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Immunolocalization of the vesicular acetylcholine transporter in larval and adult *Drosophila* neurons

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Abstract

Vesicular acetylcholine transporter (VACHT) function is essential for organismal survival, mediating the packaging of acetylcholine (ACh) for exocytotic release. However, its expression pattern in the *Drosophila* brain has not been fully elucidated. To investigate the localization of VACHT, we developed an antibody against the C terminal region of the protein and we show that this antibody recognizes a 65KDa protein corresponding to VACHT on an immunoblot in both *Drosophila* head homogenates and in Schneider 2 cells. Further, we report for the first time the expression of VACHT in the antennal lobe and ventral nerve cord of *Drosophila* larva; and we independently confirm the expression of the protein in mushroom bodies and optic lobes of adult *Drosophila*. Importantly, we show that VACHT co-localizes with a synaptic vesicle marker in vivo, confirming previous reports of the localization of VACHT to synaptic terminals. Together, these findings help establish the vesicular localization of VACHT in cholinergic neurons in *Drosophila* and presents an important molecular tool with which to dissect the function of the transporter in vivo.

Keywords

Acetylcholine; VACHT; Synaptic vesicle; Neurotransmitter; Localization

INTRODUCTION

Acetylcholine (ACh) is essential for a variety of critical functions ranging from learning to the innervation of musculature. ACh is synthesized and regulated by a machinery that includes choline acetyl transferase, choline transporter and the vesicular acetylcholine

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transporter (VACHT) [1–7]. Once synthesized in the presynaptic neuron, ACh must be packaged for exocytotic release by VACHT. VACHT belongs to the SLC18 class of vesicular neurotransmitter transporters that includes the vesicular monoamine transporter (VMAT) [8]. VACHT is critical for organismal survival in flies, mice and worms [3, 5, 7]. In mammals, VACHT is expressed in cholinergic cells in the CNS including the basal forebrain and striatum, and peripherally at the neuromuscular junction [7, 9]. In flies, VACHT expression has been observed in Kenyon Cells (KCs) where the protein is involved in the regulation of aversive olfactory learning [10]; and in monopolar neurons of the optic lobes in *Drosophila* [11]. Despite these reports, gaps remain in the current understanding about the expression of VACHT in the adult and larval brain; and the consequences of this expression pattern for the neuronal function of VACHT.

Here we sought to determine the expression of VACHT in the nervous system of both adult and larval *Drosophila* using our new anti-*Drosophila* VACHT antibody. Designed against a peptide sequence at the transporter's C-terminus, this antibody recognizes a 65KDa band on an immunoblot that corresponds to VACHT. Importantly, we find that VACHT is expressed in mushroom body KCs and in the lateral horn. VACHT co-localizes with cysteine string protein (CSP2), a synaptic vesicle marker, and is also expressed in cholinergic neuronal processes in both larval and adult CNS. Together these findings confirm localization of VACHT in cholinergic neurons in *Drosophila* and expands upon our understanding of the expression of VACHT in neurons.

MATERIALS AND METHODS

Drosophila strains and culture conditions

Fly stocks *w¹¹¹⁸CS₁₅*; *w*; *ChAT-Gal4,UAS-GFP* (ChAT-GFP, BDSC stock # 6793); *OK107-Gal4,UAS-GFP* and the *GH146-Gal4,UAS-GFP* [12] stocks were used for the immunohistochemistry assays. All stocks were maintained on standard corn meal media and raised at 25°C at a relative humidity of 50% in a 12 hour light dark cycle.

Plasmid constructs

To generate Myc-tagged VACHT, a Myc sequence GAACAGAACTGATCTCTGAAGAAGAC CTG was sub-cloned into the first luminal loop of pUAST-VACHT (gift from T. Kitamoto) consistent with a strategy previously described for VMAT [13] by site-directed mutagenesis using the QuikChange Lightning kit (Agilent Technologies, Carlsbad, CA). Untransfected cells were used as negative control. This construct was then cloned into the pUAST::attB backbone [14, 15].

S2 cell culture and transfection

Drosophila Schneider 2 (S2) were handled and transfected as described previously [13] with modifications. S2 cells and Schneider II media were purchased from Drosophila Genomics Resource Center (Bloomington, IN) and cultured in the presence of 0.5% pen/strep (Gibco, Thermo Fisher Scientific, Waltham, MA) and 10% Normal Goat Serum (Invitrogen, Santa Clara, CA). Cells transfected with pUAST::attB-VACHT and either Actin-Gal4 or pMT-Gal4 plasmids when they reached a confluency of 70–80% using Fugene® 6 (Promega, Madison,

WI). Immunocytochemistry was carried out 24 hours after induction to verify the expression of Myc and VACHT.

Western blotting

Peptide titration—Western blots were performed as described previously [16] with some modifications (see Supplementary Methods). The peptide sequence used to generate the antibody was serially-diluted from a stock that was maintained at an 8:1 molar ratio between peptide and antibody. This stock concentration was then serially diluted to 0.5x, 1x, 2x and 4x concentrations relative to the antibody. The diluted peptides were allowed to mix with anti-VACHT prior to incubation with fly head homogenates that had been transferred unto the PVDF membrane. Anti-rabbit and anti-mouse HRP conjugated secondary antibodies (Cell Signaling, Danvers, MA) were used at a dilution of 1:2000 for primary antibody detection at room temperature for 2 hours. Protein bands were visualized with an ECL substrate solution (BioRad, Hercules CA). Images were developed by exposing the PVDF membrane to a western blot scanner (LI-COR Biosciences Lincoln, NE).

S2 cells—Plasmids containing VACHT and the Gal4 driver were co-transfected into S2 cells and incubated for 36 hours. Cell were then lysed with SDS sample buffer (New England Biolabs, Ipswich, MA) and immunoblots performed as described above. Anti-Myc antibody (Cell Signaling, Danvers, MA) was used at a 1:1000 dilution.

Immunostaining and Confocal microscopy

Immunohistochemistry—Staining was performed as described previously [16] using adult or 3rd instar larval brains of experimental flies. Following staining, brains were mounted with ProLong® Gold (Thermo Fisher Scientific Waltham, MA) and confocal imaging was performed using Olympus FV10i (Center Valley, PA) or the Zeiss 880 Two-photo confocal microscope (Jena, Germany). The image was processed using Adobe Photoshop (San Jose, CA). *Immunocytochemistry*: Transfected and untransfected S2 cells were plated onto a poly-D-lysine (Sigma, St. Louis, MO) coated glass cover slips for 2 hours and fixed with 4% PFA and subsequently washed and stained using with anti-VACHT and anti-Myc primary antibodies at 1:1000 dilution; anti-CSP2 was incubated at 1:1000 (unless stated otherwise) (see Supplementary Methods for detailed procedure). Imaging was performed as described above.

RESULTS

Drosophila anti-VACHT antibody detects VACHT protein

To further understand the function of VACHT, we determined the expression pattern of the protein in vivo. A review of the literature indicates that *Drosophila* VACHT is expressed in mushroom body KCs [10] and monopolar neurons of the optic lobe [11]. However, these studies were narrow in scope. Performed in adults, they did not determine the expression of VACHT outside of the mushroom body and optic lobes. To enable us to determine the different areas of the brain where VACHT is expressed, we generated an anti-VACHT antibody capable of detecting VACHT both in cell culture and in vivo. The other *Drosophila* anti-VACHT available was developed against a large C terminal epitope [4]. In our hands, we

observed a broader expression pattern for VACHT than we expected and we sought to verify this staining pattern. We selected a unique 15-amino acid sequence in the C terminal region of VACHT (Fig. 1A) and we used this sequence to generate the antibody (Genscript, Piscataway, NJ). The affinity-purified antibody was raised using two rabbits; a cysteine residue was added to the C terminus of the peptide which was then conjugated to KLH. To test for antibody specificity, we first eliminated the possibility of a non-specific secondary antibody binding (Fig. 1 in [17]). We then performed a peptide competition experiment (Fig. 1B) in [17]) using a serial dilution of concentrations of the purified antibody epitope at 0.5x, 1x, 2x and 4x peptide concentrations relative to the antibody. Antibody binding was reduced and nearly completely abolished at 4x concentration (Fig. 1B). We note that complete elimination of the signal was challenging, presumably due to the low stability of the peptide once aliquoted. However, we were able to achieve complete inhibition of antibody binding to fly head protein when we prepared it directly from the freezer stock (not shown). We obtained similar results in whole mount brains albeit at 1x peptide (see Fig. 2 in [17]).

Following the peptide experiment, we further confirmed that the antibody detected *Drosophila* VACHT. Immunoblot assays in S2 cells for VACHT reveal a band of ~65KDa consistent with the expected size for VACHT (Fig. 1C). We assessed the expression pattern of this antibody by transfecting S2 cells with UAS-Myc-VACHT (Fig 1A) following a previous successful tagging strategy we and others have deployed for VMAT, a closely-related transporter [13, 16]. Consistent with the immunoblot results, we observed both strong anti-Myc (representative of VACHT) and anti-VACHT staining with complete overlay (yellow signal, Fig. 1D). The staining pattern was also punctate (short arrows, Fig. 1D) both near the plasma membrane and internally, akin to endocytic vesicles. We directly compared anti-VACHT staining in VACHT-transfected and untransfected cells and found little to no VACHT staining in the untransfected cells (not shown). Together, these results indicate that anti-VACHT antibody detects a protein corresponding to the mass of VACHT on western blot. Moreover, the complete co-localization of a Myc-tagged VACHT represents an independent verification of the ability of the anti-VACHT antibody to bind to its antigen. We also performed an in vivo validation study by staining for VACHT in two independent ChAT-Gal4/UAS-VACHT-RNAi constructs using our antibody, and found that VACHT expression was dramatically reduced in both VACHT RNAi constructs (Fig. 3 in [17])

Expression of VACHT in larval and adult nervous system

We next tested the usefulness of the new VACHT antibody in vivo. Expression of VACHT in the brain of *Drosophila* has been described most recently for MBs [10] and for the localization of VACHT in the lamina [11]. In our studies using ChAT-Gal4 [11, 18] to label cholinergic neurons, we also found that VACHT is expressed strongly in the lamina (large arrowheads in Fig 2A) (for description of *Drosophila* laminal architecture, see [11]). We also found that VACHT co-localizes with ChAT-GFP in distinct synaptic vesicle-like punctae (small arrowheads in Fig. 2A). By contrast, VACHT is not expressed in the cell bodies of laminal neurons (long arrows in Fig. 3A), but rather in synaptic processes, boutons and arborizations (Fig. 2A, short arrows in inset) consistent with expression reported previously for VACHT [11]. We extended our analysis of VACHT beyond [11] and we show that VACHT is also expressed in the medulla (Fig. 2A) and co-localizes with ChAT-GFP at synaptic

boutons and arborizations (inset in Fig. 2A). VACHT also appears to be expressed in the mushroom body, showing some co-localization with ChAT-GFP (long arrows in Fig. 2B; short in Fig. 2B inset).

Next, we stained the brains of 3rd instar larvae of ChAT-GFP flies, and in contrast to the adult where we see little colocalization in ChAT-GFP+ cell bodies, we find that VACHT is expressed in a subset of cholinergic neurons in the larval CNS (inset in Fig 2C, arrowhead vs. short arrow), distinct from an adjacent population of ChAT-GFP+ neurons (Fig. 2C, long arrow vs. arrowhead). We also observe VACHT expression along nerve tracks of the ventral nerve cord (VNC) where it is localized along cholinergic nerve fibers in discrete punctae (Fig. 2D, short arrows in inset). To our knowledge, this is the first time that VACHT expression has been reported in the nervous system of *Drosophila* larva.

To more clearly determine the areas of VACHT expression in the brain, we used another reporter construct, *GH146-Gal4,UAS-GFP* (GH146-GFP) that marks projection neurons (PNs), a sub-population of cholinergic neurons [12, 19, 20]. These neurons maintain their cell body in the olfactory lobe; send dendrites to the antennal lobe, and their axons to the mushroom body and the lateral horn [21, 22]. Interestingly, we found VACHT expression in both the projection neurons and the KCs that they come into contact with (short arrows in Fig. 3A). In addition, there were VACHT+ cells that did not express GH146-GFP (long arrow in Fig. 3A), reflecting the presence of an ACh neuron population distinct from projection neurons. We also found that VACHT co-localizes with GH146-GFP in the lateral horn (small arrowhead in Fig. 3A). Examination of the antennal lobe in the 3rd instar larval of GH146-GFP flies co-stained with anti-VACHT shows striking colocalization in most but not all glomeruli (short vs. long arrows in Fig. 3B). VACHT also co-localizes with the cholinergic neuron processes in the larval VNC; in particular, along axonal processes of that nerve cord (Fig. 3C). Our observation about a possible expression of VACHT in MBs was intriguing and we tested the possibility directly by staining for VACHT in OK107-GFP constructs (which label MBs) and found that VACHT is expressed in some KCs. (Fig. 4). This restricted expression differs somewhat from a previous report that shows a broader expression of VACHT in MBs [10]

Localization of VACHT to synaptic terminals

The localization of VACHT to synaptic vesicles is firmly established in rodent literature [9]. To verify that this is also the case in flies, we co-stained the VACHT antibody with anti-CSP2, a marker for synaptic vesicles in *Drosophila* [14, 16, 23]. Both VACHT and CSP2 co-localize to synaptic boutons in the lamina (Fig. 5A, short arrow in inset). A closer examination of the lamina shows that anti-VACHT (and anti-CSP2) stain distinct layers of the lamina (Fig. 5B, arrowheads vs. long arrow); both proteins co-localize strongly in synaptic boutons and along laminal neuronal processes (short arrows in Fig. 5B). Our staining suggests that VACHT is expressed in multiple monopolar neurons subtypes [11], and is intense at synaptic boutons formed both at the base and at the tip of the lamina. The presence of VACHT at synaptic terminals in the mushroom body is also confirmed by colocalization with anti-CSP2 (Fig. 5C) consistent with previous reports [10].

DISCUSSION

Studies in rodents have shown the expression of VACHT in the brain [24, 25]. However, until now, few reports have analyzed the expression of VACHT across multiple regions in the brain of *Drosophila*. We show that VACHT is expressed in the medulla in discrete punctae in adult flies. Moreover, our results also demonstrate for the first time that VACHT is expressed in the CNS and VNC of *Drosophila* larva. Importantly, we confirm previously published reports that VACHT is expressed in optic lobe lamina and in the mushroom body in adult *Drosophila*. Together, these findings delineate the expression of VACHT in the adult and shows for the first time its expression during *Drosophila* larval development.

Expression of VACHT in the *Drosophila* nervous system

Studies in mice have explored the function of VACHT in animal behavior and physiology [7, 26]. Other groups using the rodent model have exploited VACHT as a marker for cholinergic neurons without studying its role in neuronal function per se [24]. By contrast, only a handful of studies in *Drosophila* have directly assessed VACHT expression in cholinergic neurons [10, 11]. Indeed, we find that our antibody is consistent with those prior studies of neuronal VACHT expression [10, 11]; labeling cholinergic neurite projections in the lamina and forming punctae at both the basal and the top of the lamina. The identity of these neurons have been established anatomically to be L4 monopolar and Cha-Tan neurons [11]. Moreover, we confirm that VACHT is expressed both in the KCs and in the PN axons that innervate them. In addition, we report the intriguing finding that VACHT is also expressed in the lateral horn which together with MBs form higher olfaction centers in the fly brain. The expression of VACHT in this area provides spatial evidence for functional studies describes by others of a key role for ACh release in synaptic physiology [12, 27] and behavior [10] in the olfactory circuit. Importantly, our confirmation of VACHT expression in MBs contributes to helping to resolve a long standing debate about whether or not KCs themselves are cholinergic [10, 28, 29].

In larvae, the antennal lobe is among the most striking structures labelled by the anti-VACHT antibody (Fig. 4B). To our knowledge, this is the first report of the labeling of VACHT in the antennal lobe during development. Moreover, the presence of VACHT along nerve tracks of the midline in the abdominal segment has also not been described previously. The punctae-like expression of VACHT is reminiscent of the expression of synaptotagmin along the ventral nerve cord in *Drosophila* embryo and larvae [30]. From these findings, we suggest that this antibody could serve as a marker for cholinergic neurons in the optic lobe, antennal lobe and projection neuron nerve terminals.

Co-staining of anti-VACHT and anti-CSP2 confirmed the synaptic terminal localization of VACHT. Localized both along the axons and in distinct synaptic terminal sites, both antibodies strongly mark laminal neuron projections; an unsurprising finding, giving the role of ACh in the function of the photoreceptor cells of the visual system [11, 31, 32]. VACHT appears to preferentially stain neurite projections and synaptic sites while ChAT-GFP labels cell bodies more strongly than it does axonal processes. This data is in agreement with reports in rodents that VACHT more reliably labels synaptic terminals than does ChAT [33]. We note that VACHT does not co-localize with ChAT-GFP in several instances and speculate

that this is due to the limitation of ChAT-GFP as a marker for ACh neurons [32]. Similarly, we note that there appeared to be more localization of VAcHT to cholinergic cell bodies in the larvae than in the adult. This phenomenon may be due to the time it takes for synaptic vesicles to mature and migrate to the terminal [34].

CONCLUSION

We describe the expression of VAcHT in the brain of larval and adult *Drosophila*. Our finding that VAcHT is expressed in MBs provides support for the idea that ACh is the neurotransmitter released by MBs, contributing to the resolution of a long standing controversy. Moreover, strong expression of VAcHT in the larval antennal lobe suggests that the key role for cholinergic neurotransmission that has been described in the adult is likely to also occur in during larval development. Our efforts have been aided by the generation of a VAcHT antibody which we suggest as a marker for cholinergic neurons in the delineation of the neuronal function of VAcHT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- VACHT is localized to memory centers in the adult *Drosophila* brain
- Novel expression of VACHT in the CNS and ventral nerve cord of the larval brain
- Useful marker for cholinergic neurons in the optic and antennal lobes

Figure 1. Generation of anti-VACHT antibody and Myc-VACHT construct

(A) Schematic showing regions on the predicted transmembrane structure where the Myc tag (red) and antibody epitope (multicolored sequence) were generated. (B) Peptide competition experiment. Total protein from 8 adult fly heads was distributed into 5 lanes and subjected to SDS-PAGE and immunoblotted (see Methods). Increasing concentrations of the peptide eliminates anti-VACHT antibody binding; *Drosophila* neuronal marker CSP2 (42KDa) served as an internal control. (C) VACHT is detected in S2 cells using either anti-Myc antibody or anti-VACHT. (D) Immunocytochemistry in S2 cells shows that VACHT (green) and Myc (red) both detect Myc-tagged VACHT. Colocalization (yellow, overlay) is evident; an untransfected cell (arrowhead) is depicted. Each micrograph represents a single optical confocal slice. Antibody concentrations for the immunoblot were: anti-Myc 1:1000; anti-VACHT 1:1500; anti-CSP2 1:8000. Scale bars, 10 μm.

Figure 2. Expression of VAcHT in the adult and larval *Drosophila* brain

(A) VAcHT is expressed in the optic lobes. Cholinergic neurons are labelled with ChAT-GFP (green), and VAcHT is detected using the anti-VAcHT antibody (red). VAcHT is strongly expressed in the lamina (arrowhead depicts punctae) and the medulla (co-localizing with ChAT-GFP at synaptic sites, small arrowheads). VAcHT does not localize to cholinergic cell bodies (long arrows). (B) VAcHT localizes to punctae in the CNS and co-localizes with ChAT-GFP at points distal from the cholinergic neuron cell body (short arrows in inset). VAcHT also appears to label mushroom body cells (arrowhead). (C) VAcHT localizes to a sub-population of cholinergic neurons (short arrow in inset) but not in others (arrowhead in inset) and is expressed in some cell bodies long arrows). (D) VNC of 3rd instar larvae stained for anti-VAcHT shows VAcHT expression along ChAT+ nerve tracks (short arrows). VAcHT is expressed as discrete punctae along the nerve track (long arrows). Cholinergic neuron cell bodies are localized proximal to the nerve tracks (arrow head on left panel). Each micrograph represents a single optical confocal slice. Scale bar, 20µm.

Figure 3. VACHT is expressed in a subset of cholinergic projection neurons in adult and larvae
 (A) Brains of adult *GH146-GFP*, a marker for cholinergic projection neurons, PN (green) are co-stained for VACHT (red). Co-localization is seen between VACHT and PNs in the adult CNS. VACHT+ PNs also form synapses with Kenyon Cells (KCs) which do not express GFP (short arrows in inset). Some cells do not make any contact with PNs (long arrow). (B) Extensive colocalization (yellow, short arrows in inset) seen in the several glomeruli in the antennal lobe of the 3rd star larva; some glomeruli stain for VACHT but not for PNs (long arrow in inset). The nerve bundle of the glomeruli do not stain for VACHT (arrowhead) (C) Ventral nerve cord of the 3rd instar larva also express GH146-GFP in neuronal processes along the nerve track of the VNC (short arrow on left panel). VACHT co-localizes with this marker along portions of the nerve (short arrow). Several VACHT punctae however do not

co-stain with the subset of ChAT neurons (arrowheads). Each micrograph represents a single optical confocal slice. Scale bar, 20um

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Figure 4. VACHT is expressed in Mushroom Body KCs

(A) *OK107-Gal4;UAS-GFP* which labels MBs (green) co-localizes with VACHT (red) in KCs (short arrows in inset). However a larger number of KCs do not express VACHT (long arrows) (B) Some KCs express VACHT (short arrows) compared to non-VACHT expressing cells (long arrows). Each micrograph represents a single optical confocal slice. Cal, Calyx; Ped, Peduncle; scale bars, 20um.

Figure 5. *Drosophila* VACHT is localized to synaptic terminals in the adult brain

(A) VACHT (green) and the synaptic vesicle marker CSP2 (red) co-localize in the left optic lobe; specifically in the neuronal processes in a cross-section of the lamina (short arrows in inset). (B) Clear labeling of laminal neurons is apparent. VACHT and CSP2 co-localize strongly in the synaptic boutons of laminal neurons (short arrows in inset). (C) VACHT expression in the adult CNS is wide-spread; it co-localizes with CSP2. Each micrograph represents a single optical confocal slice. Scale bar, 20um.