Central cholinergic synaptic vesicle loading obeys the set-point model in *Drosophila*

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Cash F, Vernon SW, Phelan P, Goodchild J, Baines RA. Central cholinergic synaptic vesicle loading obeys the set-point model in *Drosophila*. J Neurophysiol 115: 843–850, 2016. First published December 9, 2015; doi:10.1152/jn.01053.2015.—Experimental evidence shows that neurotransmitter release, from presynaptic terminals, can be regulated by altering transmitter load per synaptic vesicle (SV) and/or through change in the probability of vesicle release. The vesicular acetylcholine transporter (VAChT) loads acetylcholine into SVs at cholinergic synapses. We investigated how the VAChT affects SV content and release frequency at central synapses in *Drosophila melanogaster* by using an insecticidal compound, 5CI-CASPP, to block VAChT and by transgenic overexpression of VAChT in cholinergic interneurons. Decreasing VAChT activity produces a decrease in spontaneous SV release with no change to quantal size and no decrease in the number of vesicles at the active zone. This suggests that many vesicles are lacking in neurotransmitter. Overexpression of VAChT leads to increased frequency of SV release, but again with no change in quantal size or vesicle number. This indicates that loading of central cholinergic SVs obeys the “set-point” model, rather than the “steady-state” model that better describes loading at the vertebrate neuromuscular junction. However, we show that expression of a VAChT polymorphism lacking one glutamine residue in a COOH-terminal polyQ domain leads to increased spontaneous SV release and increased quantal size. This effect spotlights the poly-glutamine domain as potentially being important for sensing the level of neurotransmitter in cholinergic SVs.

VESICULAR TRANSPORTERS LOAD neurotransmitter into synaptic vesicles (SVs). Classes of transporter include the vesicular acetylcholine transporter (VAChT), the transporters for glutamate (VGLUT), monoamines (VMAT), and GABA and glycine (Fei and Krantz 2009). A change in spontaneous quantal release frequency (probability of fusion of single SVs), following change in expression of VAChT or VGLUT, suggests these transporters may have a second function in neurotransmitter release (Song et al. 1997; Parsons et al. 1999; Daniels et al. 2004, 2006; de Freitas Lima et al. 2010; Rodrigues et al. 2013).

The VAChT transports acetylcholine (ACh) into SVs by exchanging two protons for one molecule of ACh (Usdin et al. 2004, 2006; de Freitas Lima et al. 2010; Rodrigues et al. 2013). Overexpression of VAChT, in *Drosophila* cholinergic premotor interneurons, is sufficient to increase frequency of spontaneous miniature events (minis) recorded in motoneurons, but does not increase their amplitude. Action potential-dependent synaptic currents are not affected. These changes mirror the proposed roles for VAChT in mammalian central nervous system (CNS) with respect to facilitating SV release but, importantly, provide evidence to support the set-point model for SV loading. We identify a poly-glutamine region in dVAChT that is seemingly important for SV filling. Expression of a dVAChT polymorphism missing one glutamine (from 13 in wild type) results in minis that are increased in both amplitude and frequency. Ultrastructural analysis shows no change to SV size. Determination of quantal content of action potential-evoked synaptic release also shows no change, indicating that this variant raises the set point of filling or alternatively switches loading to obey the steady-state model. The dVAChT poly-glutamine region is unique to some insect species and may offer an exploitable target for insecticide design.

METHODS

*Fly stocks and 5CI-CASPP application.* Flies were maintained under standard conditions at 25°C. Cha110-GAL4 (termed cha) was used to express UAS-VAChT in cholinergic neurons (Salvaterra and Kitamoto 2001). The wild-type UAS-VAChT transgene was made during the course of this study. The UAS-VAChT-ΔQ transgene was made by Syngenta. Sequencing confirmed that this was the only change to the amino acid sequence. The control used for 5CI-CASPP [(5-chloro-1’-(E)-3-(4-chlorophenyl)allyl)piperidin-3-yl]((2-chloro-4-pyridyl)methanone, made by Syngenta) was RRA-GAL4:CD8-green fluorescent protein (GFP) (termed RRA), which expresses in just the aCC/RP2 motoneurons in wall-climbing
third-instar larvae (Lin et al. 2014) and shows no differences in
synaptic excitation to true wild-type strains. UAS-Du7 (Bloomington
no. 39692) was rebalanced over a TM3::GFP balancer. Controls for
transgenic flies were parental and are indicated in the text. SCI-
CASPP (0.5 μg) was dissolved in acetone (5 μl) and added to the
surface of a grape-agar plate (50 mm) in 1 ml of aqueous dried yeast
extract (5%; Merck, Darmstadt, Germany). After being left to dry
overnight at room temperature, second instar larvae were allowed to
feed for 24 h.

qRT-PCR. CNSs were collected from third-instar wall-climbing
larvae. After RNA extraction (QIAGEN RNeasy Micro kit), cDNA
was synthesized using the Fermentas Reverse Aid H minus First
strand cDNA synthesis kit. Twenty CNSs were collected for each
sample. qRT-PCR was performed using a Roche LightCycler480 II
(Roche) with SYBRG Master reaction mix. The thermal profile used
was 5 min at 95°C followed by 45 cycles of 10 s at 95°C, followed by
10 s at 60°C, and finally 10 s at 72°C. Results were analyzed by the
ΔΔCt method and are expressed as relative RNA expression. Ct
values used were the means of three or five independent repeats.
Control gene was rp49. Primers were as follows: rp49 primers
CACCGAGCGATACA and TATAGTCGGCCATCGTTTCA. Choline
acetyltransferase (ChAT) primers ATCA-
CCAGTCGGATCGATCGATATGCTA and ACGTTGTGCACCAG-
XIV; Sigma, Dorset, UK) contained in a wide-bore (15
m) patch
pipette.
Whole cell recordings were carried out using borosilicate glass
electrodes (GC100TF-10; Harvard Apparatus, Edenbridge, UK), fire-
polished to resistances of between 8 and 12.5 MΩ. Recording
potential-induced synaptic currents. Recordings were made using an
Axopatch-1D amplifier. Cells were held at
potential-dependent currents (no TTX present), termed SRCs, to
rhythmic currents (SRCs), using pClamp 10.4 (Molecular Devices,
Sunnyvale, CA). Only neurons with an input resistance
rhythmic currents (no TTX present), termed SRCs, to
rhythmic currents (no TTX present), termed SRCs, to

Electrophysiology. Recordings were performed at room tempera-
ture (20–22°C). Third-instar larvae were dissected in saline (in mM:
135 NaCl, 5 KCl, 4 MgCl2·6H2O, 2 CaCl2·2H2O, 5
2% osmium tetroxide for 30 min. Samples were stained in aqueous
1% uranyl acetate for 1 h, dehydrated in a graded ethanol series, and
eMBEDDED in TAAB Low Viscosity resin (TAAB Laboratories Equip-
ment). Sections (70–80 nm) were cut from the ventral nerve cord
in the same area as the motoneurons. Images were observed with a FEI
tecnai 12 Biotwin Transmission Electron Microscope. Micrographs
were taken at x11,000 and random images were taken at x1,900
magnification to assess the number of active zones. 5Cl-CASPP-
treated larvae had been acutely fed for 24 h before dissection.

Image analysis. Images were coded and randomized for blind
analysis. Images were analyzed using ImageJ (National Institutes of
Health). Length of the presynaptic membrane, the shortest distance
from the center of each vesicle to the membrane and the diameter of
each vesicle, was measured. Only vesicles within 200 nm of the
presynaptic membrane were included to avoid inclusion of vesicles
closer to a neighboring synapse. In random images, taken at x1,900
magnification, the unbiased frame function was used in ImageJ to
create a randomly placed frame of 29.4 μm² within the image. The
number of active zones within each frame was counted, and only
frames that had vesicle-containing tissue in >50% of their area
were included in the analysis. Statistics. Statistical significance was calculated using a two-sample
unpaired t-test where it was relevant to compare samples with their
own parental controls. ANOVA with post hoc Bonferroni’s test was
used where multiple groups shared the same control. In both tests,
confidence intervals of P ≤ 0.05, P ≤ 0.01, and P ≤ 0.001 were used
for significance.

RESULTS

Expression level of VACHt affects only the frequency of SV release. To determine how changing levels of VACHt affect
transmitter release at central cholinergic synapses we used two
complimentary approaches to alter the activity of this trans-
porter. To downregulate, we used the insecticidal compound
5Cl-CASPP that specifically inhibits VACHt (Sluder et al.
2012). Upregulation was achieved by expression of a wild-type
VACHt transgene in all cholinergic neurons. To investigate the
consequences for cholinergic synaptic function, we undertook
patch-clamp recordings from the well-characterized aCC/RP2
motoneurons. These neurons receive identical cholinergic syn-
aptic input (Baines et al. 1999). We recorded spontaneous
release of neurotransmitter (i.e., minis) by blocking all action
potential-dependent release with addition of TTX to the extra-
cellular saline (see METHODS). We also recorded action poten-
tial-dependent currents (no TTX present), termed SRCs, to
determine the effect to multiple SV release.

Larvae fed 5Cl-CASPP for 24 h before recording showed a
marked decrease in the frequency of minis recorded in aCC/
RP2 (frequency: 27.5 ± 4.2 vs. 6.9 ± 1.0/min, P = 0.0007,
control vs. 5Cl-CASPP, Fig. 1). By contrast, no change in
amplitude was observed (amplitude: 6.7 ± 0.6 vs. 6.4 ± 0.3
pA, P = 0.8). Minis were also recorded from chA > VACHt
larvae to examine the effect increased levels of VACHt.
Larvae that overexpressed this wild-type form of VACHT showed a 50% increase in mini frequency (31.8 ± 6.4 vs. 63.9 ± 6.8/min, P = 0.003). No significant change was seen in amplitude (Fig. 1). The differences in basal values for both mini amplitude and frequency in vehicle-exposed and GAL4/ UAS parental stocks (i.e., controls) are undoubtedly due to differences in genetic backgrounds of these respective lines.

We carried out qRT-PCR to quantify overexpression of VACHT compared with controls (cha/+ ) and found a significant upregulation of transcript (2.8 ± 0.6 fold-change in RNA expression, P = 0.01, n = 3). The VACHT and ChAT genes share the same first exon, and the remainder of the VACHT gene is contained within the first intron of ChAT (Kitamoto et al. 1998). For this reason we also carried out qRT-PCR for the ChAT transcript to ensure that ChAT expression was not inadvertently changed. There was no significant increase in ChAT transcript abundance (0.1 ± 0.2-fold change, P = 1.0, n = 5). Therefore, we conclude that increased mini frequency is due to overexpression of VACHT without change to ChAT transcript level.

In the absence of TTX, larger inward synaptic currents can be recorded in Drosophila motoneurons. These SRCs are due to action potential-dependent release of multiple SVs (Baines et al. 1999, Rohrbough and Broadie 2002). SRCs recorded after 5CI-CASPP treatment were not significantly different from control in amplitude (29.8 ± 3.6 vs. 39.0 ± 6.1 pA/pF control vs. treated, P = 0.2) or duration (506 ± 32 vs. 543 ± 30 ms, P = 0.4, Fig. 2, A–C). However, SRC frequency was significantly decreased (19.2 ± 4.1 vs. 3.1 ± 1.1/min, control vs. treated, P = 0.0013, Fig. 2D). By contrast, overexpression of VACHT did not affect SRC amplitude, duration, or frequency (Fig. 2, B–D). Thus we conclude that manipulating VACHT affects only frequency of mini release, which, when reduced by blocking activity of VACHT, similarly reduces the frequency of evoked release.

The possibility exists that manipulating VACHT in presynaptic neurons evokes postsynaptic compensation, specifically change in the level of nACHR expression. Such changes, if occurring, might complicate our analysis. To test this possibility, we directly exposed the aCC motoneuron to applied ACh. The amplitude of the response to perfused ACh, under voltage clamp, was not altered in 5CI-CASPP-fed larvae (12.2 ± 1.7 vs. 11.2 ± 2.0 pA/pF, unfed vs. fed, P = 0.7, n = 6 and 7, respectively). ACh response was also unchanged in larvae that overexpressed VACHT in cholinergic neurons (8.3 ± 1.2 vs. 10.2 ± 2.9 pA/pF, controls vs. cha > VACHT, P = 0.5, n = 13 and 6, respectively). By contrast, expression of the nACHRΔ7 subunit in aCC, which has previously been linked to excitability (Ping and Tsunoda 2012), was sufficient to increase the response to applied ACh (8.8 ± 0.9 vs. 11.8 ± 0.8 pA/pF, control vs. RRa > nACHRΔ7, P = 0.02, n = 13 and 11, respectively). This confirms that the experiment is sensitive enough to detect postsynaptic changes in nACHR expression that affect the response to ACh. We conclude, therefore, that the observed change in mini frequency caused by altering active VACHT levels is a primarily presynaptic effect.

Expression of VACHT−Q affects both frequency and amplitude of SV release. Mammalian VACHT contains a di-leucine motif within the cytoplasmic COOH-terminal. This motif is involved in localization of VACHT to membranes and endocytosis after neurotransmitter release (Tan et al. 1998; Barbosa et al. 2002). By comparison, Drosophila VACHT lacks a di-leucine motif, but instead has a 13-glutamine polyQ domain. The precise number of glutamines is thought to be important (Schaefer et al. 2012), and to investigate this we overexpressed a known polymorphism that lacks one glutamine at position 549 (termed VACHT−Q; Sluder et al. 2012).

Expression of VACHT−Q in cholinergic neurons increased the frequency of recorded minis (67.5 ± 10.5 vs. 34.5 ± 6.1/min, cha > VACHT−Q vs. control, P = 0.011, Fig. 3, A and
Fig. 2. Inhibition of VChT decreases spontaneous rhythmic current (SRC) frequency. A: representative traces of SRCs recorded from aCC motoneurons in control (cha^+/+) larvae, larvae acutely fed 5CI-CASPP, and cha > VChT larve. B–D: 5CI-CASPP causes no significant change to SRC amplitude or duration but is sufficient to significantly decrease frequency. Overexpression of VChT does not affect amplitude, duration, or frequency of SRCs. Genotype for 5CI-CASPP-fed larvae and its control (exposed to acetone) is RRA-GAL4/CD8-GFP. Controls for cha > VChT are cha^+/+ and UAS-VChT^/+ (denoted GAL4/UAS), which did not differ and have been combined. Values are means ± SE, n = 10. **P < 0.01.

B) and the amplitude (14.4 ± 1.3 vs. 8.3 ± 0.6 pA, P = 0.0004, Fig. 3, A and C). In contrast to expression of wild-type VChT, expression of VChT^−Q also influenced SRC kinetics. The most notable effect was a significant increase in SRC duration (1.117 ± 125 vs. 588 ± 31 ms, P = 0.0003, Fig. 3F) and decreased frequency (18.4 ± 1.9 vs. 33.1 ± 2.9/min, P = 0.00008, Fig. 3G). By contrast, SRC amplitude was not significantly changed (41.6 ± 4.6 vs. 33.3 ± 3.0 pA/pF, P = 0.15, Fig. 3E). Analysis of the expression level of transgenic VChT^−Q compared with transgenic expression of wild-type VChT shows comparable levels (3.1 ± 0.3 vs. 2.8 ± 0.6-fold increase, VChT^−Q vs. VChT, P = 0.7, n = 5 and 3), indicating that the different effect of the former was not due to increased levels of expression.

To determine if increased duration of SRCs, produced following expression of VChT^−Q, is due to the increased amplitude of minis (i.e., larger quanta) or an increased number of SVs released per action potential, the total number of SVs released over a 20-min time period was measured. This was achieved by blocking SV recycling using bafilomycin. Bafilomycin inhibits the proton pump, preventing acidification of the SV (Bowman et al. 1988). Rundown of SRDCs was recorded for 20 min after addition of bafilomycin (Fig. 4A). Total SV number (i.e., no. of SVs released during the 20-min period) was calculated by dividing the combined area of SRDCs by mean mini area. Combined area of SRDCs is representative of total ACh release and was found to be increased following overexpression of VChT^−Q compared with control (1.0 × 10^5 ± 0.1 × 10^5 vs. 2.38 × 10^5 ± 0.4 × 10^5 pA/mS, P = 0.01, Fig. 4B). Mean mini area was also increased under these conditions (52.9 ± 6.2 vs. 141.0 ± 11.8 pA/mS, P = 0.00001, Fig. 4C), indicating that each SV released more ACh. Analysis of released quanta (SRC area/mean mini area) shows that the number of SVs released was not significantly different between cha > VChT^−Q and control (2.0 × 10^5 ± 0.2 × 10^5 vs. 1.9 × 10^5 ± 0.3 × 10^5 quanta, P = 0.8, Fig. 4D). The number of quanta per SRC was also not altered (707.3 ± 117.0 vs. 584.8 ± 116.1 quanta/SRC, P = 0.5, Fig. 4E). We conclude that longer-duration SRCs, due to expression of VChT^−Q, likely result from increased SV transmitter content, rather than increased number of SVs released.

We also tested the effect of 5CI-CASPP, which was sufficient to reduce mini frequency without change to amplitude (see Fig. 1). Exposure to this inhibitor significantly reduced the combined SRC area (1.3 × 10^3 ± 0.3 × 10^3 vs. 0.2 × 10^3 ± 0.09 × 10^3 pA/mS, P = 0.008, Fig. 4B) while mean mini area showed no difference from controls (34.4 ± 3.1 vs. 50.4 ± 10.1 pA/mS, P = 0.2, Fig. 4C). Calculation of the number of released SVs showed a significant reduction (2.5 × 10^5 ± 0.6 × 10^5 vs. 0.5 × 10^5 ± 0.2 × 10^5 total quanta, P = 0.01, Fig. 4D). However, the number of quanta released per SRC was unchanged (955.2 ± 208.5 vs. 674.27 ± 234.02 quanta/SRC, P = 0.4, Fig. 4E). Collectively, these data indicate that 5CI-CASPP prevents filling of SVs, which, in turn, limits the frequency of SRDCs that can be supported.

Altering levels of VChT or exposure to 5CI-CASPP does not affect SV size. We show that expression of VChT^−Q is sufficient to increase frequency and amplitude of minis recorded in the aCC/RP2 motoneurons. Both effects are indicative of a change in SV volume and/or an increase in the number of releasable SVs (i.e., quanta), the latter possibly due to an increase in the number of active zones.

We analyzed putative cholinergic synapses at the ultrastructural level in third-instar CNS (sections were taken from the approximate area that electrophysiological recordings were made, see METHODS). We took advantage of the fact that the majority of central neurons in Drosophila are cholinergic, and, thus, the majority of synapses in the dorsal motor neuropil will be those of cholinergic premotor interneurons (Gorczyca and
Hall 1987; Yasuyama and Salvaterra 1999). Controls (cha) were compared with CNS derived from both 5Cl-CASPP-fed larvae and from cha/H11022 VAChT/H11002 Q transgenic larvae. Measurement of SV size, distribution with respect to the active zone, and active zone length did not differ between control, cha/H11022 VAChT/H11002 Q, and WT larvae fed 5Cl-CASPP (Fig. 5). This suggests that, in cha/H11022 VAChT/H11002 Q larvae, SVs are normal in size and supports the finding that altered release of ACh is not due to an altered number of SVs at the active zone. That minis have larger amplitude in cha/H11022 VAChT/H11002 Q is perhaps consistent with SVs containing more ACh under these conditions. However, whether this is the case remains to be determined, particularly given that overexpression of wild-type VAChT does not change mini amplitude. We must also test the possibility that expression of VAChT-Q, similar to its wild-type counterpart, does not evoke postsynaptic compensation. To address the number of active zones, we analyzed random images taken at a lower magnification. Again, there was no apparent change in active zone number across the three conditions (Fig. 5B). This indicates that the change in frequency observed in minis is not likely due to a change in the number of active zones, but rather an increased release probability.

**DISCUSSION**

Using a *Drosophila* central synapse, we have investigated in vivo how VAChT regulates cholinergic transmission. We demonstrate that decreased VAChT activity leads to decreased spontaneous quantal release frequency. Increased VAChT activity, by contrast, leads to increased frequency of spontaneous release with no change to amplitude or number of SVs at the active zone, suggestive of an increased probability of SV release.

Decreased functional VAChT causes a reduction in spontaneous quantal release frequency but not quantal size. This is in agreement with studies at *Drosophila* and snake NMJs, where decreased vesicular transporter results in decreased frequency but not amplitude of miniature excitatory junctional potentials (Parsons et al. 1999; Daniels et al. 2006). However, many studies in mice and rats also link decreased VAChT with decreased transmitter load (Wilson et al. 2005; Prado et al. 2006; de Freitas Lima et al. 2010; Rodriguez et al. 2013). A possible explanation for this apparent difference is that *Drosophila* cholinergic SVs usually contain only one VAChT, and so each SV is either loaded with ACh or empty if the VAChT is blocked by 5Cl-CASPP. We report no change in vesicle number or size at the active zone, which
suggestions that there may be empty vesicles that undergo recycling, as has been previously reported (Parsons et al. 1999). This is supported by a decrease in total quanta released after bafilomycin treatment.

When VACHT activity is increased, we see a clear increase in frequency with no change in amplitude of spontaneous quantal release. An increase in mini frequency with increased VACHT in SV filling and release. Moreover, it provides evidence to suggest that the polyQ domain of VACHT seemingly results in a nonfunctional protein that cannot rescue the VACHT null (Cash and Baines, unpublished observations).

Our study presents supporting data for a dual role for VACHT at central synapses in Drosophila. A glycine-to-arginine amino acid change at position 347 disrupts an interaction with synaptobrevin, a vesicle-associated membrane protein that is pivotal for exocytosis (Link et al. 1992; Schiavo et al. 1992). The glycine at position 347 is well conserved and is present in including humans, rats, and mice, have a di-leucine motif at residues 485–486 within the cytoplasmic COOH-terminal. This di-leucine motif has been reported to be important for interaction with the AP-2 complex (Tan et al. 1998; Barbosa et al. 2006). A glycine-to-arginine substitution at this position has a role in determining SV transmitter load. Mammals, including humans, rats, and mice, have a di-leucine motif at residues 485–486 within the cytoplasmic COOH-terminal. This di-leucine motif has been reported to be important for interaction with the AP-2 complex (Tan et al. 1998; Barbosa et al. 2006).

We further show that increased spontaneous release frequency is not likely caused by an increase in the number of vesicles at the active zone or active zone density. This corroborates previous work that shows VACHT plays a second role: facilitating SV mobilization or fusion. In Caenorhabditis elegans, an interaction between VACHT and SV release machinery has been reported (Sandoval et al. 2006). A glycine-to-arginine amino acid change at position 347 disrupts an interaction with synaptobrevin, a vesicle-associated membrane protein that is pivotal for exocytosis (Link et al. 1992; Schiavo et al. 1992). The glycine at position 347 is well conserved and is present in Drosophila VACHT. However, a glycine-to-arginine substitution at this position in the Drosophila VACHT seemingly results in a nonfunctional protein that cannot rescue the VACHT null (Cash and Baines, unpublished observations).

Our study presents supporting data for a dual role for VACHT in SV filling and release. Moreover, it provides evidence to suggest that the polyQ domain of Drosophila VACHT has a role in determining SV transmitter load. Mammals, including humans, rats, and mice, have a di-leucine motif at residues 485–486 within the cytoplasmic COOH-terminal. This di-leucine motif has been reported to be important for localizing VACHT to the SV membrane and also to play a role in endocytosis after neurotransmitter release through an interaction with the AP-2 complex (Tan et al. 1998; Barbosa et al. 2002). Drosophila VACHT does not have a di-leucine motif, but, unlike the mammalian VACHT sequences mentioned, has
a 13-residue polyQ domain at the COOH-terminal. Extended polyQ domains are associated with diseases such as Huntington’s and spinocerebellar ataxia (Lievens et al. 2005; Sokolov et al. 2006). Little is known about the normal function of polyQ domains, but functions may include protein-protein interactions, transcriptional regulation, and RNA binding and signaling (Schaefer et al. 2012). This poses the possibility that the polyQ domain may be responsible for VAChT localization and endocytosis in Drosophila. It has been suggested that the number of glutamines may be of importance (Schaefer et al. 2012). It is possible, therefore, that VAChT/H11002 may be transported more efficiently to the SV membrane. In D. melanogaster the VAChT⁻Q tested in this study is a naturally occurring polymorphism identified during cloning of the Drosophila VAChT by Sluder et al. (2012, supplementary text) and subsequently confirmed by sequencing PCR products from cDNA libraries.

A BLAST search for similar amino acid sequences and predicted sequences of VAChT found that many Drosophila species contain polyQ domains in the same region as D. melanogaster, but that the length varied from 15 in D. pseudoobscura to 5 in D. grimshawi. Other insects that also have a polyQ domain in the same region include the housefly Musca domestica, which has a nine-residue polyQ domain, and three Anopheles mosquitoes (A. sinensis, A. gambiae, and A. darlingi with 7, 10, and 7 glutamines, respectively). Ant, moth, bee, and butterfly species found during the search did not contain a polyQ of more than two glutamines in the same region. The presence of the polyQ domain in three malaria-transmitting mosquito species but not other insects identifies this region as a possible target for insecticides to control these disease-carrying insects.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

F.C., P.P., J.G., and R.A.B. conception and design of research; F.C. and S.W.V. performed experiments; F.C. and S.W.V. analyzed data; F.C. and R.A.B. interpreted results of experiments; F.C. and R.A.B. prepared figures; F.C. and R.A.B. drafted manuscript; F.C., S.W.V., P.P., J.G., and R.A.B.

Fig. 5. VAChT does not influence active zone morphology. A: representative micrographs showing active zones in control, larvae-fed 5Cl-CASPP, and cha > VAChT/H11002. Black arrows, vesicles; white arrows, active zones. B: changing VAChT activity (by chemical block or transgene overexpression) has no effect on distribution of SVs at the active zone, length of the active zone, or SV number or diameter. The no. of active zones is also not affected. Values are means ± SE; n = 29, referring to the no. of active zones analyzed and n = 41 for active zone image analysis. Control is cha−/−. Scale is 200 nm.
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