A Poly-Glutamine Region in the Drosophila VAChT Dictates Fill-Level of Cholinergic Synaptic Vesicles

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Abstract
While the primary role of vesicular transporters is to load neurotransmitters into synaptic vesicles (SVs), accumulating evidence suggests that these proteins also contribute to additional aspects of synaptic function, including vesicle release. In this study, we extend the role of the VAChT to include regulating the transmitter content of SVs. We report that manipulation of a C-terminal poly-glutamine (polyQ) region in the Drosophila VAChT is sufficient to influence transmitter content, and release frequency, of cholinergic vesicles from the terminals of premotor interneurons. Specifically, we find that reduction of the polyQ region, by one glutamine residue (13Q to 12Q), results in a significant increase in both amplitude and frequency of spontaneous cholinergic miniature EPSCs (mEPSCs) recorded in the aCC and RP2 motoneurons. Moreover, this truncation also results in evoked synaptic currents that show increased duration: consistent with increased ACh release. By contrast, extension of the polyQ region by one glutamine (13Q to 14Q) is sufficient to reduce mEPSC amplitude and frequency and, moreover, prevents evoked SV release. Finally, a complete deletion of the polyQ region (13Q to 0Q) has no obvious effects to mEPSCs, but again evoked synaptic currents show increased duration. The mechanisms that ensure SVs are filled to physiologically-appropriate levels remain unknown. Our study identifies the polyQ region of the insect VAChT to be required for correct vesicle transmitter loading and, thus, provides opportunity to increase understanding of this critical aspect of neurotransmission.

Key words: acetylcholine; Drosophila; neurotransmitter; synapse; synaptic vesicle; transporter

Introduction
Vesicular loading of synaptic vesicles (SVs) is dependent on initial acidification mediated by the vATPase pump. This pump generates both a pH gradient (ΔpH) and a voltage gradient (Δψ) across the SV membrane (Edwards, 2007; Takamori, 2016). The relative requirement
for these two components for loading is dependent on neurotransmitter: anionic transmitters such as glutamate rely more heavily on $\Delta \psi$ (Maycox et al., 1988; Schenck et al., 2009; Takamori, 2016). Zwitterionic transmitters require both gradients (Edwards, 2007; Takamori, 2016), whereas, cationic transmitters (e.g., ACh) rely predominantly on $\Delta \psi$ (Parsons et al., 1993; Parsons, 2000; Takamori, 2016). Transport of ACh into a SV involves the exchange of two protons in an antiporter system using the proton-electrochemical gradient (Südhof, 2004; Lawal and Krantz, 2013). The current model suggests that one proton is used to transport ACh into the SV lumen while the second proton is needed to re-orientate the VAChT substrate binding site back toward the cytoplasm (2H$^+$ for 1ACh$^-$; Parsons, 2000). In situ, acidified SVs exhibit a pH ~1.4 units less than un-acidified SVs (Parsons, 2000). Theoretically, the cholinergic SV lumen has the capacity to concentrate ACh by 100-fold relative to cytoplasmic levels (which range between 1 and 4 mM; Parsons et al., 1993; Parsons, 2000). However, the maximal reported accumulation of ACh in SVs has been found to saturate at ~4 mM, suggesting a rather dramatic (and unknown) limiting factor impedes loading (Parsons et al., 1993; Varoqui and Erickson, 1996; Parsons, 2000).

A key limiting factor may be copy number of functional transporter per SV. Murine and Drosophila NMJ and mammalian cell culture models suggests vesicular loading is altered following either genetic and/or pharmacological manipulation of transporter activity (Varoqui and Erickson, 1996; Song et al., 1997; Daniels et al., 2004; Wilson et al., 2005; Prado et al., 2006; De Castro et al., 2009; Lima et al., 2010). However, it is notable that up-regulation of VACHT expression fails to show effects to quantal size at either snake NMJ or Drosophila motoneurons that receive cholinergic excitation (Parsons et al., 1999; Cash et al., 2016). An inability of increased transporter to affect SV loading is consistent with a set-point model of filling (Williams, 1997; Cash et al., 2016). This model posits that SVs fill to a predetermined level, independent of filling rate, which changes following manipulation of transporter expression level. We have previously reported that transgenic expression of VACHT, which carries a single glutamine truncation in a C-terminal poly-glutamine (polyQ) region (13Q to 12Q), results in increased quanta of spontaneously released SVs at identified interneuron to motoneuron synapses (Cash et al., 2016). This region, therefore, may contribute to the mechanism that regulates SV loading.

Here, we use electrophysiological characterization of cholinergic release at Drosophila larval and embryonic interneuron—motoneuron synapses to investigate the physiologic implications to SV loading when the VACHT C-terminal polyQ region is manipulated. We find, in agreement with previously published literature, that expression of a single glutamine truncation VACHT$^{12Q}$ increases both amplitude and frequency of spontaneously released cholinergic miniature EPSCs (mEPSCs; i.e., individual SV release) recorded from aCC and RP2 motoneurons. Evoked synaptic currents also show an increased duration consistent with an increased ACh load. Conversely, we further show that CRISPR induced single amino acid extension of the polyQ region (VACHT$^{13Q}$) results in the opposite effect: reduced mEPSC amplitude and frequency and, moreover, an inability to support evoked release. CRISPR mediated deletion of the polyQ region (VACHT$^{1Q}$) has no effect on mEPSC kinetics suggesting that elongation or truncation of the VACHT polyQ region is more detrimental to cholinergic functioning than its removal.

Materials and Methods

Fly stocks

Flies were maintained under standard conditions at 25°C. GAL4 drivers used to recapitulate expression of the cholinergic locus were chaB$^{979}$ (Salvaterra and Kitamoto, 2001) and ChAT-BAC (gifted by Steve Stowers, Montana State University). These lines were used to drive expression of UAS-VACHT$^{12Q}$ (Cash et al., 2016), UAS-Chr2$^{ChETA}$ (Bloomington 36354; Gunaydin et al., 2010), and UAS-Chr2 (Pulver et al., 2009). CRISPR constructs were prepared as described below and injected into cas9-expressing embryos (yw; attP40 nos-cas9/CyO;+ by BestGene Inc. Control lines were the cleaned CRISPR-injected line lacking construct insertion (w$^+$; +; +). Animals used were of either sex.

gRNA and insert design, template oligo and plasmid construction

The CRISPR Optimal Target Finder tool (http://tools.flycrispr.molbio.wisc.edu/targetFinder/) was used to specify target cut sequence specificity (GATTACGCTATCAGG-TACC). Two guide RNA constructs were made to generate cuts in 5’- and 3’-UTR of VACHT, respectively. The gRNA oligonucleotides (5’ to 3’) are: 5’-UTR: CTTC-GAGAGGAATCCCAAGGAAC and AAAAGTTGTTTGGGACTTCTCCTC; 3’-UTR: CCTCGTATTATTACAC-GACAT and AACCATATGCTATAGTAATAC; sense and antisense, respectively). A total of 100 pmol of each 5’ phosphorylated sense and antisense gRNA oligonucleotides were mixed, denatured at 95°C and then reduced to 25°C at a rate of ~0.1°C/s and ligated to the guide RNA.
expression plasmid, pU6-Bbsl-chiRNA (plasmid #45946, Addgene). Oligos used to generate PM and polyQ site mutations are shown in Table 1. Briefly, for 5' PAM site mutagenesis, PCR of primers a + b and c + d (containing TGG to TGC point mutations) were run against Drosophila genomic DNA (PM) or VACHT plasmid DNA (polyQ; PCR1). Following purification, PCR products (a + b + c + d) were used as templates for a second PCR using the most 5' and 3' primers of PCR1 (primers a and d, Table 1). This process was repeated for 3' PAM site mutagenesis utilizing primers (e + f and g + h) and VACHT\textsuperscript{14Q} (i + j) and VACHT\textsuperscript{14Q} (m + n and o + p). Full UTR sequence with PM mutations were purified, sequenced and mobilized to pHD-DsRed (plasmid #51434, Addgene) as a dsDNA donor template for CRISPR/Cas9-mediated homology-directed repair (HDR) using restriction digests (5' = Ascl and BssSi; 3' = SpeI and Xhol). PolyQ products were first mobilized to the cloning vector pJET1.2, then to the 3xP3 expression pattern. Lines were cleaned and identified by the expression of DsRed in larvae following molecular devices. Sequences were re-confirmed at the Manchester Sequencing facility before experimentation.

**Quantitative RT-PCR (qRT-PCR)**

A total of 50 late stage 17 embryos (per replicate) were collected. RNA was extracted using the RNeasy micro kit (QiAGEN). Single strand cDNA was synthesized using the Revert Aid H minus first strand cDNA synthesis kit (Fermentas). qRT-PCR was performed using a LightCycler480 II (Roche) with SYBR Green I Master reaction mix (Roche). The thermal profile used was 10 s at 72°C. Single-product amplification was completed by post-reaction dissociation analysis. PCR primers were designed with the aid of LightCycler Probe Design Software 2.0 (v1.0; Roche). Results were analyzed by the 2\(^{ΔΔCt}\) method. Ct values used were means of two to three independent replicates.

**Results**

Gene expression was normalised to actin. Primers (5' to 3') were as follows: actin, CTTCTACAATGAGCTGCGT and GAGAGCACAGCCTGGAT; VACHT, TCTCATCCTCGT-GATTGTA, and ACGGGTATGACTTTCC.

**Table 1. Primers used for creation of Drosophila VACHT UTR with modified PAM sites (5': a, b, c, d and 3’: e, f, g, h) and modified PolyQ regions (5’: i, j, k, l and 3’: m, n, o, p)**

| Sequence | Use          | PM Site |
|----------|--------------|---------|
| ATCGGGGCGCGCGCGAAATTCGCTTGGTGACTTTAGCTC | a + b | (5'PAM) |
| ACAAAGTGTTGAGCTGTTCTTTTG | b | (5'PAM) |
| CCAAAGAAGTGATGCGAAAGTITGTTT | c + d | (5'PAM) |
| CTAAATAGTCGCCCGTAAATTCCGTTACTA | d | (5'PAM) |
| GTACATGAGTTCCGGGATGCTCCTC | e + f | (3'PAM) |
| AGTACACTGGTGACTATGCTCTA | e | (3'PAM) |
| TATAGACATATGGCAAGTGATGCAGT | g + h | (3'PAM) |
| GCTACTCGAGAAGTCCGCCACAATGACAACC | h | (3'PAM) |
| GTGCCCATCGAGCGGCCGCT | i + j | VACHT\textsuperscript{14Q} |
| CAGGACCTCTGCTCGCCAGAAAGGATTGCGCCACACGG | j | VACHT\textsuperscript{14Q} |
| CCGTGGGCAATCCCTCGCCAAGCAGAGGGCTCTG | k + l | VACHT\textsuperscript{14Q} |
| GCTATATTACAATATG TAGAGATCGATCGTGCGGGCAA | l | VACHT\textsuperscript{14Q} |
| GTGCCACTCGAGCGCCGC | m + n | VACHT\textsuperscript{14Q} |
| CTGCGTCTGCTCGTGTGTTGCTGCTGCTGCTGCTGCTG | n | VACHT\textsuperscript{14Q} |
| CAGAGACAGACAGCAACAACAAACACACACAGACGGTCAGAGC | o + p | VACHT\textsuperscript{14Q} |
| GCTGATTTACAATATGATGAGATCGATCGTGCTTGCGGGCAA | p | VACHT\textsuperscript{14Q} |

**Larval and embryonic whole-cell patch-clamp recordings**

Recordings were performed at room temperature (20–22°C). Third-instar larvae were dissected in external saline (135 mM NaCl, 5 mM KCl, 4 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, H\textsubscript{2}O, 5 mM NaCl, 36 mM sucrose; pH 7.15). The CNS was removed and secured to a Sylgard (DowCorning)-coated coverslip using tissue glue (GLUture; WPI). The neurolemma surrounding the CNS was partially removed using protease (1% type XIV; Sigma) contained in a wide-bore (15 μm) patch pipette. Whole cell recordings were conducted using borosilicate glass electrodes (GC100TF-10; Harvard Apparatus), fire-polished to resistances of between 7 and 10 MΩ for L3 recordings and 14–18 MΩ for embryonic recordings. The aCC/RP2 motoneurons were identified by characteristic soma size and position within the ventral nerve cord. Cell identity was sporadically confirmed, after recording, by filling with 0.1% Alexa Fluor 488 hydrazide sodium salt (Invitrogen), included in the internal patch saline (140 mM potassium gluconate, 2 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}·H\textsubscript{2}O, 5 mM EGTA, 5 mM KCl, and 20 mM HEPES; pH 7.4). Tetrodotoxin (TTX; 2 μM, Alomone Labs) was included in the external saline to block action potential-induced SV release. Recordings were made using a MultiClamp 700B amplifier. Cells were held at –60 mV and recordings were sampled at 100 kHz and lowpass filtered at 0.5 kHz, using pClamp 10.6 (Molecular Devices). Only neurons with an input resistance of 500 MΩ (L3 recordings) or 1 GΩ (embryo) were accepted for analysis. Evoked vesicle exocytosis was elicited through driving UAS-ChR2 or UAS-ChR2\textsuperscript{ChETA} using blue light (470 nm, 10 ms, 1 Hz/0.05 Hz, light intensity 9.65 mW/cm\textsuperscript{2}).
Statistics
Statistical significance between group means was assessed using either a Student’s t test (where a single experimental group is compared to a single control group), a one-way ANOVA followed by Bonferroni’s post hoc test (multiple experimental groups). In all tests, confidence intervals of \( *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001, \) and \(**\*p \leq 0.0001\) were used for significance. Data shown are mean \( \pm \) SEM.

Results
VAChT\(^{12Q}\) increases SV loading at cholinergic synapses
We undertook patch-clamp recordings from well-characterized aCC/RP2 motoneurones, which receive identical cholinergic synaptic input (Baines et al., 1999). We recorded spontaneous mEPSCs, achieved by blocking action potential-dependent activity with TTX. We have previously shown that expression of transgenic VAChT\(^{12Q}\) in a wild-type background (i.e., VAChT\(^{13C}\)), significantly increases mEPSC amplitude and release frequency (Cash et al., 2016). It should be noted that, unlike the NMJ, mEPSCs recorded in central neurons can (and in this case do) show a range of amplitudes due to filtering of current spread through axonal and dendritic regions. In this study, we confirm that transgenic expression of VAChT\(^{12Q}\) increases mEPSC amplitude (7.9 \( \pm \) 0.5 vs 12.1 \( \pm \) 0.8 pA, GAL4/UA5 vs cha\(^{B19}\)-VASP, respectively, \( p \leq 1 \times 10^{-4} \); Fig. 1A,B) and also frequency (35.5 \( \pm \) 5.1 vs 74.3 \( \pm \) 6.2 per min, GAL4/UA5 vs cha\(^{B19}\)-VASP, respectively, \( p \leq 1 \times 10^{-4} \)). By contrast, upregulation of wild-type VAChT did not significantly increase mEPSC amplitude (7.9 \( \pm \) 0.5 vs 9.9 \( \pm \) 0.6 pA, GAL4/UA5 vs cha\(^{B19}\)-VASP, respectively, \( p \geq 0.10 \); Fig. 1A,B). However, in line with VAChT\(^{12Q}\) upregulation, frequency was increased (35.5 \( \pm \) 5.1 vs 77.7 \( \pm \) 8.5 per min, GAL4/UA5 vs cha\(^{B19}\)-VASP, respectively, \( p \leq 1 \times 10^{-4} \)). These data suggest that manipulation of the polyQ region, rather than expresional regulation of VAChT, regulates cholinergic SV loading.

To determine whether the effects we observed in mEPSCs, following expression of VAChT\(^{12Q}\), affect evoked release we recorded evoked spontaneous rhythmic currents (SRCs) in aCC/RP2 (i.e., in the absence of TTX). Figure 1C,D shows that SRCs are supported, but that they exhibit altered kinetics: significantly showing significantly increased duration [420.3 \( \pm \) 14.5, 468.8 \( \pm \) 27.9 vs 709.2 \( \pm \) 47.4 ms, cha\(^{B19}\)/+, UAS/+ vs cha\(^{B19}\)-VASP, respectively, \( p \leq 1 \times 10^{-3} \) (cha\(^{B19}\)/+) and \( 5 \times 10^{-3} \) (UAS/+)); SRC frequency was also significantly reduced [38.3 \( \pm \) 5.9, 48.1 \( \pm \) 4.2 vs 19.0 \( \pm \) 3.0 per min, cha\(^{B19}\)/+, UAS/+ vs cha\(^{B19}\)-VASP, respectively, \( p \leq 1 \times 10^{-2} \) (cha\(^{B19}\)/+) and \( 3 \times 10^{-4} \) (UAS/+)], while amplitude remained unchanged (\( p \geq 0.23 \)).

As can be seen in Figure 1C, network-driven SRCs show variability in amplitude, perhaps due to differential activity of premotor interneurones and/or filtering of current spread through the dendritic regions of motoneurones. To provide a more rigorous baseline (i.e., to reduce variability particularly in amplitude) we used an optogenetic approach. This is sufficient to produce EPSCs that are more consistent in amplitude, and are identical to SRCs (but as these are not spontaneous, we term them EPSCs). We expressed ChR2 (Pulver et al., 2009) in all cholinergic neurons using Cha\(^{B19}\)-GAL4 (this includes the excitatory premotor interneurons to aCC/RP2). Expression of VAChT\(^{12Q}\) similarly increased duration of optogenetically-evoked EPSCs (485.4 \( \pm \) 32.9 vs 625.9 \( \pm \) 49.9 ms, cha\(^{B19}\)-ChR2 vs cha\(^{B19}\)-VASP, VAChT\(^{12Q}\), respectively, \( p \leq 0.03 \)) but again did not influence amplitude (23.4 \( \pm \) 2.7 vs 24.5 \( \pm \) 3.3 pA/pF, cha\(^{B19}\)-ChR2 vs cha\(^{B19}\)-VASP, VAChT\(^{12Q}\), respectively, \( p \leq 0.81 \)). Notably, expression of wild-type VAChT also increased optogenetically-evoked EPSC duration (485.4 \( \pm \) 33.0 vs 636.4 \( \pm \) 44.6 ms, cha\(^{B19}\)-ChR2 vs cha\(^{B19}\)-VASP, VAChT, respectively, \( p \leq 0.02 \); Fig. 2C). Again, with no effect on amplitude (26.4 \( \pm \) 2.5 vs 29.0 \( \pm \) 2.4 pA/pF, \( p \leq 0.49 \)).

We also measured the amplitude ratio between the first and second EPSC evoked at a following frequency of 1 Hz. The resulting ratios (EPSC2/EPSC1) were 75.2 \( \pm \) 6.9\% vs 93.0 \( \pm \) 1.2\%, cha\(^{B19}\)-ChR2 versus cha\(^{B19}\)-VASP, VAChT\(^{12Q}\), respectively, \( p \leq 7 \times 10^{-3} \) (Fig. 2A,B). Whereas overexpression of wild-type VAChT did not statistically differ from control (75.2 \( \pm \) 6.9\% vs 87.3 \( \pm \) 2.6\%, \( p \leq 0.13 \)) this effect was abrogated when stimulation frequency was reduced to once every 20 s (0.05 Hz; 84.8 \( \pm \) 4.2\% vs 89.1 \( \pm \) 3.0\%, cha\(^{B19}\)-ChR2 vs cha\(^{B19}\)-VASP, VAChT\(^{12Q}\), respectively, \( p \leq 0.92 \)). We rationalize that this reduction represents an inability to fully refill recycled SVs and thus represents a net reduction in quantal content of the second SRC. That this reduction is greatest in wild type (Fig. 2A) is in agreement with our observations above; that expression of VAChT\(^{12Q}\) increases the fill load of SVs. This effect is mitigated using a lower frequency of stimulation (0.05 Hz), which we predict provides sufficient time to fully recycle/re-fill SVs. Taken together, and in line with previous literature (Cash et al., 2016), our data suggest that expression of VAChT\(^{12Q}\) is sufficient to increase loading of SV in the terminals of cholinergic central neurons, an effect that was not observed through upregulation of the wild-type (13Q) transporter (Cash et al., 2016). This is sufficient to produce mEPSCs exhibiting larger amplitudes, SRCS/EPSCS exhibiting longer durations and the ability of the presynaptic terminals to resist synaptic depression following continuous 1-Hz evoked vesicle release.

VAChT\(^{14Q}\) decreases SV loading at cholinergic synapses
To investigate the contribution to SV loading made by the VAChT polyQ region, we created two CRISPR knock-in gene replacements. The first extended the polyQ region by one additional glutamine (VAChT\(^{14Q}\)), while the second deleted the polyQ region (VAChT\(^{12Q}\)). A third CRISPR was attempted containing a single glutamine truncation (VAChT\(^{12C}\)) to validate our findings using the GAL4/UA5 system described above. However, despite several injection attempts (BestGene and Manchester Fly Facility) we were unable to generate transgenic progeny. CRISPR mutations were confirmed not to increase VAChT
transcript expression relative to wild type. qRT-PCR determination of expression level (using relative fold change: Log₂) was: VACHT³⁰⁻⁄⁄ VACHT¹⁴⁻⁄⁄ (0.63 ± 0.24, n = 2, p = 0.89) and VACHT¹⁴⁻⁄⁄ (0.82 ± 0.88, n = 2, p = 0.83) compared to control lines (set to 0, n = 3).

Homozygous VACHT¹⁴⁻⁄⁄ is embryonic lethal. Embryos develop normally until late stage 17, identified by the presence of inflated trachea, clearly visible mouth hooks, normal gross CNS morphology and body-wall musculature. However, no coordinated peristaltic waves of body-
Recordings from aCC/RP2, in late stage 17 embryos, showed that mEPSC amplitude was significantly reduced (4.0 ± 0.2 vs 2.9 ± 0.2 pA, control vs VAChT14Q, respectively, p = 0.05) as was frequency (26.1 ± 4.8 vs 4.56 ± 1.1 per min, control vs VAChT14Q, respectively, p = 0.007). This is not seen in wild-type VAChT expression (p = 0.13). This effect is abrogated when the second stimulus is applied at 0.05 Hz (p = 0.92). Expression of VAChT14Q increased duration of optogenetically-evoked EPSCs (p = 0.03) but did not influence amplitude (p = 0.81). Expression of wild-type VAChT also increased EPSC duration (p = 0.02), again with no effect on amplitude (p = 0.49). All data points are mean ± SEM, n is stated in each bar. *p ≤ 0.05, **p ≤ 0.01.

Figure 2. Expression of VAChT12Q increases optogenetically-evoked EPSC duration. A, Traces of EPSCs recorded from L3 aCC/RP2 in control (chaB19>ChR2) versus experimental (chaB19>ChR2; VAChT or chaB19>ChR2; VAChT12Q) conditions. EPSCs shown are composite averages derived from 9, 6 and 9 cells respectively. Scale bar: 50 pA/500 ms. B, Paired-pulse stimulations, at 1 Hz, show the presence of VAChT12Q enables presynaptic release to resist run-down that occurs in the control (p = 0.007). This is not seen in wild-type VAChT expression (p = 0.13). This effect is abrogated when the second stimulus is applied at 0.05 Hz (p = 0.92). C, Expression of VAChT12Q increased duration of optogenetically-evoked EPSCs (p = 0.03) but did not influence amplitude (p = 0.81). Expression of wild-type VAChT also increased EPSC duration (p = 0.02), again with no effect on amplitude (p = 0.49). All data points are mean ± SEM, n is stated in each bar. *p ≤ 0.05, **p ≤ 0.01.

A homozygous knock-in of VAChT14Q produces viable larvae. However larval development ceases during L1 after which lethality occurs. Recordings from late stage 17 embryonic aCC/RP2 motoneurons, in homozygous VAChT14Q, shows no obvious effects to either mEPSC amplitude (4.0 ± 0.2 vs 4.5 ± 0.3 pA, control vs VAChT14Q, respectively, p = 0.37) or frequency (26.1 ± 4.8 vs 15.1 ± 5.3 per min, control vs VAChT14Q, respectively, p = 0.24; Fig. 3A,B). Unexpectedly, we did observe a change to endogenous SRC kinetics. Specifically, SRC duration was increased (411.7 ± 38.4 vs 627.9 ± 44.5 ms, control vs VAChT14Q, respectively, p = 3 × 10⁻³), and frequency reduced (22.4 ± 4.7 vs 8.3 ± 1.6 per min, control vs VAChT14Q, respectively, p = 0.01). SRC amplitude was not affected (20.2 ± 2.8 vs 19.6 ± 4.9 pA/pF, control vs VAChT14Q, respectively, p = 0.91; Fig. 4A,B). The lack of effect to mEPSC amplitude suggests that the number of glutamines in the polyQ region is a more important deter-
minant, rather than the presence or absence of this region.

**Discussion**

We report neurophysiological consequences arising from the manipulation of the C-terminal VAChT polyQ region. We find, in agreement with previously published literature, that the presence of VAChT12Q (i.e., truncating the polyQ region by one glutamine) increases both amplitude and frequency of mEPSCs at identified central cholinergic synapses. This increase in ACh loading may explain the increased duration in evoked SRCs also observed. Conversely, we further show that a CRISPR-induced single amino acid extension of this region (13Q to 14Q) results in reduced amplitude and frequency of mEPSCs and an associated inability to support evoked release. Finally, CRISPR mediated deletion of the polyQ region (13Q to 0Q) does not affect mEPSC kinetics showing elongation or truncation of the polyQ region is more detrimental to cholinergic release than removal of this region. This work highlights the VAChT polyQ region as an important determinant mediating cholinergic release in *Drosophila*.

It is notable that although mEPSC amplitude is increased following expression of VAChT12Q the effect to SRCs is limited to increased duration. We speculate that this may be indicative that the postsynaptic nAChR receptor field is already fully saturated under endogenous conditions and heightened cholinergic tone, through VAChT12Q upregulation, is thus restricted to increasing SRC duration. Similarly, we can only speculate on why increased SRC duration is accompanied by a decrease in SRC frequency. A possible explanation is a homeostatic-type negative feedback mechanism which acts to dampen the activity of presynaptic interneurons that form the central pattern generator controlling locomotor output. Future experiments will be required to clarify these issues.

Our results suggest that the length of the polyQ domain is both deterministic for SV filling and for probability of SV release. Reducing glutamines by one residue is sufficient to increase SV load and release probability and vice versa. Moreover, addition of a glutamine (14Q) is sufficient to remove the ability of the CNS to generate a rhythmic fictive locomotor pattern, which is reliant on evoked release. We rationalize that VAChT14Q disrupts cholinergic loading, generating partially-filled SVs that, in turn, prevent evoked synaptic release. By contrast, increasing SV loading (12Q) results in evoked release events of longer duration. These observations are in agreement with recent work using a light activated vATPase pump (pHoenix) localized to SVs (Rost et al., 2015). Rost and colleagues used this tool to show that glutamatergic vesicles are only ‘nearly full’ under normal conditions (i.e., can be further filled) and, moreover, show vesicle load is proportional to release probability (Rost et al., 2015). Our data are supportive of this observation: only increased SV loading supports evoked release. Moreover, our results are also indicative of a set point model, in which vesicles can only release once they surpass a threshold load. This hypothesis, proposed by Williams in 1997, proposed two distinct models of SV loading. The set-point model proposes a mechanism restricting the amount of neurotransmitter per

Figure 3. VAChT polyQ manipulation alters spontaneous neurotransmission. A, Representative traces of mEPSCs recorded from embryonic late stage 17 aCC/RP2 between control, VAChT14Q, and VAChT12Q. Scale bar: 3 pA/30 ms. B, VAChT14Q mutants display significantly reduced mEPSC amplitude (p = 0.005) and frequency (p = 0.005). However, no obvious difference in mEPSC kinetics is observed in VAChT13Q mutants for either amplitude (p = 0.37) or frequency (p = 0.24). All data points are mean ± SEM, n stated in each bar.
vesicle to a fixed maximum, whereas, the steady state model suggests the amount of neurotransmitter that enters a SV is offset by leakage, but that both are independent variables that can autonomously change to produce SVs with variable levels of filling (Williams, 1997). The set point model is consistent with observations at the snake NMJ and Drosophila central neurons (Parsons et al., 1999; Cash et al., 2016). Whereas, the steady state model better describes loading at murine and Drosophila NMJ and in mammalian cell culture models (Varoqui and Erickson, 1996; Song et al., 1997; Daniels et al., 2004; Wilson et al., 2005; Prado et al., 2006; De Castro et al., 2009; Lima et al., 2010).

Analysis of related Drosophila spp. reveal polyQ regions of differing lengths (e.g., nine in D. willastomi, 11 in D. simulans, and 15 in D. pseudoobscura). It is tempting to speculate that evolution may have manipulated the length of the polyQ region to alter SV content in these related species. However, recordings from aCC/RP2 in these related species show mEPSC amplitude is remarkably conserved (S. W. Vernon and R. A. Baines, unpublished data). Thus, the predicted effect of SV loading due to change in polyQ length, across these related species, may have been abrogated by compensatory mutations in other regions of the VACHT. A comparative analysis may thus be useful to identify such regions for future study.

The VACHT polyQ region is specific to insects. A BLAST search comparison shows no other insect neuronal vesicular transporter possesses a C-terminal polyQ domain (S. W. Vernon and R. A. Baines, unpublished data). Mammalian VACHT possesses a di-leucine motif in the same approximate location to the insect polyQ domain. The di-leucine motif is well established as a trafficking region (Bonifacino and Traub, 2003). Removal of the mammalian VACHT C-terminal tail, or specific mutation of the di-leucine motif, results in mislocalization of the transporter to the neuronal membrane (Colgan et al., 2007). Mutant Htt protein containing a polyQ expansion from 20Q to

Figure 4. VACHT polyQ manipulation alters evoked neurotransmission. A, Representative traces of SRCs recorded from aCC/RP2 between control, VACHT14Q, and VACHT14Q. Scale bar: 50 pA/300 ms. Data points are mean ± SEM, n stated in each bar. B, VACHT14Q mutants lack any observable SRCs. By contrast, VACHT14Q mutants show SRCs with no observable change in amplitude (p = 0.91). However, VACHT14Q mutants exhibit increased SRC duration (p = 0.003) and reduced SRC frequency (p = 0.01). Control: C, Representative traces (from a total of four experiments) of ChR2ChETA evoked EPSCs recorded from RP2 between control (upper trace) and VACHT14Q (lower trace). Scale: 50 pA/300 ms (upper), 2 V/300 ms (lower).
120Q was found to preferentially bind to SVs in murine axon terminals and, further, to displace the binding of Huntington-associated protein (HAP1) usually co-localized to SVs (Li et al., 2003). 120Q mutants were also shown to reduce glutamate release suggesting a direct interaction between extended polyQ domains and synaptic release (Li et al., 2003). HAP1 has also been shown to bind synapsin 1 (Mackenzie et al., 2016) which is critical for SV pool mobilization and formation (Rosalh et al., 1995; Akbergenova and Bykhovskaia, 2010). We therefore theorize that the polyQ region in VACHT may play a similar role in trafficking the transporter to the SV, plasma membrane and/or SV pool formation.

It is notable that complete removal of the VACHT polyQ region does not influence mEPSCs, although does alter SRC kinetics (increasing their duration). This dichotomy may mirror an increasingly accepted molecular distinction between spontaneous (mEPSCs) and synchronous (SRC) release modalities (Sara et al., 2005; Ramirez and Kavalali, 2011; Kavalali, 2015). Other work has shown, for example, that mEPSC release is maintained in the absence of the vesicle associated SNARE protein synaptobrevin, while evoked release is halted (Schoch et al., 2001). Munc-13 has also been shown to influence the spatial localization of evoked release while having no effect on mEPSCs at C. elegans NMJ (Zhou et al., 2013). These observations are predictive of a model in which multiple fusion complexes are physiologically separate and dependent on the modality of release. Moreover, a role for VACHT in SV release is indicated by a reported interaction between synaptobrevin and VACHT. A glycine to arginine substitution (G342R) in VACHT is sufficient to reduce cholinergic mediated larval motility in C. elegans, an effect that is rescued by a complimentary substitution of an isoleucine to an aspartate in synaptobrevin (Sandoval et al., 2006).

VACHT12Q mutants show early larval mortality (L1) despite being able to produce SRCs. This is further confused by the similarity in SRC kinetics with chaB19 > VACHT12Q which produce viable L3 larvae and adults. We attribute early VACHT12Q mortality to the lack of wild-type transporter present in the VACHT14Q genetic background and may be consistent with cholinergic deficiencies presented in wider physiologic function. In humans, ChAT immunoreactivity and nACHR/mACHR expression is observed in non-neuronal epithelial, endothelial, mesothelial and immune cells (Wessler and Kirkpatrick, 2008) and are shown to modulate multiple cellular processes including but not exclusive to, cellular migration and apoptosis (Grando et al., 2006), proliferation (Metzen et al., 2003), anti/proinflammatory responses (Shytte et al., 2004; Pavlov and Tracey, 2005) and histamine release (Reinheimer et al., 2000; Wessler and Kirkpatrick, 2008). In insects, non-neuronal ACh has been shown to be heavily influential in reproduction and larval development (Wessler et al., 2016; Wessler and Kirkpatrick, 2017) and so it remains possible that VACHT modulation may alter wider, and currently unknown, physiologic aspects of larval development.

The effects we report here relating to expression of VACHT12Q (truncation) versus VACHT14Q (expansion) were achieved using different experimental conditions. VACHT12Q was tested using Gal4-based overexpression in an otherwise wild-type VACHT background, while VACHT14Q was tested using a CRISPR mutant. This was because our attempt to make VACHT12Q via CRISPR was unsuccessful. Thus, the results we report here must be tempered. Indeed, the co-presence of wild-type VACHT in VACHT12Q upregulation may, to some extent, reduce the observed phenotype. Moreover, protein level, nor protein localization, was measured and thus the possibility remains that the VACHT14Q mutation may affect expression levels and/or vesicular localization, which makes it difficult to reach firm conclusions about results obtained. However, we do not believe this detracts from the interpretation of the data presented within this study.

Since the first demonstration of fixed quanta that describes spontaneous release of SVs, a key question of “how does a SV know when it is full” remains to be answered. The polyQ region of the Drosophila VACHT, that we report here, seemingly orchestrates the filling of cholinergic SVs at central synapses. Future studies to identify the function of this region, including identification of binding partners, provide optimism for understanding how SVs monitor their fill state.

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