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

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Submitted 23 November 2015; accepted in final form 9 December 2015

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.

acetylcholine; central synapse; vesicular transporter

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. 1995; Nguyen et al. 1998). The number of transporters per SV is unknown but has been estimated to be between one and three (Van der Kloot 2003). This has led to speculation that even small reductions in VAcHT expression level may result in SVs devoid of transporter (Prado et al., 2013). Partial knockdown of VAcHT in mice results in a reduction in both frequency and amplitude of quantal miniature end-plate potentials at the neuromuscular junction (NMJ) (de Freitas Lima et al. 2010; Rodrigues et al. 2013). Electron microscopy showed no reduction in total SV number but altered SV distribution in the synaptic terminals of the NMJ (Rodrigues et al. 2013). By contrast, overexpression of VAcHT, in Xenopus spinal motoneurons, results in increased quantal size and also frequency at the NMJ (Song et al. 1997). The increase in amplitude has been used to argue for a “steady-state” model of SV filling, where inflow is balanced by outflow rather than an alternate “set-point” model (Williams 1997). Alteration of VAcHT activity at the NMJ is seemingly sufficient to increase ACh loading into SVs, an effect that is not countered by a compensatory change in outflow. The consequence is increased ACh per SV. However, whether this model applies to central cholinergic synapses has yet to be determined.

Here we show that overexpression of VAcHT (dVAcHT), 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 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. ChaB19-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 [(S)-1-[(E)-3-(4-chlorophenyl)allyl]spiro[indoline-3,4'-piperidine-1-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 SYBR 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 CACGTGGATCTTTCC; choline acetyltransferase (ChAT) primers ATCA-GAA; VAChT primers CTCATCCTCGTGATTGTA and ACGGG-CCAGTCGGATCGATCGATATGCTA and ACGTTGTGCACCAG-

Electrophysiology. Recordings were performed at room temperature (20–22°C). Third-instar larvae were dissected in saline (in mM: 135 NaCl, 5 KCl, 4 MgCl2·6H2O, 2 CaCl2·2H2O, 5 NaHPO4, 2 KH2PO4, pH 7.4) and fixed in a wide-bore (2% osmium tetroxide for 30 min). Samples were stained in aqueous 0.1 M cacodylate buffer for 1 h. Samples were then incubated in 1% aqueous uranyl acetate for 1 h, dehydrated in a graded ethanol series, and embedded in TAAB Low Viscosity resin (TAAB Laboratories Equipment). Sections (70–80 nm) were cut from the nerve ventral cord in the same area as the motoneurons. Images were observed with a FEI Tecnai 12 Biotwin Transmission Electron Microscope. Micrographs were taken at ×11,000, and random images were taken at ×1,900 magnification to assess the number of active zones. SCI-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 ×1,900 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 transporter. 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 synaptic 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 extracellular saline (see METHODS). We also recorded action potential-dependent currents (no TTX present), termed SRCs, to determine the effect to multiple SV release.

Larvae fed SCI-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 of increased levels of VACHT.

J Neurophysiol • doi:10.1152/jn.01053.2015 • www.jn.org
Larvae that overexpressed this wild-type form of VAChT showed a 50% increase in mini frequency (31.8 \pm 6.4 vs. 63.9 \pm 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 \pm 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 level (2.8 \pm 0.9 vs. control, \( P = 0.02, n = 5 \)). 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 \textit{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 5Cl-CASPP treatment were not significantly different from control in amplitude (29.8 \pm 3.6 vs. 39.0 \pm 6.1 pA/pF control vs. treated, \( P = 0.2 \)) or duration (506 \pm 32 vs. 543 \pm 30 ms, \( P = 0.4 \), Fig. 2, A–C). However, SRC frequency was significantly decreased (19.2 \pm 4.1 vs. 3.1 \pm 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 5Cl-CASPP-fed larvae (12.2 \pm 1.7 vs. 11.2 \pm 2 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 \pm 1.2 vs.10.2 \pm 2.9 pA/pF, controls vs. cha > VAChT, \( P = 0.5, n = 13 \) and 6, respectively). By contrast, expression of the nAChR\(_{\beta 7}\) 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 \pm 0.9 vs. 11.8 \pm 0.8 pA/pF, control vs. RRa > nAChR\(_{\beta 7}\), \( P = 0.02, n = 13 \) 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, \textit{Drosophila} VAChT lacks a di-leucine motif, but instead has a 13-glutamine polyQ domain in the COOH-terminal. Little is known about the normal function of polyQ domains, but functions may include protein-protein interactions, transcriitional regulation, and RNA binding and signaling (see DISCUSSION). Therefore, the polyQ domain may substitute the role of the mammalian di-leucine motif. 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 \pm 10.5 vs. 34.5 \pm 6.1/min, cha > VAChT\(^{−Q}\) vs. control, \( P = 0.011 \), Fig. 3, A and 3B) and increased the amplitude of the response to perfused ACh (8.8 \pm 0.9 vs. 11.8 \pm 0.8 pA/pF, controls vs. cha > VAChT, \( P = 0.02, n = 13 \) 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.
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 VAChT, expression of VAChT<sup>Q</sup> also influenced SRC kinetics. The most notable effect was a significant increase in SRC duration (1.117 ± 0.125 vs. 0.588 ± 0.31 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 VAChT<sup>Q</sup> compared with transgenic expression of wild-type VAChT shows comparable levels (3.1 ± 0.3 vs. 2.8 ± 0.6-fold increase, VAChT<sup>Q</sup> vs. VAChT, 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 VAChT<sup>Q</sup>, 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 SRCs 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 SRCs by mean mini area. Combined area of SRCs is representative of total ACh release and was found to be increased following overexpression of VAChT<sup>Q</sup> compared with control (1.0 × 10<sup>5</sup> ± 0.1 × 10<sup>5</sup> vs. 2.38 × 10<sup>4</sup> ± 0.4 × 10<sup>4</sup> pA/pF, 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 > VAChT<sup>Q</sup> and control (2.0 × 10<sup>5</sup> ± 0.2 × 10<sup>5</sup> vs. 1.9 × 10<sup>5</sup> ± 0.3 × 10<sup>5</sup> 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 VAChT<sup>Q</sup>, likely result from increased SV transmitter content, rather than increased number of SVs released.

We also tested the effect of 5Cl-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<sup>3</sup> ± 0.3 × 10<sup>3</sup> vs. 0.2 × 10<sup>3</sup> ± 0.09 × 10<sup>3</sup> 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<sup>5</sup> ± 0.6 × 10<sup>5</sup> vs. 0.5 × 10<sup>5</sup> ± 0.2 × 10<sup>5</sup> 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 5Cl-CASPP prevents filling of SVs, which, in turn, limits the frequency of SRCs that can be supported.

**Altering levels of VAChT or exposure to 5Cl-CASPP does not affect SV size.** We show that expression of VAChT<sup>Q</sup> 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/VaChT-Q transgenic larvae. Measurement of SV size, distribution with respect to the active zone, and active zone length did not differ between control, cha/VaChT-Q, and WT larvae fed 5Cl-CASPP (Fig. 5). This suggests that, in cha/VaChT-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/VaChT-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
suggests 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 is in good agreement with a study in Xenopus spinal neurons (Song et al. 1997). Moreover, VGLUT overexpression in Drosophila NMJ results in a modest increase in quantal release (Daniels et al. 2004). However, these studies, and another in rat (Wilson et al. 2005), consistently report an increase in transmitter load of the SVs, which supports the second role: facilitating SV mobilization or fusion. In Cae-rothorhabditis elegans, an interaction between VAChT and SV release machinery has been reported (Sandau 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 norhabditis elegans, an interaction between VAChT and SV 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/Q 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.

ACKNOWLEDGMENTS

We are grateful to Marcus Allen who originated this project. We thank members of the Baines group for help and advice during the course of this work. We also thank Dr. Aleksandr Mironov (University of Manchester Electron Microscopy Unit) for assistance and advice with TEM sample preparation and imaging.

GRANTS

This work was supported by a Biotechnology and Biological Sciences Research Council CASE studentship to F. Cash and by a BBSRC project grant to R. A. Baines (BB/J005002/1).

DISCLOSURES

Work on this project benefited from the Manchester Fly Facility, established through funds from University and the Wellcome Trust (087742/Z/08/Z). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. No conflicts of interest, financial or otherwise, are declared by the authors.

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.
approved final version of manuscript; P.P. and R.A.B. edited and revised manuscript.

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