

## METHODS &amp; TECHNIQUES

# Transgenic line for the identification of cholinergic release sites in *Drosophila melanogaster*

Katarina Pankova<sup>1,2,\*</sup> and Alexander Borst<sup>1</sup>**ABSTRACT**

The identification of neurotransmitter type used by a neuron is important for the functional dissection of neuronal circuits. In the model organism *Drosophila melanogaster*, several methods for discerning the neurotransmitter systems are available. Here, we expanded the toolbox for the identification of cholinergic neurons by generating a new line FRT-STOP-FRT-VAcHT::HA that is a conditional tagged knock-in of the vesicular acetylcholine transporter (*VAcHT*) gene in its endogenous locus. Importantly, in comparison to already available tools for the detection of cholinergic neurons, the FRT-STOP-FRT-VAcHT::HA allele also allows for identification of the subcellular localization of the cholinergic presynaptic release sites in a cell-specific manner. We used the newly generated FRT-STOP-FRT-VAcHT::HA line to characterize the Mi1 and Tm3 neurons in the fly visual system and found that VAcHT is present in the axons of both cell types, suggesting that Mi1 and Tm3 neurons provide cholinergic input to the elementary motion detectors, the T4 neurons.

**KEY WORDS:** VAcHT, Acetylcholine, Neurotransmitter, Motion vision, Mi1 neurons, Tm3 neurons

**INTRODUCTION**

Understanding of the information processing in neuronal circuits requires knowledge about connectivity and properties of the cells involved. The type of neurotransmitter released by a cell defines, to a large extent, the role of a cell and the range of logical operations that are performed within a circuit. Thus, the identification of the cellular neurotransmitter phenotype is of crucial importance for the functional dissection of neuronal circuits.

Various techniques have been described for the identification of neurotransmitter systems in the *Drosophila melanogaster* nervous system. The most common approach is the detection of neurotransmitter molecules (Monastirioti et al., 1995; Yuan et al., 2005; Kolodziejczyk et al., 2008), neurotransmitter-synthesizing enzymes (Takagawa and Salvaterra, 1996; Featherstone et al., 2000; Blanco et al., 2011) or vesicular neurotransmitter transporters (Kitamoto et al., 1998; Daniels et al., 2004; Greer et al., 2005; Romero-Calderón et al., 2008; Fei et al., 2010) with an antibody. In *Drosophila* neurons, the major disadvantage of this strategy is that

either cell bodies or larger neuropil areas are examined for the antibody staining. Because of the small diameter of the neuronal processes, reliable localization of the antibody staining within the individual neurites is beyond the resolution threshold of traditional confocal microscopy. Therefore, the markers that localize to the presynaptic regions and are not present ubiquitously in the cytoplasm or at the cytoplasmic membrane of soma cannot be easily detected in individual neurons.

The second approach for the identification of neurotransmitter phenotype is the detection of mRNA transcripts for neurotransmitter-synthesizing enzymes or neurotransmitter vesicular transporters. The *in situ* hybridization technique has been used to study gene expression mainly in fly embryos but also in other tissues, including the nervous system. Nevertheless, the demanding process of probe optimization poses a challenge and therefore this technique is not routinely used to assess neurotransmitter phenotype. With respect to the specificity and dynamic range, the current method of choice for transcript profiling is RNA-seq of a single cell or a homogeneous population of cells (Henry et al., 2012; Thomas et al., 2012). In addition, other techniques such as RT-PCR or gene expression microarrays have been successfully used to study gene expression in *Drosophila* neurons (Nagoshi et al., 2010; Takemura et al., 2011). Regardless of the specific technique, the cell-type-specific transcriptome profiling requires isolation of labeled somata, nuclei or ribosomes in sufficient quantity and purity, which is labor-intensive. Also, contamination of the analyzed sample with mRNA from other cell types may occur during this process.

The third approach relies on genetic labeling of neurons expressing the neurotransmitter-synthesizing enzymes or neurotransmitter vesicular transporters via insertion of a transgene into 5' UTR or a coding intron of the respective gene (Venken et al., 2011; Diao et al., 2015). When the inserted transgene is a transcription factor of a binary expression system such as Gal4/UAS (Brand and Perrimon, 1993) or LexA/lexAop (Lai and Lee, 2006), the complete expression pattern of a particular gene can be easily identified throughout the whole nervous system. Recently, a set of LexA knock-in lines for the neurotransmitter vesicular transporter genes was generated by ends-out homologous recombination (Simpson, 2016).

Acetylcholine is a major excitatory neurotransmitter in the *Drosophila* nervous system. Synthesis of acetylcholine is catalyzed by the enzyme choline acetyltransferase (ChAT) and its loading into synaptic vesicles is mediated by the vesicular acetylcholine transporter (VAcHT). Currently, the available tools for identification of the cholinergic neurons are ChAT antiserum (Takagawa and Salvaterra, 1996), ChAT Trojan-MiMIC driver lines (Venken et al., 2011; Diao et al., 2015) and VAcHT-LexA knock-in line (Simpson, 2016).

In the present study, we describe a newly generated FRT-STOP-FRT-VAcHT::HA allele for the reporting of the endogenous

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expression of VACHT that not only identifies neurons with the cholinergic phenotype but also provides information about the subcellular localization of the cholinergic presynaptic release sites.

## MATERIALS AND METHODS

### Fly stocks and genotypes

The flies were raised on a standard cornmeal-agar food at 25°C. The following stocks were used: *yw*, *Act5C-cas9*, *lig4* (provided by F. Schnorrer, Max Planck Institute of Neurobiology, Germany) (Zhang et al., 2014), *UAS-FLP* (BDSC 4539 and 8208), *UAS-mCD8::GFP* (BDSC 5137) (Lee and Luo, 1999), *VT25965-Gal4* (T4/T5 line) (provided by B. Dickson, Janelia Research Campus, USA); *R20D01-Gal4* (LPi3-4 line) (BDSC 48889) (Jenett et al., 2012); *VT7747-AD*, *VT49371-DBD* (Mi1 line) (Ammer et al., 2015), *GMRSS00300-split Gal4* (Tm3 line) (provided by A. Nern, Janelia Research Campus, USA), *MB008B* (Aso et al., 2014), *MB112C* (Aso et al., 2014), *UAS-nsyb::GFP* (BDSC 6921) (Zhang et al., 2002) and *Act5C-Gal4* (BDSC 4414).

The genotypes of flies used in this study are detailed in Table 1.

### Generation of the FRT-STOP-FRT-VACHT::HA allele with the CRISPR/cas9 system

The target sites for CRISPR/cas9-induced cleavages in the *VACHT* gene were designed using a web-based software tool (<http://crispr.mit.edu/>; Hsu et al., 2013). The efficiency of individual guide RNAs (gRNAs) was tested in S2 cells stably expressing *cas9* (provided by F. Schnorrer) (Böttcher et al., 2014) as described previously (Zhang et al., 2014). The CRISPR target sites used for genome editing were AGAGGAAGTCCCAAAGAAAG (TGG) and GGGCTATCGAT-ACAATCACG (AGG). The target-specific sequences were cloned into pU6-BbsI-gRNA plasmid (provided by M. Harrison, K. O'Connor-Giles and J. Wildonger; Addgene plasmid 45946) (Gratz et al., 2013) such that the first base of both sequences was replaced by G. The gRNA-expressing plasmids and the donor plasmid for the homology-directed repair were injected into fly embryos of the genotype *yw*, *Act5C-cas9*, *lig4*. The embryo injections were performed by BestGene Inc. (<https://www.thebestgene.com/>).

The donor fragment for the generation of FRT-STOP-FRT-VACHT::HA allele was assembled by PCR fusion of the following

sequences: (1) flippase recognition target (FRT)-flanked cassette containing transcriptional terminator (*hsp70Ab* polyadenylation signal) and the sequence for a screenable eye marker (3xP3-DsRed- $\alpha$ 1tub\_3'UTR), synthesized *de novo*; (2) DNA fragment containing the Kozak sequence followed by an open reading frame (ORF) of the *VACHT* gene with the sequence for the HA tag inserted after the first 14 codons from translational start, synthesized *de novo*; and (3) two 1 kb homology arms flanking the CRISPR cleavage sites, amplified from genomic DNA of *yw*, *Act5C-cas9*, *lig4* flies. The resulting donor fragment consisted of the upstream homology arm fused to the FRT cassette, followed by the Kozak sequence, the ORF with tag sequence and the downstream homology arm. The donor fragment was blunt-end cloned into pJet1.2 vector (Thermo Fisher Scientific). The nucleotide sequence of the HA tag was TAC CCA TAC GAT GTT CCA GAT TAC GCT.

### Immunohistochemistry

Fly brains were dissected in PBS and fixed in 4% PFA with 0.1% Triton X for 25 min. Brains were washed in 0.3% PBT and incubated first with primary (24–72 h) and then secondary (24–48 h) antibodies in 0.3% PBT supplemented with 5% NGS. The brains were mounted in Vectashield mounting medium (Vector Laboratories) and imaged on a Leica TCS SP5 or SP8 laser-scanning confocal microscope. The following antibodies were used: rabbit anti-GFP (Torrey Pines TP401, 1:400), rat anti-HA (Sigma-Aldrich, clone 3F10, 1:100), mouse anti-ChAT (DSHB, deposited by P. Salvaterra, 1:50) (Takagawa and Salvaterra, 1996), goat anti-rabbit Alexa 488 (Thermo Fisher Scientific A-11008, 1:200), goat anti-rat Alexa 647 (Thermo Fisher Scientific A-21247, 1:200) and goat anti-mouse Alexa 647 (Thermo Fisher Scientific A-21235, 1:200).

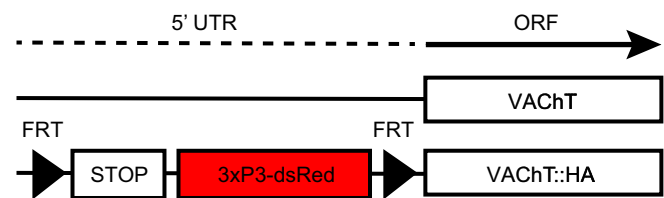
## RESULTS AND DISCUSSION

Using CRISPR/cas9-based genome editing (Jinek et al., 2012; Gratz et al., 2013), we generated a new allele of the *VACHT* gene that carried an additional HA tag. This new allele was positioned in the original genomic locus of the *VACHT* gene and therefore its expression depended on the endogenous regulatory sequences of *VACHT*. The HA tag was placed after the first 14 amino acids from the N terminus, within the cytoplasmic domain of the VACHT protein. The position of the tag was chosen such that it would not interfere with protein folding or signaling sequences known to participate in the intracellular trafficking of VACHT (Fei et al., 2008).

Our aim was to restrict the expression of VACHT::HA to a population of neurons defined by the expression pattern of a Gal4 line of choice. Therefore, we included a transcriptional stop cassette into the 5' UTR of the *VACHT* gene that was flanked by two FRT sites (Fig. 1). The expression of the VACHT::HA was, as a result,

**Table 1. Genotypes of flies used in the study**

Experiment	Genotype
Adult viability	<i>w<sup>-</sup>/w<sup>-</sup></i> ; <i>Act5C-Gal4/UAS-FLP</i> ; FRT-STOP-FRT-VACHT::HA/FRT-STOP-FRT-VACHT::HA
Fig. 2A	<i>w<sup>-</sup></i> ; <i>UAS-FLP/UAS-mCD8::GFP</i> ; FRT-STOP-FRT-VACHT::HA/VT25965-Gal4
Fig. 2B	<i>w<sup>-</sup></i> ; <i>UAS-FLP/UAS-mCD8::GFP</i> ; FRT-STOP-FRT-VACHT::HA/R20D01-Gal4
Fig. 2C	<i>UAS-FLP/w<sup>-</sup></i> ; MB008B-split Gal4/ <i>UAS-mCD8::GFP</i> ; MB008B-split Gal4/FRT-STOP-FRT-VACHT::HA
Fig. 2D	<i>UAS-FLP/w<sup>-</sup></i> ; MB112C-split Gal4/ <i>UAS-mCD8::GFP</i> ; MB112C-split Gal4/FRT-STOP-FRT-VACHT::HA
Fig. 3A	<i>UAS-FLP/w<sup>-</sup></i> ; VT7747-AD/ <i>UAS-mCD8GFP</i> ; VT49371-DBD/FRT-STOP-FRT-VACHT::HA
Fig. 3B	<i>UAS-FLP/w<sup>-</sup></i> ; GMRSS00300-split Gal4/ <i>UAS-mCD8GFP</i> ; GMRSS00300-split Gal4/FRT-STOP-FRT-VACHT::HA
Fig. 3C	<i>UAS-FLP/w<sup>-</sup></i> ; VT7747-AD/ <i>UAS-nsyb::GFP</i> ; VT49371-DBD/FRT-STOP-FRT-VACHT::HA
Fig. 3D	<i>UAS-FLP/w<sup>-</sup></i> ; GMRSS00300-split Gal4/ <i>UAS-nsyb::GFP</i> ; GMRSS00300-split Gal4/FRT-STOP-FRT-VACHT::HA
Fig. 3E	<i>w<sup>-</sup></i> ; VT7747-AD/ <i>UAS-mCD8GFP</i> ; VT49371-DBD/+
Fig. 3F	<i>w<sup>-</sup></i> ; GMRSS00300-split Gal4/ <i>UAS-mCD8GFP</i> ; GMRSS00300-split Gal4/+



**Fig. 1. Original VACHT allele and FRT-STOP-FRT-VACHT::HA allele.** FRT-flanked transcriptional stop signal in the 5' UTR constrains the expression of VACHT::HA. Removal of the stop cassette requires FLP recombinase, introduced by the Gal4/UAS system. Expression of the VACHT::HA is therefore restricted to cells with active endogenous regulatory sequences of *VACHT* that are, in addition, part of the Gal4 expression pattern. The 3xP3-dsRed sequence encodes a screenable eye marker. ORF, open reading frame.



confined to cells that were expressing flippase (FLP) recombinase introduced by the Gal4/UAS system and contained an active endogenous promoter of *VAcHt*.

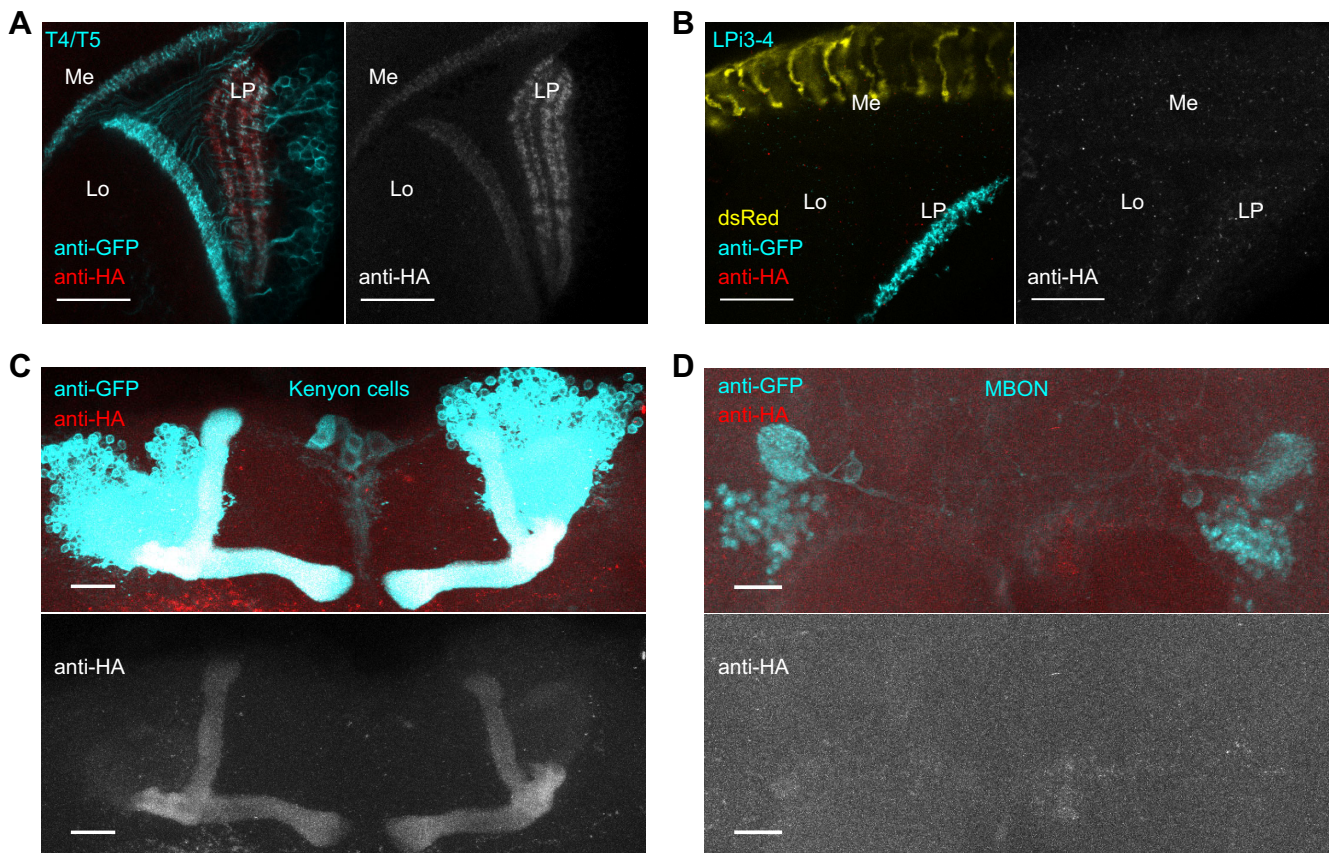
Disruption of both copies of the *VAcHt* gene causes lethality during embryonic or larval development (Kitamoto et al., 2000). We did not observe any adult flies homozygous for the newly generated FRT-STOP-FRT-VAcHt::HA allele, confirming that the stop cassette efficiently disrupts transcription of the VAcHt::HA. When the stop cassette was removed by expressing the FLP recombinase ubiquitously with Act5C-Gal4 driver line, the flies homozygous for FRT-STOP-FRT-VAcHt::HA allele were viable. This suggests that the tagged transporter VAcHt::HA can fully substitute the original VAcHt transporter at the synapse.

To test the functionality of the FRT-STOP-FRT-VAcHt::HA allele, we chose the T4/T5 neurons. T4/T5 neurons are the elementary motion detectors of the fly, sensitive to motion of bright (T4) and dark (T5) edges (Maisak et al., 2013). These cells have been shown previously to synthesize and release acetylcholine (Mauss et al., 2014; Shinomiya et al., 2014). In accordance with these prior findings, we detected VAcHt::HA in the axon terminals of the T4/T5 neurons in the lobula plate (Fig. 2A). A weaker HA signal was present also in the dendrites of T4/T5 neurons in medulla and lobula. This finding is in line with a previous study reporting existence of the dendritic presynaptic release sites in the T4 neurons (Takemura et al., 2013). To show that the expression of VAcHt::HA is absent in non-cholinergic neurons, we looked at the expression of

VAcHt::HA in the LPI3-4 neurons that have been previously identified as glutamatergic (Mauss et al., 2015). As expected, we could not detect any expression of the VAcHt::HA in the LPI3-4 neurons (Fig. 2B).

To demonstrate that FRT-STOP-FRT-VAcHt::HA allele reliably captures the endogenous expression pattern of the *VAcHt* gene in a variety of neuronal populations, we additionally examined the expression of VAcHt::HA in the mushroom body neurons. We detected VAcHt::HA in the Kenyon cells in  $\alpha/\beta$  lobes of the mushroom body (Fig. 2C) that have recently been shown to release acetylcholine (Barnstedt et al., 2016). On the contrary, we did not observe any HA signal in the GABAergic mushroom body output neurons  $\gamma$ 1pedc> $\alpha/\beta$  (Aso et al., 2014) (Fig. 2D).

Mi1 and Tm3 neurons synapse onto dendrites of T4 neurons (Takemura et al., 2013) and are involved in visual detection of the moving bright edges (Behnia et al., 2014; Ammer et al., 2015). Despite the functional characterization of the responses of Mi1 and Tm3 neurons (Behnia et al., 2014) and the reported effects of the synaptic silencing of Mi1 and Tm3 on the motion vision circuit (Ammer et al., 2015), the exact contribution of the Mi1 and Tm3 neurons to direction-selective responses of the T4 neurons is not clear (Maisak et al., 2013; Haag et al., 2016), nor is it known whether the synaptic input that Mi1 and Tm3 neurons provide to T4 neurons is excitatory or inhibitory. Therefore, we employed the newly generated FRT-STOP-FRT-VAcHt::HA allele to investigate the neurotransmitter system used by Mi1 and Tm3 neurons.



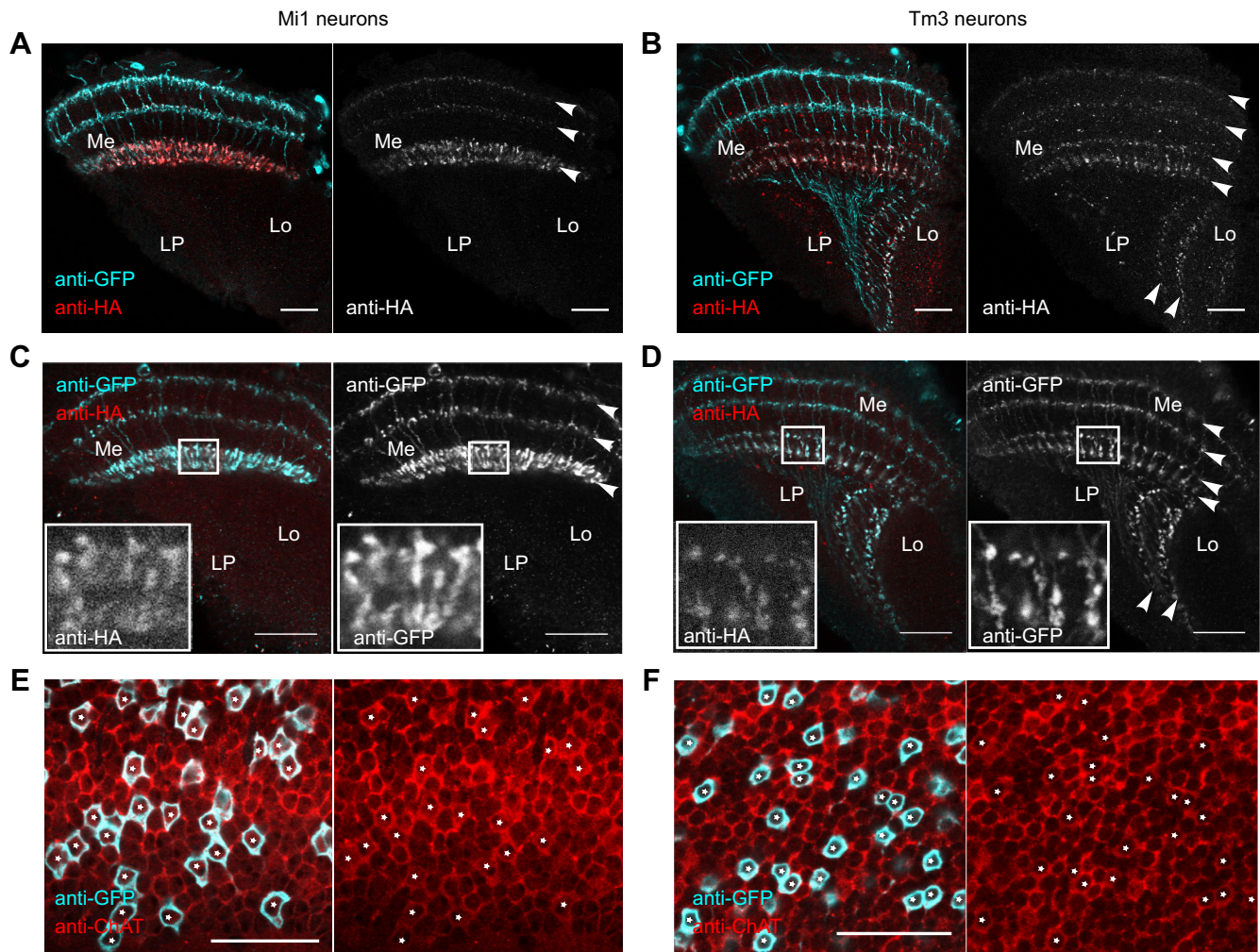
**Fig. 2. VAcHt::HA is detectable exclusively in the cholinergic neurons.** (A) The expression of VAcHt::HA in T4/T5 neurons is localized to the axons in the lobula plate and dendrites in the medulla and lobula. (B) In the LPI3-4 neurons, no expression of VAcHt::HA can be detected. The fluorescence of DsRed in the R7/R8 photoreceptor terminals in the medulla confirms the presence of the FRT-STOP-FRT-VAcHt::HA allele in the fly genome. (C) Kenyon cells in the  $\alpha/\beta$  lobes of the mushroom body show expression of VAcHt::HA. (D) No co-localization of the HA signal and GFP staining in the mushroom body output neurons (MBON)  $\gamma$ 1pedc> $\alpha/\beta$  can be detected. All scale bars: 20  $\mu$ m. Me, medulla; Lo, lobula; LP, lobula plate.



Using the FRT-STOP-FRT-VAcHT::HA line, we identified the Mi1 and Tm3 neurons as cholinergic. The expression of VAcHT::HA could be detected in all medullar and lobular layers where Mi1 and Tm3 neurons laterally extend their neurites (Fig. 3A,B). The VAcHT::HA signal was strongest for both the Mi1 and Tm3 neurons in the medullar layer 9/10, where Mi1 and Tm3 neurons synapse on the dendrites of T4 neurons (Takemura et al., 2013). In order to examine whether the localization of VAcHT::HA corresponds to the presynaptic release sites in the Mi1 and Tm3 neurons, we expressed a marker for presynaptic sites, the GFP-tagged neuronal synaptobrevin (*nsyb::GFP*) (Zhang et al., 2002), in Mi1 and Tm3 neurons. We observed that the subcellular localization of *nsyb::GFP* in both Mi1 and Tm3 neurons shows the same pattern as VAcHT::HA (Fig. 3C,D), confirming that the subcellular distribution of VAcHT::HA corresponds to the presynaptic release sites. The expression of *nsyb::GFP* in Mi1 and Tm3 neurons was stronger than the expression of VAcHT::HA and could be detected also in the neuronal fibers. This is likely due to overexpression of the *nsyb::GFP* transgene with the Gal4/UAS system. To prove the cholinergic phenotype of the Mi1 and Tm3 neurons by another line of evidence,

we stained fly brains with ChAT antiserum (Takagawa and Salvaterra, 1996) and looked at the presence of ChAT immunostaining in the cell bodies of Mi1 and Tm3 neurons. We detected the presence of ChAT immunoreactivity in the cell bodies of both Mi1 and Tm3 neurons (Fig. 3E,F), confirming that Mi1 and Tm3 neurons use acetylcholine as their neurotransmitter.

When using the FRT-STOP-FRT-VAcHT::HA allele, one important aspect to consider is choosing a Gal4 line with as specific an expression pattern as possible. Even very weak expression of the FLP can lead to genomic excision of the FRT-flanked transcriptional stop cassette and, as a result, to expression of the VAcHT::HA. As the expression level of VAcHT::HA depends on the endogenous regulatory sequences and not on the amount of Gal4 molecules present, it may occur that the expression of VAcHT::HA is stronger than that of Gal4-driven GFP. When using a Gal4 line containing cells with various strength of Gal4 expression, we noticed the presence of VAcHT::HA also in the neurons that were barely detectably labeled by GFP. We believe that this is the reason for the unspecific dotted pattern of the anti-HA staining in the optic lobe of the Tm3 line (Fig. 3B).



**Fig. 3. The Mi1 and Tm3 neurons are cholinergic.** The VAcHT::HA can be found in all layers of the medulla and lobula in which Mi1 (A) and Tm3 (B) neurons laterally extend their processes (white arrowheads). The subcellular localization of the presynaptic marker *nsyb::GFP* in the Mi1 (C) and Tm3 (D) neurons (white arrowheads) corresponds to that of VAcHT::HA. The insets in C and D show close-ups of the medulla regions containing presynaptic release sites of Mi1 and Tm3 neurons labeled by VAcHT::HA and *nsyb::GFP*. Anti-ChAT staining co-localizes with the GFP-labeled somatic cytoplasmic membrane of the Mi1 (E) and Tm3 (F) neurons. White asterisks mark the position of the GFP-labeled cell bodies of the Mi1 and Tm3 neurons. All scale bars: 20  $\mu$ m. Me, medulla; Lo, lobula; LP, lobula plate.

The decision about which approach to use for the identification of cholinergic neurons should be based on the driver line inspected. For the Gal4 lines with a narrow expression pattern and the split-Gal4 lines, the FRT-STOP-FRT-VAcHT::HA allele is the tool of choice. When using the FRT-STOP-FRT-VAcHT::HA allele, there is no need for further experiments to determine which neurites contain presynaptic release sites. In contrast, for the Gal4 lines with a broader expression pattern, the examined neurons should instead be tested for co-localization with the expression pattern of the ChAT Trojan-MiMIC driver line (Venken et al., 2011; Diao et al., 2015), the VAcHT-LexA knock-in line (Simpson, 2016) or with ChAT antiserum (Takagawa and Salvaterra, 1996).

A previously reported method for synaptic tagging with recombination using bruchpilot protein as a general marker of presynaptic release sites (Chen et al., 2014) served as an inspiration for the generation of the transgenic allele described in this study. The combination of the conditionally tagged bruchpilot protein and the conditionally tagged VAcHT might enable enumeration of the total presynaptic release sites and cholinergic release sites simultaneously in a single neuron, assuming that a specific and sparse Gal4 line is provided.

#### Acknowledgements

We would like to thank B. Dickson, M. Harrison, A. Nern, K. O'Connor-Giles, F. Schnorrer, J. Wildonger, Bloomington *Drosophila* Stock Center and Developmental Studies Hybridoma Bank for the flies and reagents. Fly embryo injections were performed by BestGene Inc. We would like to thank W. Essbauer and M. Sauter for technical assistance.

#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

K.P. conceived and performed the experiments and wrote the manuscript. A.B. revised the manuscript.

#### Funding

This work was supported by the Max-Planck-Gesellschaft. Deposited in PMC for immediate release.

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