al. 2008, Prashad and Charlton 2014).

All CNTs ultimately lead to disruption of neurotransmitter release at synapses. However, they attack the components of the SNARE apparatus at distinct molecular target sites, which causes specific effects on synaptic properties during the decay of synaptic function. For instance, toxin injection into the calyx of Held revealed that BoNT-A-mediated SNAP-25 cleavage primarily reduces the Ca$^{2+}$ sensitivity of neurotransmitter containing vesicles (Sakaba et al. 2005). The same study shows that TNT-mediated Synaptobrevin cleavage seems to modify the coupling of release competent vesicles to Ca$^{2+}$-channels whereas BoNT-C-mediated Syntaxin cleavage neither seems to affect the sensitivity to Ca$^{2+}$ nor the coupling to Ca$^{2+}$-channels of remaining release competent vesicles. Other studies on crayfish motoneurons could show that although BoNT-B and TNT cleave n-Syb at the identical peptide bond, their effectiveness in blocking neurotransmitter release varies greatly depending on the activity state of the synapse (Hua and Charlton 1999, Prashad and Charlton 2014): TNT-mediated SNARE cleavage depends on ongoing neurotransmitter release, whereas BoNT-B cleaves its substrate independent of synaptic activity. The base for these different properties are putative differently located CNT binding sites on neuronal Synaptobrevin. At inactive synapses, the SNARE complex is supposed to be tightly zipped exposing only the binding site for BoNT-B, but not for TNT (Hua and Charlton 1999). As these studies exemplify, comparison of the distinct effects of different CNTs can provide valuable insight into the molecular processes governing neurotransmitter release.

To date, TNT is available as a genetic tool only in *Drosophila* (Sweeney et al. 1995). The *Drosophila* genetic toolbox allows expression of effectors in a spatially and temporally restricted manner using binary expression systems such as the UAS/GAL4 couple (Brand and Perrimon 1993) and the temperature sensitive suppressors, e.g. the GAL4-suppressive *tub-GAL80* (McGuire et al. 2003). By that, UAS-TNT has been successfully used in *Drosophila* in a multitude of studies to silence groups of neurons in order to identify
their role in network function (Neuser et al. 2008). However, UAS-TNT appears ineffective in blocking neurotransmitter release in specific cell types, such as mushroom body neurons (Thum et al. 2006), photoreceptor cells (Rister and Heisenberg 2006) and lamina monopolar neurons (Zhu et al. 2009) giving away the chance to analyze SNARE-mediated processes in these neurons. Adding botulinum toxins to the Drosophila toolbox could provide potent alternatives for silencing neuronal cells where UAS-TNT is ineffective. Furthermore, using botulinum toxins in addition to TNT could allow to examine molecular properties of the SNARE apparatus. It would therefore greatly enlarge the spectrum of possibilities to analyze the molecular requirements of neurotransmission. In addition, SNARE protein isoforms are involved in non-neuronal vesicular secretion and membrane trafficking (DiAntonio et al. 1993, McMahon et al. 1993, Schulze et al. 1995). Botulinum toxins thus also represent potential effectors for disruption of non-neuronal cell functions.

In our study, we generated transgenic UAS-BoNT flies that allow for expression of different botulinum toxin light chains. We performed biochemical assays to assess the susceptibility of Drosophila SNARE to BoNT-mediated proteolysis. Further, we analyzed the facility of BoNTs to induce lethality after expression in neuronal and non-neuronal cells and quantified the impact of induced toxin expression on larval motor behavior. We found that UAS-BoNT-B and UAS-BoNT-C are effective in interfering with neuronal and non-neuronal cellular processes in Drosophila. We also tested whether the newly generated BoNTs could advantageously supersede UAS-TNT as effective tools for the blockade of neurotransmitter release in cells where UAS-TNT was not effective. For this purpose, we expressed UAS-BoNTs in adult photoreceptor cells and assessed the influence of BoNT-expression on vision-dependent motion behavior (Optomotor response, OMR). We found that contrary to UAS-TNT, UAS-BoNT-B expression in photoreceptor cells influences OMR, although no complete block of photoreceptor function could be achieved.

Conclusively, BoNTs constitute valuable tools for the analysis of synaptic function. In the future, they can be conveniently combined with various other genetic manipulations including gene knock-outs, RNAi-mediated cell-specific expression knock-downs (Dietzl et al. 2007) or specific mutations of proteins that interact with the SNARE apparatus to further gain insight into the complex molecular interactions of presynaptic proteins.

Materials and methods

Fly strains

Flies were raised on a standard cornmeal-based medium under a 12h/12h light–dark cycle and were held at 25 °C. For experiments with the heat sensitive GAL4-suppressor GAL80	extsuperscript{f}, flies were crossed and raised at 18 °C and incubated at 31 °C. The motoneuron-specific driver line OK6-GAL4 (Sanyal 2009), the pan-neuronal driver line clav-GAL4 (Luo et al. 1994), the muscle-specific driver G7-GAL4 (Zhang et al. 2001) and the photoreceptor-specific driver GMR-GAL4 (Freeman 1996) were used in the experiments. For comparison with the generated UAS-BoNT lines, we chose the most common UAS-TNT insertion UAS-TNT-E (Sweeney et al. 1995). For conditional experiments, UAS-TNT-E; tub-GAL80	extsuperscript{f} (Thum et al. 2006) was used. The same tub-GAL80	extsuperscript{f} insertion was combined with UAS-BoNT-B. An insertion of tub-GAL80	extsuperscript{f} on the second chromosome was used for combination with UAS-BoNT-C (BDSC, #7108). For a detailed description of utilized crosses and genotypes see Supplemental Tables 1, 2 and 3.

Plasmid preparation

cDNAs for all BoNTs were synthesized by GeneArt after adaptation to the Drosophila melanogaster codon table with GeneOptimizer (http://www.lifetechnologies.com/de/de/home/life-science/cloning/gene-synthesis/geneart/gene-synthesis/geneoptimizer.html?cid=fl-gene Optimization) (Graf et al. 2000). Plasmids for embryonic injections and expression in cell cultures were generated using the Drosophila Gateway system (Invitrogen) as follows: Synthetic cDNAs encoding the light chains of BoNT-A, -B, -D, -F and -G were digested with XhoI and EcoRV and ligated into pENTR1Dual (Invitrogen, #A10462), which was previously digested with XhoI and Drai. Drosophila n-syb (BDGP, #GH04664) was amplified with tl_23F and tl_24R, digested with SalI and EcoRV and ligated into pENTR1Dual that was digested with SalI and EcoRV. Drosophila syntaxin-1A cDNA (BDGP, #LD43943) was amplified using tl_27F and tl_28R, digested with SalI and EcoRV and ligated into pENTR1Dual, that was digested with SalI and EcoRV. Drosophila SNAP-25 cDNA (BDGP, #GH28821) was amplified with pb_21F and pb_22R, digested with Xhol and Drai and ligated into pENTR1Dual that was digested with Xhol and Drai. Entry clones were recombined with the destination clones through a LR reaction according to the manufacturer’s instructions (Invitrogen).

For BoNT-expression in S2 cells, we generated a destination vector containing a Gateway cassette preceded by 20 Codon-optimized UAS-repeats, followed by a C-Terminal 3xFlag-tag. The plasmid also encoded an Actin-5C-GAL4 element placed in opposite direction to the Gateway element pAWF (Murphy, T (2003-)). The Drosophila gateway vector collection (DGVC, http://www.ciemblab.edu/labs/murphy/Gateway%20vectors.html) was amplified using primers tl_346F and tl_347R, digested with Ascl and HindIII and ligated into pJFRC7-20XUAS-IVS-mCD8-GFP (Pfeiffer et al. 2010) that was digested with Ascl and HindIII. Product was digested with Xhol and Xbal and ligated with PCR-product of pTWF (DGVC) with tl_348F and tl_349R that was digested with AvrII and Xhol (intermediate product pPB2). Product was digested with Ascl and EcoRV and ligated with PCR product of pBPGAL4.2::p65Uw (Pfeiffer et al. 2010) with pb_19F and pb_20R that was digested with PvuII and Ascl.

For SNARE protein expression in S2-cells, we generated a UAS-driven expression clone containing a N-terminal V5-tag and a C-terminal HA-Tag: PCR of pTWH_attB (Bischof et al. 2007) with pb_23F and pb_24R. Digest from PCR product and pTWH_attB with NolI and BglII and subsequent ligation.
For phiC31-mediated transformation of BoNTs, we generated an expression clone containing the respective recombination sites and a C-terminal 3xFlag-tag: Digest of pTW H attB and pPB1 with NotI and AgeI and subsequent ligation.

For P-element dependent integration, pTWF (DGVC) was the destination clone.

See Supplemental Table 4 for linker sequences and tags, Supplemental Table 5 for primer sequences and Supplemental Table 6 for a list of used protein isoforms including accession numbers. Plasmids used for integration of BoNT-A, -B, -C and -G were deposited into GenBank (Accession numbers: KR260972, KR260973, KR260974 and KR260975).

**Integration of UAS-BoNTs**

Embryonic injections were performed by TheBestGene. BoNT-A, -C and -G were integrated using phiC31-mediated transformation (Bateman 2006) into an attP landing site on the third chromosome (BDSC #8622; 2, 3 and 5 transformants, respectively). For BoNT-B and -F additional integration attempts were performed in BDSC strains #24885, #9743, #9725, #9738 and #34763. In addition, BoNT-B, -D and -F were selected for random P-element dependent integration (Spradling & Rubin, 1982) into w1118 flies yielding 4 transformants for BoNT-B: UAS-BoNT-B1 (second), UAS-BoNT-B2 (X), UAS-BoNT-B3 (second) and UAS-BoNT-B4 (second). UAS-BoNT-A2, UAS-BoNT-B1, UAS-BoNT-C1 and UAS-BoNT-G1 were used for experiments in this publication.

**Protein analysis**

For Western blot analysis, Drosophila S2 cells were transfected with two plasmids: One plasmid contained the UAS-V5::SNARE::HA construct, the other contained an UAS-BoNT::Flag and Actin5C-GAL4 driver. This ensured that SNARE substrate and respective botulinum toxin were co-expressed in S2 cells. Control groups were co-transfected with UAS-V5::SNARE::HA and Actin5C-GAL4, with the UAS-BoNT::Flag-Actin5C-GAL4 construct or Actin5C-GAL4 alone. After transfection, cells were incubated at 31°C for 48 h and subsequently lysed. Upon protein preparation and denaturation protease inhibitors (1:1000, P8340-1ML, Sigma) were added. Electrophoresis was performed in 15% polyacrylamide gel and proteins were transferred to a polyvinylidene fluoride membrane (Merck Millipore, Immobilon-P). Membranes were blocked and incubated with primary antibodies over night: mouse anti-V5 (for SNAP-25, 1:5000, monoclonal, Invitrogen #R960-25) or mouse anti-HA (1:2500 for N-SYB + BoNT-D or 1:5000 for remaining blots, monoclonal, Covance HA.11 Clone 16B12). Membranes were incubated with secondary goat anti-mouse HRP conjugated antibodies (1:2500, monoclonal, Dianova #115-035-166) for 2 h at room temperature. After development of the first immunoreaction, membranes were stripped and incubated with primary mouse anti-beta-tubulin antibody for BoNT-D (1:1000, monoclonal, DSHB #E7) or rabbit anti-alpha-tubulin antibody (1:1000, polyclonal, Santa Cruz Biotechnology (E-19)-R #sc-12462-R) for remaining blots and afterwards incubated with secondary goat anti-mouse HRP conjugated antibodies (1:5000, polyclonal, Dianova #115-035-166) and goat anti-rabbit HRP conjugated antibodies (1:5000, polyclonal, Dianova #111-035-144), respectively.

**Cell cultures**

Drosophila S2 cells (Invitrogen, #R690-07) were held at 27°C and regularly split every 3–4 days. Cells were raised in Schneider’s Drosophila medium (Gibco)+10% fetal calf serum (Biochrom). Cells were disseminated on surface 24-well multidishes (Nunclon). S2 cells were transfected in accordance with the appropriate transfection protocol of Invitrogen using Lipofectamine 2000 (Invitrogen, #11668027). Used DNA concentrations were modified in order to transfect with equal plasmid quantities.

**Lethality**

For lethality assays, either the GALA-line or the UAS-CNT-line was crossed to a balancer chromosome with a dominant marker (CyO or TM3-Sb for adult flies, CyO-GFP for larvae). These balanced GALA- or UAS-lines were then crossed to the respective homozygous UAS- or GALA-lines. This way only balancer-negative progeny carry both, the GALA-line and the UAS-transgenes required for toxin expression. Expression of an effective toxin led to progeny only consisting of balancer-positive flies, whereas ineffective toxin expression led to a Mendelian ratio of balancer-positive and balancer-negative flies. The progeny was collected at eight (elav-GALA and OK6-GALA) consecutive days after the first adult flies hatched. For G7-GALA, third instar larvae were counted twice in two consecutive crossings for each genotype.

**Larval behavior**

After distinct length of incubation (1 h, 5 h, 10 h, 15 h, 20 h, 25 h, 40 h) third instar larvae were transferred to a Petri dish (Sarstedt, 92 mm) filled with 1% agarose (Biozym, #84004). Front and back peristaltic movements (Suster and Bae 2002) were equally counted by eye for 90 sec for each larva. Experiments were performed at 25 – 27°C ambient temperature. All genotypes were tested at the same day in an alternating manner.

**Optomotor response (OMR)**

Flies were measured in a behavioral setup adapted from Buchner et al. (1976). Per trial, one fly was tethered with a hook glued to its thorax right above a styrofoam ball, allowing the fly to walk on the styrofoam ball that is floating on an air stream. A high contrast vertical zebra-striped pattern is rotated around the fly at different speeds resulting in different “pattern frequencies”, which expresses how many pairs of stripe passes one point in the view field of the fly in one second. A single measurement took 140–155 min and
involved repeated randomized stimulations with different pattern frequencies. The rotation of the styrofoam ball was monitored by an optical mouse sensor and information was stored by a self-constructed computer software. The OMR units are relative, whereas one expresses perfect syn-directional turning and zero expresses random turning of the fly, independent of pattern rotation. Please see the Supplemental Material for detailed materials and methods on OMR behavior.

**Statistics**

For pairwise comparison of larval motor behavior and OMR experiments the Mann-Whitney-U-Test was used (Prism 5). The multiple comparisons represent an explorative statistical analysis. Thus no corrections as the Bonferroni correction were performed. For calculation of the incubation length required for a 50% suppression of larval motor behavior, a logistic curve fit was applied. The respective values represent the time points where the curve reaches half of its maximum. For data analysis of lethality tests, the fraction of balancer positive flies has been calculated for each genotype. A fraction of 0.5 indicates no influence on lethality. Two-tailed binomial test based on a success probability of 0.5 (http://graphpad.com/quickcalcs/binomial1). Percentage ID of sequence alignments were calculated with Jalview (Waterhouse et al. 2009). * reflects \( p < 0.05 \), ** \( p < 0.01 \) and *** \( p < 0.001 \).

**Results**

**BoNT-B, -D and -F cleave Drosophila neuronal SNARE with high efficiency**

For each BoNT light chain, we generated synthetic cDNAs with codon usage and GC content adapted to increase expression yield in *Drosophila melanogaster*. We selected six different BoNTs for analysis: BoNT-A, -B, -C, -D, -F and -G. BoNT-E was not considered, as previous studies had shown its inability to cleave *Drosophila melanogaster* SNAP-25 (Washbourne et al. 1997). All BoNTs were C-terminally tagged with a Flag-tag to allow histochemical detection. The primary structure of *Drosophila* neuronal SNAREs n-Syb, SNAP-25 and Syx-1A is moderately conserved in *Drosophila melanogaster* in comparison to their vertebrate homologues (*Drosophila melanogaster* (Dm) n-Syb vs. *Rattus norvegicus* (Rn) VAMP2: Percentage ID = 61%; *Dm* Syx-1A vs. *Rn* Syx-1A: Percentage ID = 69%; *Dm* SNAP-25 vs. *Rn* SNAP-25: Percentage ID = 60%, Figure 1). To assess the efficiency of BoNTs to cleave *Drosophila* neuronal SNARE isoforms, we performed cleavage assays in cultured *Drosophila* S2 cells. Notably, Western blot analysis revealed that each BoNT cleaves its *Drosophila* SNAP-25 substrate, although the efficiency was variable. Complete cleavage of SNAP-25 substrate was observed for BoNT-B, -D and -F, while BoNT-A, -C and -G only partially catalyzed proteolysis of SNAP-25 (Figure 2, Table 1).

**Successful insertion of BoNT-A, -B, -C and -G into Drosophila**

Initially, integration of UAS-BoNT constructs was performed using *Drosophila* strains with specific insertion sites in their genome (phi-C31) (Bateman 2006). Transformation yielded transgenic fly strains for UAS-BoNT-A, -C and -G. However, no transgenic flies were recovered for UAS-BoNT-B and -F, even though a multitude of different insertion sites were selected for integration (see Materials and methods for details). Therefore, a P-element-mediated transformation protocol was subsequently employed to randomly integrate constructs of UAS-BoNT-B, -D and -F (Spradling and Rubin 1982), but only UAS-BoNT-B transformants could be additionally recovered (Table 1).

**Pan-neuronal and motoneuronal expression of BoNT-B and -C is lethal**

Previous investigations had shown that pan-neuronal or motoneuronal TNT expression leads to death at early stages.
developmental stages (Sweeney et al. 1995). Following this observation, we tested the biological effect of BoNT expression in vivo. We assessed the ability of BoNTs to induce lethality by driving expression with the pan-neuronal elav-GAL4 and the glutamatergic motoneuron-specific OK6-GAL4 enhancers (Table 1). To quantify the potency of the toxins, we crossed the respective GAL4-line to a UAS-BoNT line whereas either the GAL4-line or the UAS-BoNT line were heterozygous to a balancer chromosome with a dominant marker. This way, progeny of an effective toxin contained only marker-positive flies, whereas progeny of an ineffective toxin contained a balanced ratio of marker-positive and negative flies. In accordance with the results from previous studies (Sweeney et al. 1995), pan-neuronal and motoneuronal UAS-TNT-E expression effectively interfered with fly development. Amongst the botulinum toxins UAS-BoNT-B and -C were equally effective and led to complete lethality with both driver lines. UAS-BoNT-A led to (incomplete) lethality only after pan-neuronal, but not motoneuronal expression, whereas UAS-BoNT-G expression did not show any effect with both GAL4-lines. In the next set of experiments, we thus focused on UAS-BoNT-B and -C.

**Toxicity of UAS-BoNT-C is not confined to neurons**

To assess the neuron-specificity of the toxins’ actions we expressed them using the muscle-specific driver line G7-GAL4 (Table 1). Only UAS-BoNT-C expression resulted in lethality, indicating that it does not solely interfere with neurotransmitter release, but also disrupts non-neuronal functions. For expression of UAS-TNT-E and UAS-BoNT-B, we observed a minor influence on larval survival, suggesting effects that are confined to neurons.

**BoNT-B is a potent effector on larval behavior after induced neuronal expression**

The previous experiments demonstrated that UAS-BoNT-B and -C crucially affected neuronal function in developing animals. To measure the acute effect of BoNT expression in developed neurons, we combined UAS-BoNT-B and -C with the temperature-sensitive tub-GAL80ts construct that inhibits UAS-activation at low temperatures (18°C) (McGuire et al. 2003). We then measured the course of spontaneous larval

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**Table 1. Overview on BoNT in vitro cleavage, transgenesis and induced lethality.**

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Cleavage efficiency</th>
<th>Transformants</th>
<th>Lethality with elav-GAL4</th>
<th>Lethality with OK6-GAL4</th>
<th>Lethality with G7-GAL4</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNT</td>
<td>+</td>
<td>phi-C31: BDSC #8622, third chromosome</td>
<td>+ (f = 1, p &lt; 0.0001, n = 79)</td>
<td>+ (f = 1, p &lt; 0.0001, n = 108)</td>
<td>- (f = 0.67, p &gt; 0.0001, n = 148)</td>
</tr>
<tr>
<td>BoNT-A</td>
<td>++</td>
<td>phi-C31: BDSC #8622, second chromosome</td>
<td>(+) (f = 0.97, p &lt; 0.0001, n = 29)</td>
<td>- (f = 0.48, p = 0.65, n = 122)</td>
<td></td>
</tr>
<tr>
<td>BoNT-B</td>
<td>++</td>
<td>P-element: X and second chromosome</td>
<td>+ (f = 1, p &lt; 0.0001, n = 46)</td>
<td>+ (f = 1, p &lt; 0.0001, n = 59)</td>
<td>- (f = 0.59, p = 0.05, n = 129)</td>
</tr>
<tr>
<td>BoNT-C</td>
<td>++</td>
<td>phi-C31: BDSC #8622, third chromosome</td>
<td>+ (f = 1, p &lt; 0.0001, n = 37)</td>
<td>+ (f = 1, p &lt; 0.0001, n = 106)</td>
<td>+ (f = 1, p &lt; 0.0001, n = 134)</td>
</tr>
<tr>
<td>BoNT-D</td>
<td>+</td>
<td>phi-C31: BDSC #8622, second chromosome</td>
<td>- (f = 0.47, p = 0.86, n = 34)</td>
<td>- (f = 0.49, p = 1, n = 95)</td>
<td></td>
</tr>
<tr>
<td>BoNT-G</td>
<td>+</td>
<td>phi-C31: BDSC #8622, third chromosome</td>
<td>- (f = 0.47, p = 0.86, n = 34)</td>
<td>- (f = 0.49, p = 1, n = 95)</td>
<td></td>
</tr>
</tbody>
</table>

Cleavage efficiency was estimated by Western blots (Figure 2). Complete cleavage is indicated by ++ and incomplete cleavage by +. Primarily, BoNT-A, -B, -C, -F and -G were phi-C31 dependent injected into targeted gene loci. After five unsuccessful attempts to integrate into different loci, P-element-mediated injections were performed. For lethality assays balanced UAS-CNT flies were cell-specifically over-expressed in either motoneurons (OK6-GAL4), in all neurons (elav-GAL4) or larval muscles (G7-GAL4). Effective toxins led to a fraction (f) of marker positive progeny of one, whereas ineffective toxins led to an even proportion of marker positive and marker negative progeny. – indicates unaltered survival, (–) slightly reduced survival, (+) incomplete lethality with few survivors and + complete lethality. G7-GAL4 controls did not show an effect on larval survival (f = 0.52, p = 0.61, n = 184).
peristaltic movement frequency (Suster and Bate 2002) as a function of time after a temperature shift to 31 °C that leads to expression induction (Figure 3). Again UAS-TNT-E served as a reference to classify the efficiency of UAS-BoNTs. For quantification of the decay of larval activity we calculated the length of incubation necessary for a 50% suppression of movement frequency on the basis of fitted curves (mf50-values). Further, we made pairwise statistical comparisons of the experimental groups with the respective controls at each time point (Figure 3, Supplemental Table 7). In addition to the genetic controls, flies of the same genotype that were held at 31 °C for 1 h served as un-induced controls. After 25 h induction, pan-neuronal expression of UAS-BoNT-C led to significantly reduced locomotion in larvae of the experimental group compared to control larvae (elav-GAL4 > UAS-BoNT-C mf50 = 22.78 h), but no complete paralysis occurred in the observed time window of 40 h. Elav-GAL4 driven BoNT-B expression already yielded significant reduced locomotion after 1 h incubation (elav > BoNT-B vs elav-GAL4; p = 0.0097 and UAS-BoNT-B: p = 0.0027; Mann-Whitney U-Test), whereas the peristaltic of larvae transgenic for the UAS-BoNT-B construct only was not significantly altered. However, pan-neuronal expression of either UAS-TNT-E or UAS-BoNT-B led to significantly weaker performance than controls after 15 h and 10 h and complete paralysis after 25 h and 20 h, respectively (elav-GAL4 > UAS-TNT-E mf50 = 12.09 h, elav-GAL4 > UAS-BoNT-B mf50 = 8.75 h). Using the motoneuron specific driver line OK6-GAL4, even after 40 h induction at high temperature (31 °C) no significant alteration of the behavior of UAS-BoNT-C expressing larvae was detected. In contrast, UAS-TNT-E and UAS-BoNT-B motoneuronal expression led to a robust suppression with significantly weaker performance after 25 h (OK6-GAL4 > UAS-TNT-E mf50 = 22.08 h) and 15 h (OK6-GAL4 > UAS-BoNT-B mf50 = 15.24 h), respectively, without inducing complete paralysis in the observed time window. In conclusion, UAS-BoNT-C takes longer than UAS-TNT-E to impair larval activity after pan-neuronal expression and is ineffective after induced motoneuronal expression. UAS-BoNT-B on the other side decreases larval peristaltic activity faster than UAS-TNT-E for both tested GAL4-lines.

BoNT-B expression in photoreceptor cells influences motion vision dependent behavior

We tested the ability of walking flies to syn-directionally turn with a rotating striped pattern (Buchner 1976). This behavior is referred to as “Optomotor response” (OMR) and accurate photoreceptor function has been shown to be a prerequisite for it (Heisenberg and Buchner 1977). To detect even subtle deficits of photoreceptor function, we examined OMR not only under favorable pattern turning frequencies (0.1–10 Hz), but also at the limits of temporal motion vision resolution (30–45 Hz) for Drosophila. Flies were tested after tub-GAL80 mediated induced expression of different CNTs in the photoreceptor cells of adult flies using the driver line GMR-GAL4.
(Freeman 1996). Regardless of expression restriction with tub-GAL80	extsuperscript{ts}, the combination of GMR-GAL4 and UAS-BoNT-C did not yield viable progeny. In accordance with the results of Rister and Heisenberg (2006), UAS-TNT-E expression induction in adult photoreceptor cells did not significantly alter the flies OMR at the tested spectrum of different pattern frequencies ($p > 0.05$ for all pairwise comparisons, Mann-Whitney U-Test; Figure 4(A)). Expression of BoNT-B using GMR-GAL4 yielded a slight leaky effect at a pattern frequency of 45 Hz even at low temperature ($GMR > BoNT-B$ (not incubated) vs. GMR: $p = 0.0483$ and BoNT-B: $p = 0.0023$). However, in contrast to TNT, expression of UAS-BoNT-B led to a significant decrease in vision dependent motion behavior at the fastest frequencies of 30–45 Hz, compared to the not incubated control (30 Hz: $p = 0.0012$; 45 Hz: $p = 0.026$) and to the respective GAL4- and UAS- controls ($p < 0.001$ for each comparison; Figure 4(B)). Therefore, using BoNT-B as effector alters photoreceptor function at the limits of temporal motion vision resolution.

**Discussion**

We introduce four new transgenes for botulinum toxin expression in *Drosophila*: UAS-BoNT-A, -B, -C and -G. Whereas insertions of UAS-BoNT-A, -C and -G directly led to transgenic progeny, UAS-BoNT-B insertion yielded transgenic flies only after several trials and no transgenic flies could be generated for UAS-BoNT-D and -F. Notably, the difficulties to integrate UAS-BoNT-B, -D and F correlates with a high cleavage efficiency of these toxins as estimated by our biochemical data (Figure 2, Table 1). This suggests toxic effects on SNARE targets, already in the absence of a GAL4 transcription factor, due to leaky expression. A possible higher affinity of BoNT-F and -D to either n-Syb or its ubiquitously expressed homolog Synaptobrevin (Syb) (Chin et al. 1993, DiAntonio et al. 1993, Hua et al. 1998) could provide a possible explanation for the fact that insertions of UAS-BoNT-B yielded transgenic flies, while those of UAS-BoNT-D and -F did not.

UAS-BoNT-G expression only led to week cleavage of n-Syb in Western blots and consequently does not affect fly survival (Figure 2, Table 1). This likely results from a critical amino acid substitution present in *Drosophila* n-Syb (A90G) in respect to its vertebrate homologue (Figure 1). Generally, mutations at the first amino acid after the BoNT-G cleavage site are critical for BoNT-mediated substrate cleavage (Vaidyanathan et al. 1999, Schiavo et al. 2000, Sikorra et al. 2008). A previous study suggested that *Drosophila* SNAP-25 was not susceptible to BoNT-A (Washbourne et al. 1997). In our experiments, UAS-BoNT-A led to incomplete cleavage of its *Drosophila* substrate and to partial lethality after neuronal expression (Figure 2, Table 1). Altogether UAS-BoNT-A and -G appear to be insufficient effectors in *Drosophila*.

UAS-BoNT-C partially cleaves its substrates SNAP-25 and Syntaxin-1A in our biochemical assays (Figure 2). It efficiently interferes with fly survival after expression in neurons and also in muscles and is thus not a neuron-specific effector (Table 1). Since BoNT-A seems to cleave SNAP-25 equally effective as BoNT-C, but does not sufficiently interfere with fly survival, the observed UAS-BoNT-C effect is more likely to be mediated by cleavage of Syntaxin-1A than of SNAP-25. Despite its role in neuroexocytosis, Syntaxin-1A plays a key role in synaptic vesicle recycling (Belluzzi et al. 2008).
role in non-neuronal secretion in *Drosophila* (Schulze et al. 1995). Another potential target for UAS-BoNT-C-mediated cleavage is SNAP-24, a SNAP-25 homologue, which is also involved in neurotransmission and non-neuronal cell function (Niemeyer and Schwarz 2000, Vilinsky et al. 2002). Together potential UAS-BoNT-C-mediated cleavage of Syntaxin-1A or SNAP-24 provide a sufficient explanation for the observed non-neuronal toxicity of expressed BoNT-C. A recent study has shown that GMR-GAL4 also promotes expression in non-neuronal tissues as tracheae, leg and wing discs (Liet al. 2012). This explains the observed lethality after UAS-BoNT-C expression with the photoreceptor-specific” driver line GMR-GAL4 and underlines the potency of BoNT-C to interfere with the viability of developing cells. Persistence of the lethal phenotype in combination with tub-GAL80ts implies leakiness of expression restriction in these non-neuronal cells, as the GAL4-suppressor successfully prohibited UAS-BoNT-C induced lethality with the neuronal-specific driver lines elav-GAL4 and OK6-GAL4.

Compared to UAS-BoNT-B and UAS-TNT-E, pan-neuronal UAS-BoNT-C expression requires longer incubation time to interfere with larval motor behavior (88% longer than UAS-TNT-E, 160% longer than UAS-BoNT-B, based on fm50-values) and is ineffective after induced expression in motoneurons (Figure 3). Although these characteristics limit the potential usage of UAS-BoNT-C, it could prove a useful tool where general, non-neuronal cell toxicity is desired. Thus it represents an alternative for established tools, such as transgenes carrying the apoptosis activating gene reaper (White et al. 1996) or the mutated Diphtheria toxin A-chain DTI (Han et al. 2000).

In contrast to UAS-BoNT-A, C and –G, UAS-BoNT-B led to complete cleavage of its substrate n-Syb in Western blots (Figure 2). Similar to UAS-TNT-E, pan-neuronal and motoneuronal expression of UAS-BoNT-B causes lethality, while expression in muscle cells only exerts a minor influence on survival. Both toxins thus seem to act equally neuron-specifically. UAS-BoNT-B, however, appeared to be more potent than UAS-TNT-E, as it affected larval locomotion behavior faster, irrespective of the GAL4-line used (33% faster for OK6-GAL, 28% for elav-GAL4, based on fm50-values, Figure 3). This should prove useful for neuroscience analyses, especially with GAL4-lines exhibiting low transgene expression levels.

The higher potency of UAS-BoNT-B compared to UAS-TNT-E led to a coherent more pronounced effect of leaky toxin expression. This was observed for larval motor behavior where un-induced UAS-BoNT-B led to slight but significant decrease in larval peristaltic with elav-GAL4. In optomotor response experiments, UAS-BoNT-B led to a minor phenotype at the fastest pattern frequency in the un-induced control group despite expression restriction using tub-GAL80ts (Figures 3(B) and 4(B)).

UAS-BoNT-B significantly influences vision dependent motion behavior after expression in the TNT-insensitive photoreceptor cells, but no complete blockade of photoreceptor function could be observed. These results reflect a common obstacle of UAS-TNT-E and UAS-BoNT-B to abolish photoreceptor function in *Drosophila*. Rister and Heisenberg (2006) discussed that n-Syb might be protected from TNT cleavage because of the calcium binding messenger protein Calmodulin, that appears to be involved in synaptic transmission in *Drosophila* photoreceptor cells (Xu et al. 1998).

N-Syb features a binding site for Calmodulin overlapping with the cleavage site for TNT and BoNT-B (De Haro et al. 2003). Following this hypothesis, UAS-BoNT-B seems to somehow better overcome the assumed competitive Calmodulin “shielding” of the cleavage site. This could be simple due to a higher synaptic concentration or more favorable binding kinetics as reflected by the observed higher potency of UAS-BoNT-B compared to UAS-TNT-E. Another explanation is provided by the putative different dependence on the active state of the synapse for substrate cleavage of BoNT-B and TNT. As a result of the differentially located binding sites on n-Syb of these two neurotoxins, TNT requires ongoing exocytosis events to cleave its SNARE substrate whereas BoNT-B is also effective at inactive synapses (Hua and Charlton 1999). Since Calmodulin binding critically depends on a high Ca$^{2+}$ concentration, the blockade of the n-Syb cleavage site should break down at inactive synapses thus restricting n-Syb cleavage to BoNT-B (Di Giovanni et al. 2010).

The different requirements on synaptic activity of the two toxins may constitute a general advantage of UAS-BoNT-B in *Drosophila*. Before experiments, flies are usually raised under stable environmental stimulus conditions. In this period, TNT would be unable to cleave n-Syb in resting neuron populations, whereas BoNT-B could potentially interfere with synaptic silent nerve cells.

Distinct zippering states of neuronal SNARE in different stages of presynaptic cycling are supposed to account for the different features of BoNT-B and TNT. This could provide a new approach for the analysis of active zone processes in *Drosophila*. A recent study made use of these toxins by injection into *Crayfish* motoneurons. The authors tested whether the vesicle release probabilities at tonic and phasic synapses are determined by different SNARE zippering states (Prashad and Charlton 2014). The concept of employing these toxins as reporters for zippering states of SNARE proteins could potentially be translated into research in *Drosophila*. A combination of these reporters with genetic alterations of other molecular players that are known to interact with SNARE proteins could help to identify the mechanisms and molecular interactions that underlie SNARE zippering.

**Conclusion**

In this study, we introduce two novel effector transgenes in *Drosophila*: UAS-BoNT-C and UAS-BoNT-B. UAS-BoNT-C exerts toxicity in neurons and muscles, and alters behavior after induced pan-neuronal but not motoneuronal expression. In contrast, UAS-BoNT-B is a specific suppressor of neuronal function and interferes with behavior most effectively compared to the other tested CNTs. Although cleaving n-Syb at the identical peptide bond, BoNT-B and TNT are supposed to harbor distinct features cleaving its substrate differently dependent on the state of SNARE zippering. This may provide the possibility to utilize these toxins for analysis of the
molecular interactions that govern SNARE function during transmitter release. Correspondingly, BoNT-B should be a useful addition to the Drosophila toolkit interfering with the SNARE machinery in resting neurons. It thus represents a means – complementary to TNT – to specifically suppress neuronal activity in Drosophila.

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