A Neurotransmitter Atlas of the *Caenorhabditis elegans* Male Nervous System Reveals Sexually Dimorphic Neurotransmitter Usage

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ABSTRACT The nervous system of most animals is sexually dimorphic but such dimorphisms are generally poorly mapped on an anatomical, cellular, and molecular level. The adult nervous system of the nematode *Caenorhabditis elegans* displays a number of clearly defined anatomical sexual dimorphisms, but molecular features of sexually dimorphic neurons remain sparse. In this resource paper, we provide a comprehensive atlas of neurotransmitters used in the nervous system of the male and compare it to that of the hermaphrodite. Among the three major neurotransmitter systems, acetylcholine (ACh) is the most frequently used, followed by glutamate (Glu), and lastly γ-aminobutyric acid (GABA). Many male-specific neurons utilize multiple neurotransmitter systems. Interestingly, we find that neurons that are present in both sexes alter their neurotransmitter usage depending on the sex of the animal. One neuron scales up its usage of ACh, another becomes serotonergic in males, and another one adds a new neurotransmitter (glutamate) to its nonsex-specific transmitter (ACh). In all these cases, neurotransmitter changes are correlated with substantial changes in synaptic connectivity. We assembled the neurotransmitter maps of the male-specific nervous system into a comprehensive atlas that describes the anatomical position of all the neurons of the male-specific nervous system relative to the sex-shared nervous system. We exemplify the usefulness of the neurotransmitter atlas by using it as a tool to define the expression pattern of a synaptic organizer molecule in the male tail. Taken together, the male neurotransmitter atlas provides an entry point for future functional and developmental analysis of the male nervous system.

KEYWORDS *C. elegans*; neurotransmitter; sexual dimorphisms

ONE of the most fascinating questions in neurobiology is how the sexual identity of an organism affects the development, structure, and function of a brain. In all organisms in which this problem has been studied to date, sexual dimorphisms in the brain are either evidenced by the presence of sex-specific neurons or by the existence of sex-specific traits of neurons that are shared between both sexes (Yang and Shah 2014).

The lineage analysis and anatomical reconstruction of the nervous system of both sexes of the nematode *Caenorhabditis elegans* has revealed unprecedented insights into sexual dimorphisms of nervous system anatomy (Sulston and Horvitz 1977; Sulston *et al.* 1980, 1983; White *et al.* 1986; Jarrell *et al.* 2012; Emmons 2014; Sammut *et al.* 2015). Apart from 294 sex-shared neurons, hermaphrodites contain eight hermaphrodite-specific neurons while males contain 93 male-specific neurons (Figure 1A). Most male-specific neurons are generated by male-specific divisions of sex-shared blast cells during larval development. The electron micrographical (EM) analysis of the male nervous system has revealed that, apart from the existence of sex-specific neurons, neurons that are shared between both sexes adopt sex-specific traits during sexual maturation. Sex-specific traits of sex-shared neurons include sexually dimorphic axo/dendritic anatomy,

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sexually dimorphic synaptic connections (Jarrell *et al.* 2012; Oren-Suissa *et al.* 2016; Serrano-Saiz *et al.* 2017), and sexually dimorphic expression of molecular markers (Jarrell *et al.* 2012; Ryan *et al.* 2014; Oren-Suissa *et al.* 2016; Hilbert and Kim 2017; Serrano-Saiz *et al.* 2017).

Contrasting the depth of description of male-specific anatomy, the molecular landscape of the mature male nervous system is remarkably unexplored, thereby limiting our understanding of both the function and development of the male-specific nervous system. For example, while genetic specification mechanisms have been identified for ~3 quarters of all neurons of the sex-shared nervous system (Hobert 2016), the development of only a limited fraction of male neurons has been studied (most notably, the ray sensory neurons) (Chow and Emmons 1994; Sutherlin and Emmons 1994; Chow *et al.* 1995; Zhao and Emmons 1995; Portman and Emmons 2000; Lints *et al.* 2004; Jia and Emmons 2006; Yu *et al.* 2009; Siehr *et al.* 2011). To better understand the function and development of sexual dimorphisms in the nervous system, the identification of molecular features of anatomically dimorphic structures in the *C. elegans* nervous system is required. However, compared to the sex-shared and the hermaphrodite-specific nervous system, few genes have been characterized for their patterns of expression in the male-specific nervous system, and no systematic efforts have yet been undertaken to provide a comprehensive and comparative map of sexually dimorphic molecular features of the *C. elegans* nervous system.

We provide here a molecular map of the male nervous system in the form of a neurotransmitter atlas that encompasses maps of individual neurotransmitter systems. A neurotransmitter atlas provides an invaluable resource to explore the development and function of the nervous system. As neurotransmitter usage is a key identity feature of a neuron, a neurotransmitter atlas also represents an entry point for studying how neuronal identity is genetically controlled, *i.e.*, initiated, maintained, and modified. For example, the generation of a map of GABAergic, cholinergic, and glutamatergic neurons of the nervous system of the *C. elegans* hermaphrodite was used to define transcription factor codes that define neurotransmitter identity in distinct neuron classes (Serrano-Saiz *et al.* 2013; Pereira *et al.* 2015; Gendrel *et al.* 2016). Furthermore, defining the means by which neurons communicate permits the manipulation of neuronal circuit activity with the ultimate goal of understanding information processing in the male nervous system.

Neurotransmitter maps of all major neurotransmitter systems have been charted for the hermaphroditic form of *C. elegans* (Sulston *et al.* 1975; McIntire *et al.* 1993; Rand and Nonet 1997; Chase and Koelle 2007; Duerr *et al.* 2008; Serrano-Saiz *et al.* 2013; Pereira *et al.* 2015; Gendrel *et al.* 2016). However, the male nervous system has remained much less explored. Specifically, male-specific neurons have been examined for the expression of the neurotransmitter GABA (Gendrel *et al.* 2016) and acetylcholine (ACh) (Pereira *et al.* 2015), but not glutamate (Glu). In addition, the expression of some monoamines (serotonin and dopamine) (Loer and Kenyon 1993; Lints and Emmons 1999; Lints *et al.* 2004) but not all monoamines (octopamine and tyramine) has been previously reported, albeit incompletely. In this resource paper, we fill these gaps by analyzing the expression of fosmid-based reporters for genes specific for each neurotransmitter pathway (Figure 1B). By establishing a comprehensive neurotransmitter atlas of the male-specific nervous system, we also discovered novel sexual dimorphisms of neurotransmitter usage in sex-shared neurons. Lastly, we provide a proof-of-principle analysis for how the male neurotransmitter atlas provides landmarks for the much-needed elucidation of expression patterns of genes that were previously only examined in the hermaphrodite.

Materials and Methods

Reporter transgenes

Previously published: *otIs518 (eat-4^{fosmid}::sl2::mCherry::h2b)* (Serrano-Saiz *et al.* 2017)
otIs388 (eat-4^{fosmid}::sl2::yfp::h2b) (Serrano-Saiz *et al.* 2013)
otIs576 (unc-17^{fosmid}::gfp) (Pereira *et al.* 2015)
otIs544 (cho-1^{fosmid}::sl2::mCherry::h2b) (Pereira *et al.* 2015)
otIs354 (cho-1^{fosmid}::sl2::yfp::h2b) (Stefanakis *et al.* 2015)
otIs564 (unc-47^{fosmid}::sl2::yfp::h2b) (Gendrel *et al.* 2016)
nIs107 III (tbh-1^{prom}::gfp) (Alkema *et al.* 2005)
kyIs693 (pNP502(tdc-1^{prom}::HisCl1::sl2::mCherry) (Jin *et al.* 2016)
otIs450 (oig-1^{fosmid}::sl2::gfp) (Howell *et al.* 2015)
otEx4452 (odr-2::DsRed) (Pereira *et al.* 2015)
ynIs67 (flp-6^{prom}::gfp) (Kim and Li 2004)
wgIs73 (ceh-14^{fosmid}::gfp) (kindly provided through by V. Reinke)
otIs549 (unc-25::mCherry) (Gendrel *et al.* 2016)
otIs570 (snf-11^{fosmid}::sl2::yfp::h2b) (Gendrel *et al.* 2016)
unc-25 (ot867[unc-25::sl2::nls::gfp::h2b]) (Gendrel *et al.* 2016)

Newly generated in this study: *otIs517 (tph-1^{fosmid}::sl2::yfp::h2b)*
otIs625 (cat-1^{fosmid}::sl2::mCherry::h2b)
otIs644 (tdc-1^{prom}::ChR2::yfp)

Fosmid reporters

Fosmid reporters were generated as previously described (Tursun *et al.* 2009), using pBALU23 (a gift from B. Tursun and Í. Carrera) for *otIs517* and pBALU24 (a gift from Í. Carrera) for *otIs625*. The genomic clones used to generate the *tph-1* and *cat-1/VMAT* (vesicular monoamine transporter) fosmid reporters were WRM064dC01 and WRM0628dC03, respectively. In all fosmid reporters, the sequence encoding the respective fluorescent protein was inserted at the 3′-end of the respective locus and separated from the coding sequence of the locus with an SL2 sequence (Tursun *et al.* 2009).

Antibody staining

A previously-described antibody staining protocol (McIntire *et al.* 1993) was modified in the following manner. L4/young

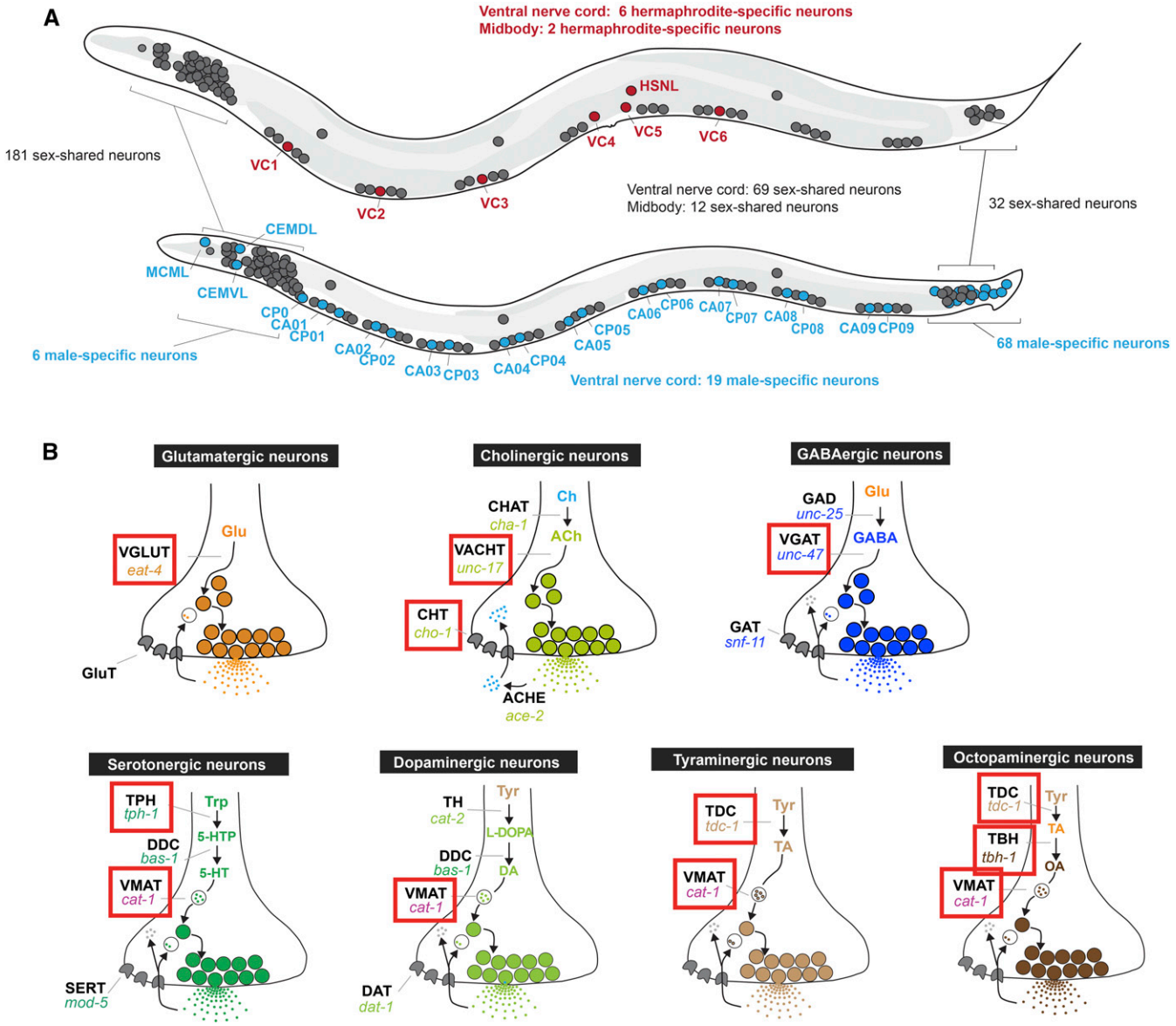

Figure 1 Overview of *C. elegans* nervous systems and neurotransmitter system. (A) Overview of the adult male and hermaphroditic nervous system. Gray circles indicate shared neurons between the two sexes; red circles indicate hermaphrodite-specific neurons; blue circles indicate male-specific neurons. (B) Neurotransmitter pathway genes. The expression pattern of fluorescent fosmid-based reporters was analyzed in the male nervous system for the genes boxed in red. VGLUT, vesicular glutamate transporter; VACHT, vesicular ACh transporter; CHT, choline transporter; VGAT, vesicular GABA transporter; TPH, tryptophan hydroxylase; VMAT, vesicular monoamine transporter; TDC, tyrosine decarboxylase; TBH, tyramine hydroxylase.

adult hermaphrodites or males were fixed for 24 hr at 4° in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4) and 4% paraformaldehyde. After being washed three to four times in PBS/0.5% Triton X-100, the worms were rocked gently for 18 hr at 37° in a solution of 5% β-mercapto-ethanol with 1% Triton X-100 in 0.1 M Tris-HCl (pH 7.5) (as opposed to 0.125 M Tris-HCl, pH 6.9). The worms were washed four times in 1% Triton X-100/0.1 M Tris-HCl (pH 7.5) and one time in 1 mM $CaCl_2$/1% Triton X-100/0.1 M Tris-HCl (pH 7.5). A worm pellet of 20–50 μl was shaken vigorously in 1 ml of 1 mM $CaCl_2$/1% Triton X-100/0.1 M Tris-HCl (pH 7.5) and 1 mg/ml of collagenase type IV (C5138; Sigma [Sigma Chemical], St. Louis, MO) for 30 min. The worms were then washed three times in PBS/0.5% Triton X-100. An extra step was added to quench the paraformaldehyde-induced auto-fluorescence by incubating worms for 1 hr at 4° in a freshly made solution of PBS and 1 mg/ml of $NaBH_4$ (71321; Sigma).

Samples were blocked for 30 min at room temperature with 0.2% gelatin from fish (Sigma). Anti-5HT (5-hydroxytryptamine) antibodies (S-5545, serum; Sigma) were used at a 1:100 concentration. For double labeling, anti-GFP (A10262; Thermo Fisher) or anti-mCherry (M11217; ThermoFisher) were used at a 1:1000 and 1:500 concentrations, respectively. Incubations were done overnight at 4°. Secondary antibodies included Alexa-488-labeled goat anti-chicken

Table 1 Summary of glutamatergic, male-specific neurons

Class	Neuron	Neuron Function	Glu (*eat-4/VGLUT*)	ACh (*unc-17/VACHT*, *cho-1/CHT*)	Aminergic Neurotransmitter	Other Neurotransmitter
Ventral nerve cord						
CP	CP0	Inter/motor	+			
	CP05	Inter/motor	+		Serotonin (*tph-1*, *cat-1*)	*unc-47/VGAT* only [anti-GABA(−)][a]
	CP06	Inter/motor	+		Serotonin (*tph-1*, *cat-1*)	*unc-47/VGAT* only [anti-GABA(−)][a]
	CP07	Inter	+++			
Tail: preanal ganglion						
HOA	HOA	Sensory	+++		Tyramine[b] (*cat-1/VMAT*, *tdc-1*)	*unc-47/VGAT* only [anti-GABA(−)][a]
PVV	PVV	Inter	+++	+++		
Tail: lumbar ganglion						
RnA	R5AL	Sensory	++		Dopamine (*cat-1/VMAT*, *cat-2/TH*)	low *unc-47/VGAT* only [anti-GABA(−)][a]
	R5AR	Sensory	++		Dopamine (*cat-1/VMAT*, *cat-2/TH*)	low *unc-47/VGAT* only [anti-GABA(−)][a]
	R9AL	Sensory	+		Dopamine (*cat-1/VMAT*, *cat-2/TH*)	low *unc-47/VGAT* only [anti-GABA(−)][a]
	R9AR	Sensory	+		Dopamine (*cat-1/VMAT*, *cat-2/TH*)	low *unc-47/VGAT* only [anti-GABA(−)][a]
RnB	R2BL	Sensory	+			
	R2BR	Sensory	+			
	R6BL	Sensory	+			
	R6BR	Sensory	+			
Tail: cloacal ganglion						
PCA	PCAL	Sensory/motor	+++			
	PCAR	Sensory/motor	+++			

See text for information on reporter transgenes. *cat-2/TH* data from (Lints and Emmons 1999). Glu, glutamate; ACh, acetylcholine; VGAT, vesicular GABA transporter; VMAT, vesicular monoamine transporter; GABA, γ-aminobutyric acid. Number of "+" indicate relative levels of reporter expression.
[a] *unc-47/VGAT* expression in the absence of anti-GABA staining indicates usage of another neurotransmitter, perhaps glycine (see text).
[b] *tdc-1* is only detected in young adult males.

(A11039; Invitrogen), Alexa-488-labeled goat anti-rabbit (A21206; Invitrogen, Carlsbad, CA), Alexa-594-labeled goat anti-rabbit (A21207; Invitrogen), or Alexa-594-labeled donkey anti-rat (A21209; Invitrogen).

Cell ablation

Cell ablations were performed as previously described (Bargmann and Avery 1995). A Spectra-Physics VSL-337ND-S Nitrogen Laser (Mountain View, CA) was used, attached to an Olympus BX51 microscope (Olympus, PA). Ablations were done on 2.5% agar pads. To remove the DVE and DVF male-specific neurons, the B.pp was ablated in L3 males placed in 15 mM NaN_3. Nonablated control males were placed on pads for the same amount of time.

Microscopy

Worms were anesthetized using 100 mM of sodium azide (NaN_3) and mounted on 5% agarose on glass slides. All images were acquired using a Zeiss confocal microscope (LSM880; Zeiss [Carl Zeiss], Thornwood, NY). Acquisition of several z-stack images was performed with Micro-Manager software (Version 3.1) (Edelstein *et al.* 2010). Image reconstruction was performed using the ZEN software tool.

For the quantification of fluorescence intensity of the *unc-17/VACHT* (vesicular ACh transporter) fosmid and the *odr-2* reporter, a stack of images was acquired using the Zeiss confocal microscope. The acquisition parameters were maintained constant among all samples (same pixel size and laser intensity). The fluorescence intensity mean was obtained with the ZEN software tool for mCherry and for GFP separately. The values were normalized between each other where the highest value for each color signal was considered to be one.

Data availability

Strains are available at the *Caenorhabditis* Genetics Center or upon request. The authors state that all data necessary for confirming the conclusions presented in the article are represented fully within the article.

Results

Expression pattern of eat-4/VGLUT in the male nervous system

Expression of the vesicular Glu transporter *VGLUT* defines glutamatergic neurons (Takamori *et al.* 2000) (Figure 1B). The pattern of expression of the *C. elegans VGLUT* ortholog *eat-4* in the hermaphrodite nervous system has recently been reported (Serrano-Saiz *et al.* 2013), but its expression in the male nervous system has only been minimally explored (Correa *et al.* 2015). Using previously described *eat-4/VGLUT* fosmid-based reporter transgenes (*otIs388* – *eat-4*^{fosmid}::*yfp*; *otIs518* – *eat-4*^{fosmid}::*mCherry*), we examined *eat-4/VGLUT* expression in male-specific neurons. We observed *eat-4/VGLUT* expression in 16/93 male-specific neurons (6/27 neuron classes), including sensory, inter-, and motorneurons located in different ganglia of the male tail (Figure 2 and Supplemental Material, Figure S1, and Table 1). Specifically, we observed robust expression in the unpaired HOA tail

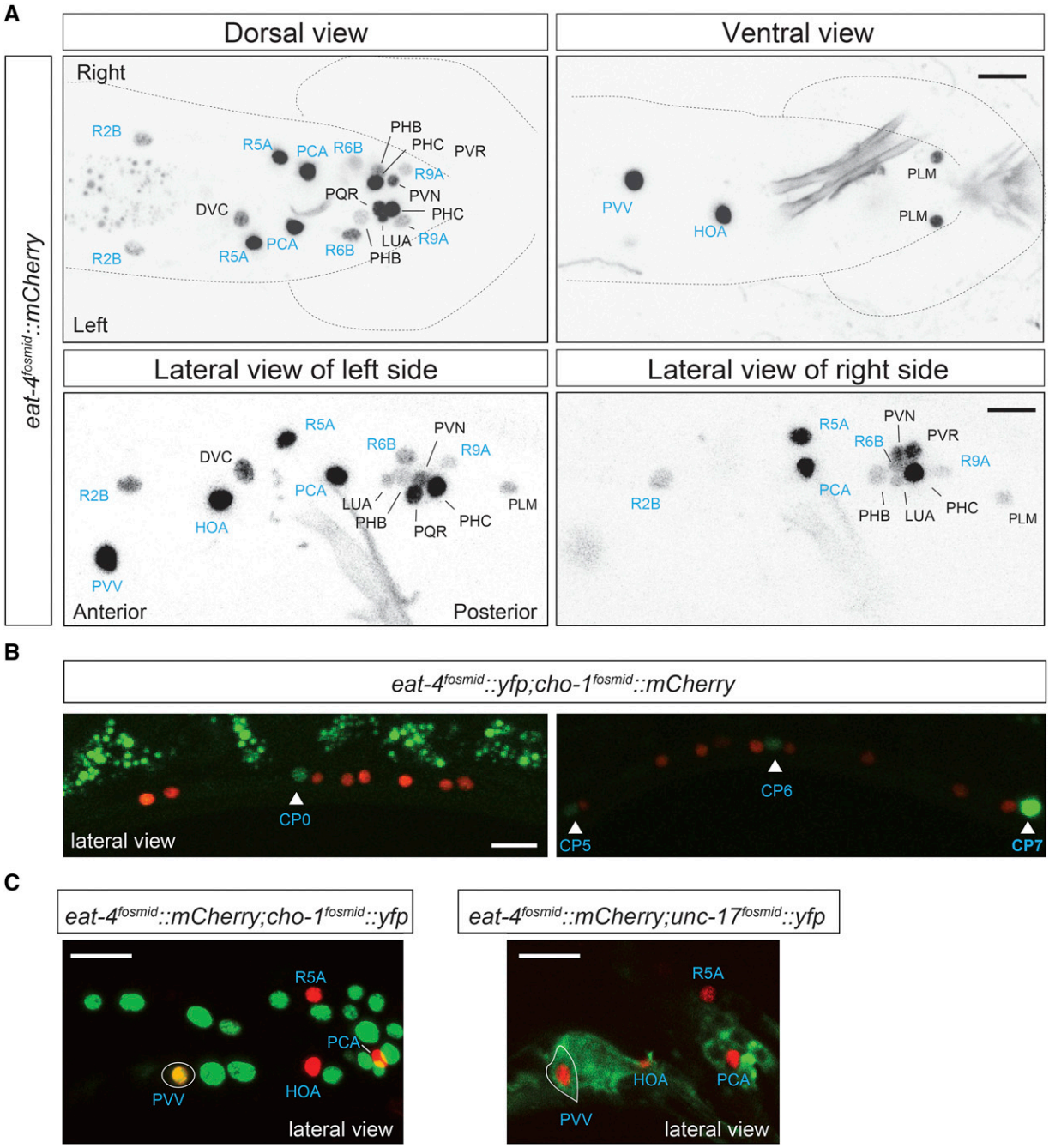

Figure 2 Expression pattern of *eat-4/VGLUT* in the adult *C. elegans* male. (A) *eat-4/VGLUT* fosmid-based reporter expression (*otIs518*) in an adult male tail. R6B was identified by colocalization of *eat-4/VGLUT* fosmid with *flp-6* (*ynIs67*) [expressed in the B-type rays 2, 5, 6, and 7 (Lints *et al.* 2004)]. (B) *eat-4/VGLUT* (*otIs388*) and *cho-1/CHT* fosmid reporter expression (*otIs354*) pattern in the ventral nerve cord. White arrows indicate *eat-4/VGLUT* expression in CP0 (left panel) and CP05, CP06, and CP07 (right panel). The other, red neurons are *cho-1/CHT*(+) ventral cord motor neurons. (C) *eat-4/VGLUT* fosmid-based reporter (*otIs518*) overlaps with *cho-1/CHT* (*otIs354*) and *unc-17/VACHT* (*otIs576*) fosmid-based reporters in PVV in the preanal ganglion of an adult male. The nature of the other *cho-1/CHT*-expressing cells (green) are detailed in Figure 5. In all panels, sex-shared neurons are indicated in black letters. Male-specific neurons are indicated in blue letters. Bar, 10 μm. CHT, choline transporter. VACHT, vesicular ACh transporter; VGLUT, vesicular glutamate transporter.

sensory neuron, the PCA sensory/motor neuron pair, the PVV interneuron, and in subsets of ray sensory neurons and CP ventral nerve cord neurons (Figure 2 and Figure S1). Within the A-type ray sensory neurons, robust expression is observed in the R5A neuron pair and weak expression in the R9A neuron pair (R5A and R9A are also dopaminergic) (Table 1) (Lints and Emmons 1999). Within the B-type ray neurons, expression is observed in the R2B neuron pair and the R6B neuron pair (Figure 2A). In the ventral nerve cord, robust expression is observed in the CP07 interneuron and weaker

expression is present in CP0, CP05, and CP06 (Figure 2B). No expression is observed in CP01-04, CP08, or CP09. We note that the PVV neuron has been previously reported to be cholinergic (Pereira *et al.* 2015) and we confirmed that it coexpresses *eat-4/VGLUT*, *cho-1/CHT*, and *unc-17/VACHT* (Figure 2C and Figure S1).

Apart from its expression in male-specific neurons, we also discovered the male-specific induction of *eat-4/VGLUT* expression in the sex-shared cholinergic PVN interneuron, which we will describe in the context of other sexual dimorphisms of neurotransmitter usage in sex-shared neurons in a later section of this manuscript.

Expression pattern of cat-1/VMAT in the male nervous system

To comprehensively map all monoaminergic neurons, we first examined the expression of the sole *C. elegans* ortholog of the vesicular monoamine transporter, encoded by *cat-1/VMAT* (Figure 1B) (Duerr *et al.* 1999). Previous work had described *cat-1/VMAT* expression by antibody staining in several neurons of the hermaphrodite nervous system (Duerr *et al.* 1999), including serotonergic (NSM, ADF, RIH, AIM, HSN, and VC04/05), dopaminergic (ADE, CEP, and PDE), tyraminergic (RIM), and octopaminergic (RIC) neurons, as well as one neuron class (CAN) to which no known monoamine has yet been assigned. Expression of *cat-1/VMAT* in the male nervous system has not been previously described.

We generated a fosmid-based reporter to study expression of *cat-1/VMAT* (Figure 3A). The *cat-1/VMAT* reporter recapitulates previously described CAT-1 antibody staining in the hermaphrodite (Duerr *et al.* 1999), except for the interneuron AIM in which we have not been able to detect expression of the *cat-1/VMAT* fosmid (we believe that the reported CAT-1/VMAT antibody-staining signal is from tyraminergic RIM, not AIM). In the male nervous system, *cat-1/VMAT* fosmid-based reporter expression was observed in the sex-shared serotonergic (NSM, ADF, and RIH), dopaminergic (ADE, CEP, and PDE), tyraminergic (RIM), and octopaminergic (RIC) neurons. In the male-specific nervous system, *cat-1/VMAT* fosmid reporter was observed in the following neurons (Figure 3A for primary data; summarized Table 2 and Table S1):

1. A subset of CP ventral nerve cord neurons. Of the 10 CP neurons (CP0 to CP09), six express *cat-1/VMAT* (CP01–06) (Figure 3A). This matches previously described anti-5HT antibody staining (Loer and Kenyon 1993).
2. A subset of A-type ray sensory neurons. R5A, R7A, and R9A express the *cat-1/VMAT* fosmid reporter (Figure 3A). These neurons were previously shown to be dopaminergic, based on dopamine staining via formaldehyde-induced fluorescence and the expression pattern of the dopamine-synthesizing enzyme tyrosine hydroxylase/*cat-2* (Sulston *et al.* 1975; Lints and Emmons 1999).
3. A subset of B-type ray sensory neurons. R1B, R3B, and R9B express the *cat-1/VMAT* fosmid reporter, albeit at a lower level than the A-type rays (Figure 3A). These neurons were previously shown to be serotonergic based on anti-5HT staining (Loer and Kenyon 1993; Lints *et al.* 2004). To confirm the previously reported neuronal neurotransmitter identity, we performed anti-5HT staining on a strain carrying the *cat-1/VMAT* fosmid-based reporter. Our results confirmed the serotonergic identity of the R1B, R3B, and R9B neuron classes (Figure 3A).
4. The PGA neuron. As we will show in the next section, this neuron likely reuptakes serotonin but does not synthesize the neurotransmitter because it lacks the expression of the tryptophan hydroxylase enzyme, encoded by *tph-1* (Figure 3A).
5. In addition, we observed the expression of *cat-1/VMAT* in two neuron classes that had not been previously reported as monoaminergic. The hook sensory neuron HOA located in the preanal ganglion, in addition to its glutamatergic identity, shows robust expression of the *cat-1/VMAT* fosmid-based reporter. As we will describe below, we find this neuron to express the tyrosine decarboxylase enzyme, encoded by *tdc-1/TDC*, thereby assigning this neuron a tyraminergic identity (Figure 3D). Another neuronal pair in the male tail showed low levels of *cat-1/VMAT* expression that were only detected when using antibody staining against the *cat-1/VMAT* fosmid tag. As we will show below, this neuron is a sex-shared neuron (PVW) that becomes serotonergic only in males.

Serotonergic neurons of the male nervous system

The identification of the HOA and PVW neuron classes as *cat-1/VMAT*-positive prompted us to reexamine the expression pattern of previously reported serotonin-producing neurons. To this end, we generated a fosmid-based reporter for the rate-limiting step of 5HT synthesis, tryptophan hydroxylase (TPH), encoded by *tph-1* in *C. elegans* (Figure 1B). In the hermaphrodite nervous system, we observed expression in NSM, ADF, and HSN; these neurons were previously shown to stain with anti-5HT and express *tph-1* reporter constructs that encompassed significantly smaller genomic intervals (Horvitz *et al.* 1982; Sze *et al.* 2000). No *tph-1* fosmid reporter expression was observed in the RIH, AIM, and VC04/VC05 neurons, which were previously shown to also contain 5HT by antibody staining and take up extracellular 5HT via the 5HT reuptake transporter *mod-5/SERT* (Jafari *et al.* 2011). While we confirmed RIH and AIM staining with anti-5HT antibodies, we did not observe staining in VC04/05. Instead, we noted weak 5HT staining, but no *tph-1* fosmid expression, of the URX and I5 neurons (Figure 3B).

In the male nervous system, we observed *tph-1* fosmid reporter expression in the previously identified subset of anti-5HT antibody-stained CP ventral cord neurons (CP01 through CP06) and in the B-type ray neurons 1B, 3B, and 9B (all *cat-1/VMAT*-positive as described above) (Figure 3C) (Loer and Kenyon 1993; Lints *et al.* 2004). Our anti-5HT

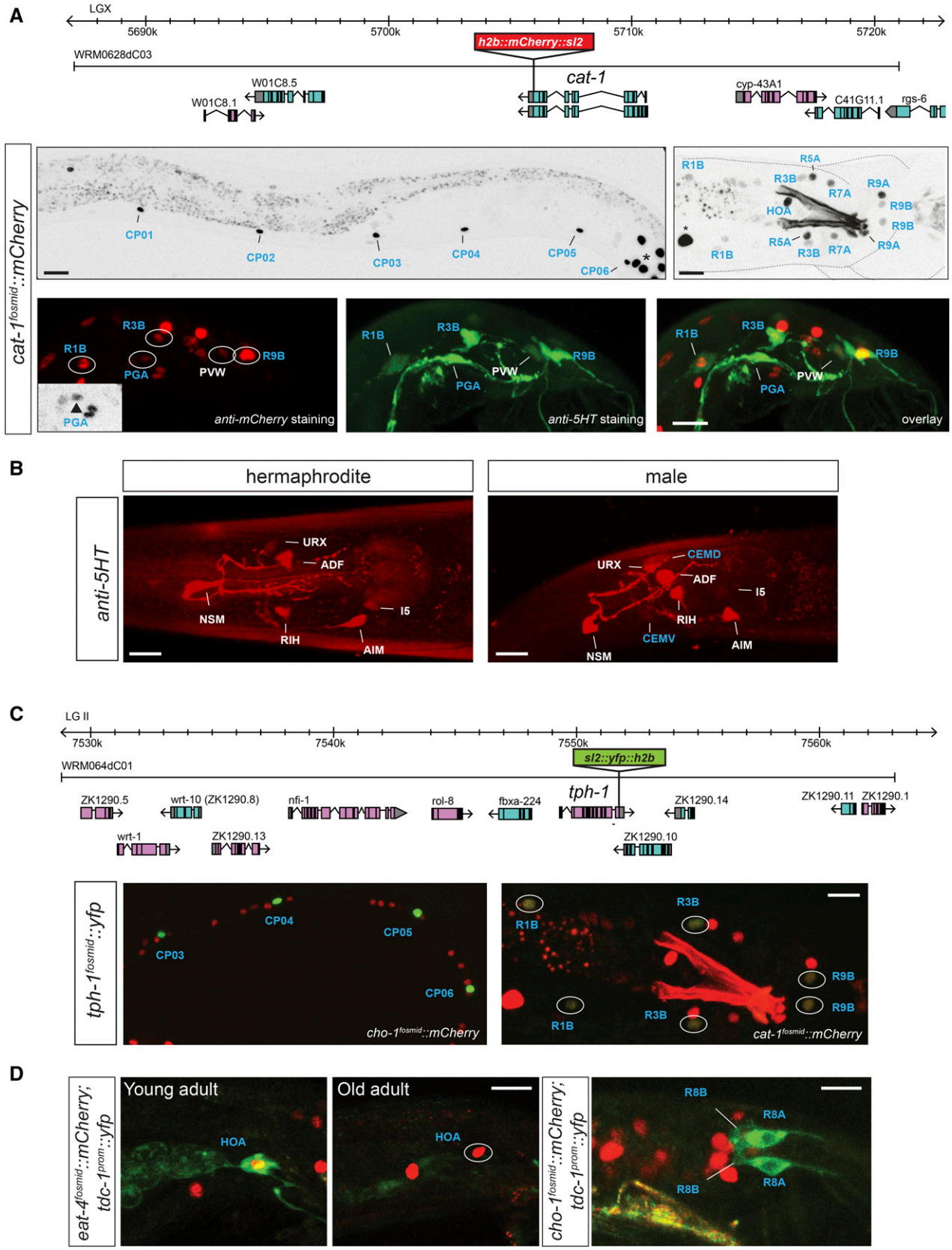

Figure 3 Monoaminergic neurons in the male *C. elegans* nervous system. (A) Expression of a *cat-1/VMAT* fosmid-based reporter (*otIs625*; structure of the reporter is schematized in upper part of the panel) in the ventral nerve cord in CP neurons (upper left panel). *cat-1/VMAT* expression in the adult male tail (upper right panel). Anti-mCherry staining in *cat-1/VMAT* fosmid-based reporter (*otIs625*) and anti-5HT (green) in the adult male tail (lower

Table 2 Monoaminergic neurons

Neuron Class	*cat-1/VMAT*[a]	5HT Ab[b]	*tph-1*	DA[c]	*tdc-1*[d] (Tyramine)	*tbh-1*[d] (Octopamine)	Aminergic Neurotransmitter Usage	Other Small Neurotransmitter
NSM	+	+++	+	—	—	—	5HT	Unknown
ADF	+	+++	+	—	—	—	5HT	Unknown
HSN[e]	+	+++	+	—	—	—	5HT	ACh
CP01-06[e]	+	+++	+	—	—	—	5HT	Glu (CP05-06)
R1B[e]	+	+	+	—	—	—	5HT	Unknown
R3B[e]	+	+++	+				5HT	Unknown
R9B[e]	+	+++	+				5HT	GABA
AIM	—	+++	—	—	—	—	5HT clearance?[h]	Glu/ACh
URX	—	+	—	—	—	—	5HT clearance?[h]	Ach
I5	—	±	—	—	—	—	5HT clearance?[h]	Glu
CEM[e]	—	±	—	—	—	—	5HT clearance?[h]	ACh
RIH	+	++	—	—	—	—	5HT uptake[i]	ACh
VC04/05[e]	± (?)[f]	± (?)[f]	—	—	—	—	5HT uptake[i]	ACh
PGA[e]	±[g]	+++	—	—	—	—	5HT uptake[i]	ACh
PVW	±[g]	+	—	—	—	—	5HT uptake[i]	Unknown
ADE	+	—	—	+	—	—	Dopaminergic	Unknown
CEP	+	—	—	+	—	—	Dopaminergic	Unknown
PDE	+	—	—	+	—	—	Dopaminergic	Unknown
R5A/7A/9A[e]	+	—	—	+	—	—	Dopaminergic	Glu (R5A, R9A)
RIM	+	—	—	—	+	+	Octopaminergic	Glu
RIC	+	—	—	—	+	—	Tyraminergic	Unknown
HOA[e]	+	—	—	—	+	—	Tyraminergic	Glu
R8A/8B[e]	—	—	—	—	+	—	Tyraminergic (release?)	ACh
CAN	+	—	—	—	—	—	?	Unknown

VMAT, vesicular monoamine transporter; 5HT, 5-hydroxytryptamine; Ab, antibody; DA, dopamine; ACh, acetylcholine; Glu, glutamate; GABA, γ-aminobutyric acid.
[a] CAT-1/VMAT expression by antibody staining was previously described in the hermaphrodite in AIM, NSM, ADF, RIH, HSN, VC04/05, ADE, CEP, PDE, RIM, and RIC (Duerr *et al.* 1999).
[b] 5HT antibody staining was previously detected in AIM, RIH, VC04/05, NSM, ADF, and HSN (Horvitz *et al.* 1982; Sze *et al.* 2000; Jafari *et al.* 2011). In the male, 5HT was detected in the rays R1B, 3B, and 9B, in the CP01-06, and in one neuron in the tail (Loer and Kenyon 1993).
[c] Dopaminergic staining was previously described (Sulston *et al.* 1975; Lints and Emmons 1999).
[d] *tdc-1* expression was reported in the hermaphrodite before in RIC and RIM; *tbh-1* was reported to be expressed in RIM (Alkema *et al.* 2005).
[e] Sex-specific neurons.
[f] Duerr *et al.* (1999) reported CAT-1/VMAT antibody staining and weak and variable anti-5HT in VC04/05. We do not observed *cat-1/VMAT* fosmid reporter expression or 5HT antibody staining.
[g] Expression only visible upon mCherry-antibody staining of *cat-1/VMAT* fosmid reporter expression strain.
[h] Does not synthesize or synaptically release 5HT.
[i] Does not synthesize 5HT, but can synaptically release 5HT that was taken up.

staining showed that, in the male head, the CEM neurons are also weakly 5HT-positive but do not express *cat-1/VMAT* (data not shown); these neurons are likely to be uptake neurons that do not employ 5HT for neurotransmission.

Previous work had also reported 5HT staining in one single/unpaired neuron that was considered to be either the cholinergic PGA or PDC interneuron (Loer and Kenyon 1993). We identified the 5HT-positive neuron as PGA [based on *unc-17/VACHT*(+) and *cho-1/CHT*(−) expression; Figure 3A]. As mentioned above, *cat-1/VMAT* is also expressed in PGA and therefore we have assigned this neuron a serotonergic identity (Figure 3A). Since we detect no *tph-1/TPH* fosmid expression in this neuron, we presume that PGA takes up extracellular 5HT synthesized by other neurons.

panels). The inset in the lower left panel shows a color-inverted, black/white image to illustrate weak expression in PGA. The 5HT-positive neurons are marked, the other red fluorescent cells are the dopaminergic *cat-1/VMAT*(+) neurons and the tyraminergic HOA neuron. *Consistent nonneuronal *cat-1/VMAT* expression in the posterior gut nuclei. (B) 5HT staining in the head is detected in NSM, ADF, the reuptake neuron RIH [*tph-1*(−)], and the putative 5HT-clearance neurons URX, AIM, and I5 [*tph-1*(−);*cat-1/VMAT*(−)] in the hermaphrodite (right panel) and male heads (left panel). Additionally, 5HT is detected in the putative 5HT-clearance neurons CEM. (C) Expression of a *tph-1* fosmid-based reporter (*otIs517*; structure of the reporter is schematized in upper part of the panel). Only *tph-1*-expressing neurons are labeled. Expression in the CP neurons of the ventral nerve cord is shown in the left panel. The other red-expressing neurons are *cho-1/CHT*(+) ventral nerve cord neurons. *tph-1* and *cat-1/VMAT* (*otIs625*) fosmid reporters colocalized in the rays R1B, R3B, and in R9B (right panel). The coexpression of the two genes confer these neurons a serotoninergic identity. The other red-expressing neurons are *cat-1/VMAT*(+) dopaminergic and the tyraminergic HOA neurons. (D) Expression of *tdc-1* reporter (*otIs644*) in the adult male tail. *tdc-1* is detected in the hook sensory HOA neuron in young adult animals but is no longer visible in old adults (left and central panels; the other *eat-4/VGLUT*(+)-expressing neurons in these panels are PVV, PCA, and several ray neurons). *tdc-1* is also expressed in two pairs of ray neurons R8A and R8B (right panel). Only *tdc-1* male-specific neurons that colocalize with *eat-4/VLGUT* (*otIs518*) in HOA, and *cho-1/CHT* (*otIs544*) in R8A and R8B, are labeled (right panel: the other *cho-1/CHT*(+) neurons anterior to R8A and R8B are R9B and R6A). Sex-shared neurons are indicated in white. Male-specific neurons are indicated in blue. Bar, 10 μm. 5HT, 5-hydroxytryptamine; VGLUT, vesicular glutamate transporter; VMAT, vesicular monoamine transporter; CHT, choline transporter; TPH, tryptophane hydroxylase.

Tyraminergic neurons in the male nervous system

To identify tyraminergic and octopaminergic neurons in the male, we examined the expression of two biosynthetic enzymes: *tdc-1*, which encodes the major *C. elegans* tyrosine decarboxylase that synthesizes tyramine, and *tbh-1*, which encodes the sole *C. elegans* tyramine β-hydroxylase that synthesizes octopamine out of the tyramine precursor (Alkema *et al.* 2005) (Figure 1D). In the hermaphrodite nervous system, *tdc-1* has previously been shown to be expressed in the sex-shared RIC and RIM neurons (Alkema *et al.* 2005). Of these two neurons, the RIC neuron expresses *tbh-1*, indicating an octopaminergic identity. The expression of neither of these two enzymes has been formerly reported in the male. Using previously described reporter constructs (Alkema *et al.* 2005; Jin *et al.* 2016), we find the same expression in the sex-shared RIC and RIM neurons in the male head, but no other male head neurons. We observed no *tbh-1* reporter expression in male-specific neurons in the entire animal, indicating that octopamine is not used by male-specific neurons. In contrast, three sensory neuron classes, HOA and the rays R8A and R8B, express *tdc-1* (Figure 3D). Therefore, this analysis assigns a tyraminergic identity to the HOA neuron, which we had described above as expressing *cat-1/VMAT*. When analyzing the expression pattern of *eat-4/VGLUT* we had also identified HOA as a glutamatergic neuron (Figure 2A and Figure 3D). In contrast to *eat-4/VGLUT* expression or *cat-1/VMAT* expression, *tdc-1* expression fades in HOA after 4 days in adult males to undetectable levels (Figure 3D). R8A and R8B do not appear to express the *cat-1/VMAT* fosmid reporter and it is not clear if or how these neurons use the tyramine that they may generate. We summarize the patterns of expression of monoamines, synthesizing enzymes, and vesicular transporters in Table 2.

Expression pattern of unc-47/VGAT (vesicular GABA transporter) and reassessment of GABA(+) neurons in the male nervous system

In the course of mapping all GABAergic neurons in the hermaphrodite and male, the vesicular GABA transporter *unc-47/VGAT* was found to be expressed not only in GABA-positive neurons (*i.e.*, neurons stained by anti-GABA antibody), but also in GABA-negative neurons (Gendrel *et al.* 2016), suggesting that *unc-47/VGAT* may be a vesicular transporter for another neurotransmitter, perhaps glycine (see *Discussion*). We examined *unc-47/VGAT* expression systematically throughout the male nervous system and found that it is expressed in all the male-specific GABAergic neurons, *i.e.*, the rays R2A, R6A, and R9B and the interneurons CP09 and EF1–4, but also in 16 different non-GABAergic male-specific neuron classes (Figure 4 and Table S1). R2A and R9B expression is not readily detectable by reporter fluorescence (Gendrel *et al.* 2016) but becomes apparent through immunostaining using an antibody against the *unc-47/VGAT* fosmid fluorescent tag. In addition to what has been described previously in both males and hermaphrodites (Gendrel *et al.* 2016), the antibody staining also detected expression of *unc-47/VGAT* in the cholinergic motorneurons of the ventral nerve cord (DA2-3, VA2-6, and VB10) and in the glutamatergic PVD, PQR (only in hermaphrodites), and PLM neurons (Figure 6E). None of these neurons show anti-GABA antibody staining.

In addition to the GABAergic neurons mentioned above, *unc-47/VGAT* is expressed in 16 different non-GABAergic male-specific neuron classes, including CA and a subset of CP ventral nerve cord neurons (Figure 4 and Table S1). Some of these cells show very low fosmid reporter fluorescence and require anti-mCherry immunostaining for unambiguous detection. Among them, four neurons, DVE, DVF, and R5BL/R, express no other known neurotransmitter system. We also observed sexually dimorphic *unc-47/VGAT* expression in sex-shared neurons, as we will describe below.

Our *unc-47/VGAT* expression analysis also prompted us to reexamine the expression of a GFP-tagged *unc-25/GAD* locus (*ot867*) (Gendrel *et al.* 2016). In addition to the previously described, male-specific *unc-25/GAD*(+) neurons EF1–4, we also observed expression in the CP09 interneuron in the preanal ganglion (which also expresses *unc-47/VGAT*) (Figure S2). CP09 also stains with anti-GABA antibodies (Figure S2). The underlying cause for previously missing the GABAergic identity of CP09 is the nonstereotyped generation of the EF4 neuron, already noted by Sulston *et al.* (1980). Using *unc-25*::*gfp* (*ot867*), we confirmed this nonstereotypy of EF4 generation, which is present in $< 50\%$ of animals (Figure S2). CP09 expression of *unc-25*::*gfp* made us also reconsider what we reported to be male-specific expression of *snf-11/GAT*, the GABA reuptake transporter, in VD12. Reanalysis of these images leads us to conclude that *snf-11/GAT* is expressed in CP09, not VD12 (Figure S2). In summary, the CP09 interneuron is a "conventional" GABAergic neuron that stains for GABA and expresses *unc-25/GAD*, *unc-47/VGAT*, and *snf-11/GAT*.

Expression pattern of cho-1/CHT and unc-17/VACHT in the male nervous system

Cholinergic neurotransmitter identity is defined by the expression of the enzyme choline acetyltransferase (*cha-1/CHAT*) and the vesicular ACh transporter (*unc-17/VACHT*). The *cho-1/CHT* gene encodes for the reuptake transporter of choline, which is the substrate for CHA-1/CHAT substrate to synthesize ACh. It has recently been shown that *cho-1/CHT* expression is present in most but not all cholinergic neurons (Pereira *et al.* 2015). The expression of cholinergic pathway genes was previously analyzed in the male-specific nervous system, and it was shown that *cho-1/CHT* and *unc-17/VACHT* fosmid-based reporters overlapped in the CEM sensory neurons in the adult male head (Pereira *et al.* 2015). The presence of *cho-1/CHT* and *unc-17/VACHT* was also detected in the CA01−06 ventral nerve cord interneurons (CA07–09 showed lower levels of *unc-17/VACHT* and no *cho-1/CHT*) and 14 additional neuronal classes in the male tail (Pereira *et al.* 2015).

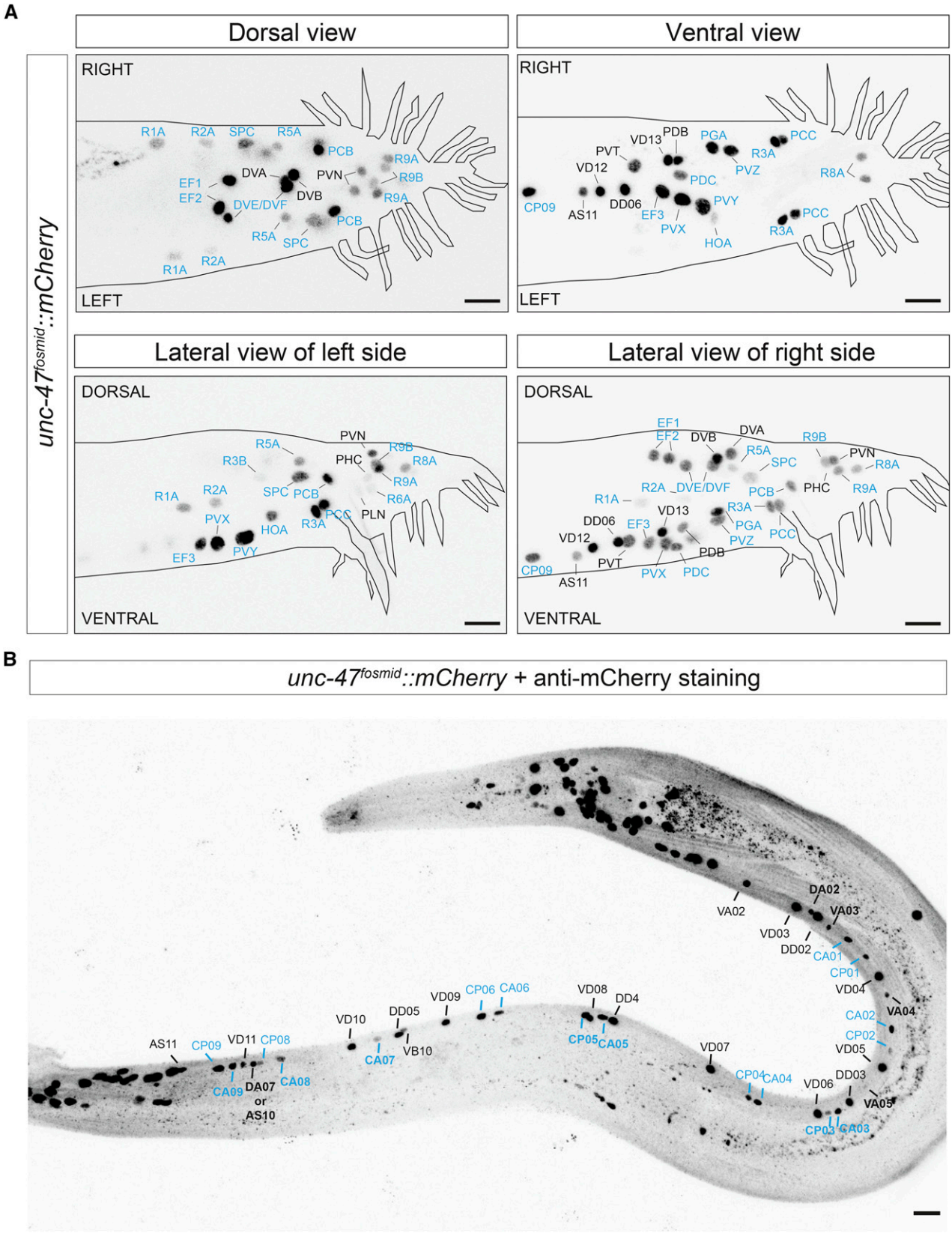

Figure 4 Fosmid-based reporter expression of *unc-47*/VGAT in the male nervous system. (A) *unc*-47/*VGAT* fosmid-based reporter (*otIs564*) expression in the adult male tail. ALN, PLM, and R5B are not visible on those pictures. (B) α-mCherry immunostaining in *unc-47*/*VGAT* fosmid-based reporter (*otIs564*) adult male. Only the ventral nerve cord neurons are labeled [for tail neurons refer to (A) and for the head neurons to Gendrel *et al.* (2016)]. Shared neurons are indicated in black. Male-specific neurons are indicated in blue. Bar, 10 μm. VGAT, vesicular GABA transporter.

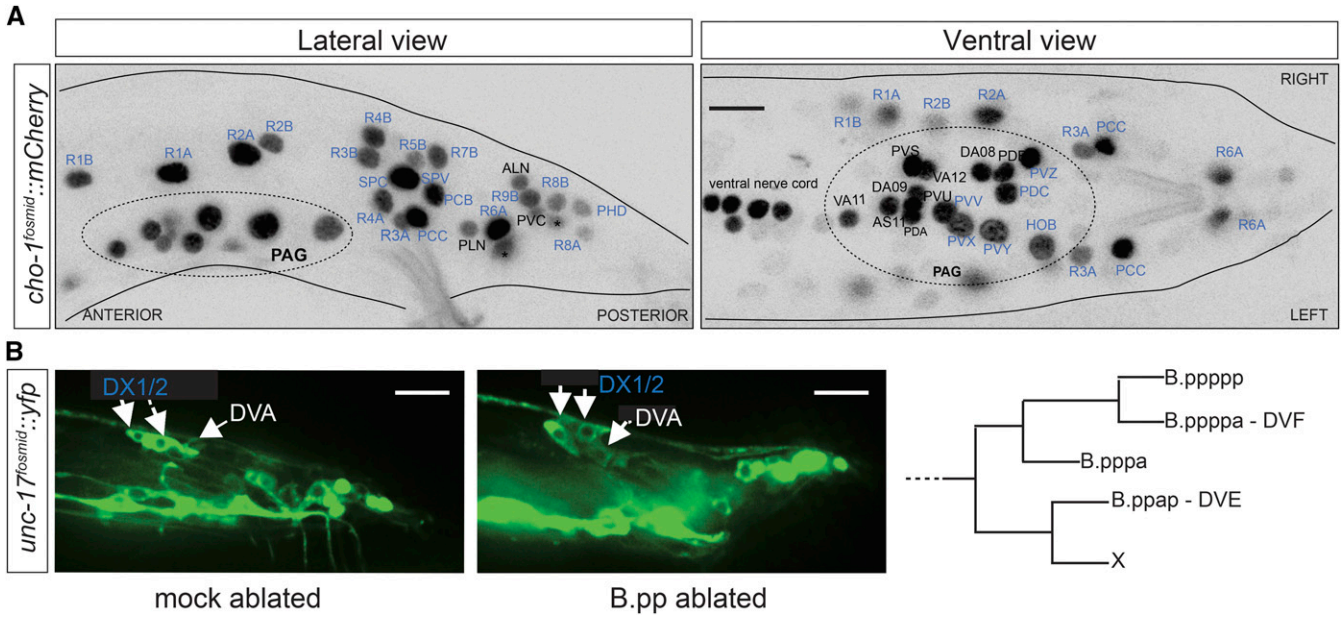

Figure 5 Expression pattern of cholinergic genes in the adult *C. elegans* male. (A) *cho-1/CHT* fosmid-based reporter expression in an adult male tail (*otIs544*). The position of the male-specific neurons (labeled in blue) was determined relative to the sex-shared neurons (labeled in black). *Left/right neuronal pairs are visible for these neurons. Preanal ganglion (PAG). (B) Laser ablations of the B.pp progenitor in the *unc-17/VACHT* fosmid reporter strain (*otIs576*) did not affect the number of *unc-17/VACHT*-positive neurons in the dorsorectal ganglion. DX1/2 neurons were identified by the morphology of the projections. Diagram of the B lineage last divisions is shown in the right panel. Sex-shared neurons are indicated in black (in black and white panels) and in white (in color panels). Male-specific neurons are indicated in blue. Bar, 10 μm. CHT, choline transporter; VACHT, vesicular ACh transporter.

We extended these findings by observing *cho-1/CHT* expression in male-specific neurons of the ventral nerve cord and tail that do not show *unc-17/VACHT* expression. Specifically, we observed *cho-1/CHT* fosmid expression in B-type ray neurons R1B, R2B, R3B, R4B, R5B, R7B, R8B, and R9B (Figure 5A and Table S1). Due to elimination of problems with a co-injection marker that partially obscured expression in the tail, we also extended the expression of the *unc-17/VACHT* fosmid-based reporter in male-specific neurons, observing weak expression in CP08 and CP09 as well as in R8A and R8B (CP08 and CP09 neurons did not express the *cho-1/CHT* fosmid) (Table S1). We also detected a male-specific neuronal pair labeled by both *unc-17/VACHT* and *cho-1/CHT* in the very posterior dorsal end of the tail. This cholinergic neuronal pair has been identified as a new, previously unknown neuronal pair, termed PHD (B. Kim, R. Poole, and A. Barrios, personal communication). Lastly, we revised neurotransmitter identity of DVE and DVF, which we had previously identified as expressing *unc-17/VACHT* but not *cho-1/CHT* (Pereira *et al.* 2015). We performed laser ablations of the B lineage that gives rise to both DVE and DVF in strains carrying the *unc-17/VACHT* fosmid and *unc-25/GAD* reporter (*otIs549*). We found that in the B.pp-ablated animals, the two male-specific *unc-17/VACHT* and *unc-25/GAD* neurons persisted, indicating that the previous identification was mistaken and that these neurons are either DX1/2 or EF1/2 (Figure 5C and data not shown). Based on projections of axons toward the tail, we have now identified the *unc-17/VACHT*-positive neurons in the dorsorectal ganglion as DX1/2 (the GABAergic EF neurons do not display such projections).

Sexual dimorphisms in neurotransmitter usage in the sex-shared nervous system

Apart from examining the expression of neurotransmitter pathway genes in male-specific neurons, we also examined whether sex-shared neurons may display sexual dimorphisms in neurotransmitter usage. We have previously shown that the sex-shared interneuron AIM alters its neurotransmitter usage in males, switching from glutamatergic to cholinergic (Pereira *et al.* 2015). Moreover, the sex-shared PHC neuron scales up the expression of *eat-4/VGLUT* specifically in males (Serrano-Saiz *et al.* 2017). Examining all major neurotransmitter systems (ACh, Glu, GABA, and monoamines) we find other examples of sexual dimorphisms in sex-shared neurons (summarized in Table 3):

1. An additional sexually dimorphic scaling of a neurotransmitter pathway. Previous work showed an upregulation of *eat-4/VGLUT* expression in the sex-shared PHC neuron, a phenomenon we called "scaling" (Serrano-Saiz *et al.* 2017). We observed a similar scaling phenomenon in the AVG head interneuron, which upregulates expression of *unc-17/VACHT* specifically in males (Figure 6A). We compared the levels of *unc-17/VACHT* in AVG relative to the adjacent RIG neurons in L1 and young adult animals and found that, while *unc-17/VACHT* expression is the

Table 3 Sexual dimorphisms of sex-shared neurons

Neuron Class	Neuron Type	Inferred Neurotransmitter Change in the Male	Hermaphrodites		Males	
			Synaptic Output Partner[a]	Number of Sections	Synaptic Output Partner[a]	Number of Sections
AIM[b]	Interneuron	switch (Glu→ACh)	17	128	6	138
PHC[c]	Inter/sensory	*eat-4* up *unc-47* on	10	135	30	774
AVG	Interneuron	*unc-17* up	14	67	18	103
PVN	Interneuron	*eat-4* on *unc-47* on	23	192	33	317
PVW	Interneuron	5HT on	3	15	0	0
ADF	Sensory	*unc-47* on	20	338	12	195
PQR	Sensory	*unc-47* off	12	102	16	212
PDB	Inter/motorneuron	*unc-47* on	3	36 (+12 NMJ)	24	237 (+50 NMJ)
AS10[d]	Motorneuron	*unc-47* on (?)	2 (+10 NMJ)	30	2 (+7 NMJ)	28
DA07[d]	Motorneuron	*unc-47* on (?)	6 (+38 NMJ)	75	7 (+38 NMJ)	76
AS11	Motorneuron	*unc-47* on	3	35 (+9 NMJ)	3	46 (+23 NMJ)

Glu, glutamate; ACh, acetycholine; NMJ, neuromuscular junction.
[a] Only connections observed in more than one section were considered in this calculation. Connectivity data is from www.wormwiring.org (Jarrell *et al.*, 2012).
[b] Pereira *et al.* (2015).
[c] Serrano-Saiz *et al.* (2017).
[d] AS10 and DA07 could not be disambiguated. Only one of the two is sexually dimorphic for *unc-47/VGAT* expression.

same in both sexes at the L1 stage, it is five times higher in males compared to hermaphrodites after sexual maturation. This sex-specific, temporally controlled upregulation correlates with an increase in synaptic output that AVG makes in the male: 67 EM sections show presynaptic contacts to 14 target cells in hermaphrodites, while 103 EM sections are observed with contacts to at least 18 different target cells in the male (Table 3) (www.wormwiring.org) (White *et al.* 1986; Jarrell *et al.* 2012).

2. In the male tail, the PVN interneurons acquire a glutamatergic identity during sexual maturation. It has previously been reported that the PVN interneurons are cholinergic in the hermaphrodite (Pereira *et al.* 2015). PVN interneurons maintain their cholinergic identity in the male while they turn on *eat-4/VGLUT* expression during sexual maturation (Figure 6C). PVN was identified based on the colocalization of the *eat-4/VGLUT* fosmid reporter and the *ceh-14* fosmid reporter expressed nondimorphically in DVC, PHA, PHB, PHC, PVQ, PVN, PVR, PVW, and PVT in the two sexes (Figure 6C). According to EM reconstruction (Jarrell *et al.* 2012), PVN is poorly connected in the hermaphrodite while it shows a threefold increase in the number of synaptic outputs in the male (Table 3).
3. The PVW interneurons acquire a serotonergic identity in the adult male. We detect anti-5HT staining and *cat-1/VMAT* fosmid expression in PVW exclusively in the male (Figure 3A and Figure 6D). We have identified this pair of neurons based on *ceh-14* fosmid expression and the lack of *cho-1/CHT*, *eat-4/VGLUT*, and *unc-47/VGAT* expression. The absence of *tph-1* expression in PVW suggests that this cell, like several others in the worm, may serve as a reuptake 5HT neuron which is able to subsequently utilize 5HT via *cat-1/VMAT*.
4. The *unc-47/VGAT* fosmid reporter shows multiple sites of dimorphic expression. Specifically, we found that *unc-47/VGAT* is expressed in ADF, AS11, PHC, PDB, and PVN neurons only in the male (Figure 6, B and E). In addition, expression was observed in a cholinergic motorneuron next to VD11, but we could not discriminate its identity between AS10 and DA7. According to the EM reconstruction, most of these neurons show an increase of synaptic outputs in the male (Table 3). The onset of *unc-47/VGAT* expression in these neurons appears to occur at sexual maturation (Figure 6, B and E), as previously observed for other neurotransmitter dimorphisms in sex-shared neurons (Pereira *et al.* 2015; Serrano-Saiz *et al.* 2017).
5. Apart from male-specific *unc-47/VGAT* expression in sex-shared neurons, we also detected hermaphrodite-specific *unc-47/VGAT* expression in the sex-shared PQR neuron (Figure 6E). PQR is glutamatergic in both sexes. *unc-47/VGAT* is expressed at the L3 stage in PQR in both sexes, and during sexual maturation *unc-47/VGAT* expression is downregulated specifically in the male (Figure 6E).

In summary, we define a number of dimorphisms in neurotransmitter usage and we found that all occur during sexual maturation (we reiterate that the nature of the neurotransmitter transported by *unc-47/VGAT* remains unclear). The changes in neurotransmitter usage correlate with changes in synaptic connectivity and, specifically, in most cases a substantial increase in number of target neurons innervated by the respective neurons in males, as well as number of synaptic contacts (Table 3).

Applications of the male neurotransmitter map: neurotransmitter landmarks as tools for expression pattern determination

In *C. elegans*, gene expression patterns are most often determined via analysis of reporter transgenes (Boulin *et al.* 2006). Since cell identification based on position is nontrivial in the

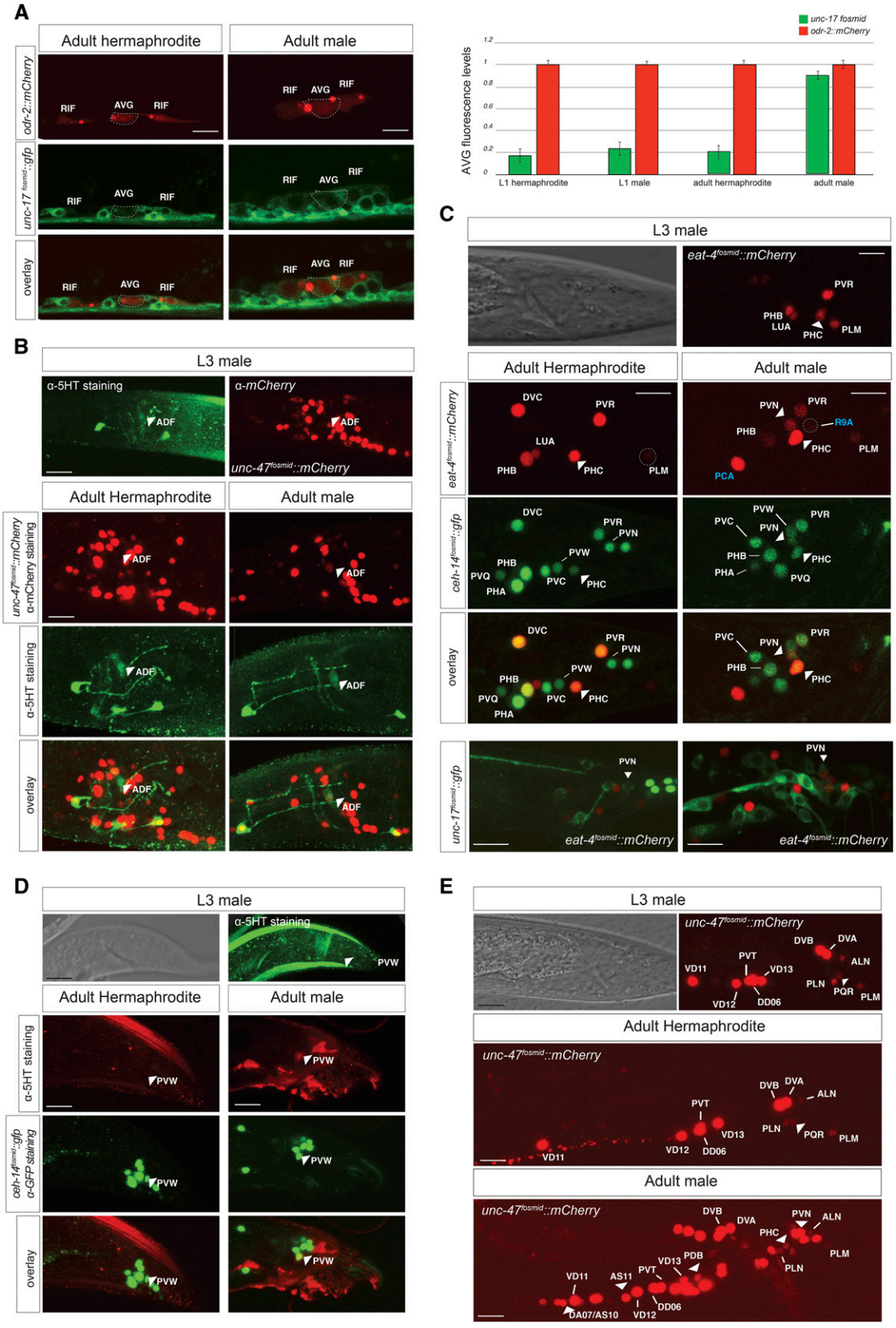

Figure 6 Sexually dimorphic expression patterns in sex-shared neurons. (A) *unc-17/VACHT* scaling in AVG neurons in adult male. The AVG neurons were identified in hermaphrodite and males by expression of *unc-17/VACHT* fosmid-based reporter (*otIs576*) overlapping with the *odr-2*::*dsRed* reporter (*otEx4452*). AVG neuron (dashed line traces the AVG soma) is localized in between the RIG neurons that also express both reporters. The AVG neurons

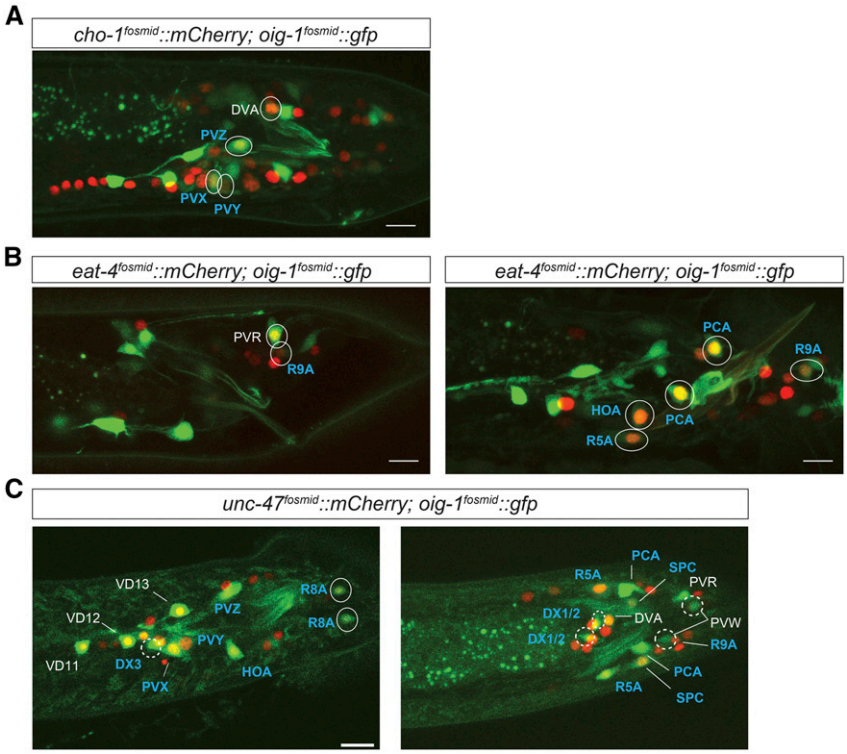

Figure 7 Cell identification of expression of an *oig-1* fosmid-based reporter in the *C. elegans* male tail using neurotransmitter landmarks. *oig-1* fosmid-based reporter (*otIs450*) colocalization patterns in young adult animals. (A) Colocalization with the *cho-1/CHT* fosmid-based reporter (*otIs544*) in the sex-shared neuron DVA, and in the male-specific neurons SPC, PVX, PVY, and PVZ. (B) Colocalization with the *eat-4/VGLUT* fosmid-based reporter (*otIs518*) in the sex-shared neuron PVR and in the male-specific neurons HOA, PCA, R5A, and R9A. (C) Colocalization with the *unc-47/VGAT* fosmid-based reporter (*otIs564*) in the VD neurons and the male-specific neurons R8A. The lack of colocalization with any of the three neurotransmitter landmarks indicates that *oig-1* is expressed in the share neurons PVW and the male-specific DX neurons. White solid circles indicate the neurons that have been identified with the corresponding fosmid. White dashed circles indicate neurons that have been identified because they do not colocalize with any of the three neurotransmitter landmarks. Sex-shared neurons are indicated in white letters. Male-specific neurons are indicated in blue letters. Bar, 10 μm. CHT, choline transporter. VGLUT, vesicular glutamate transporter; VGAT, vesicular GABA transporter.

male tail, few reporter gene expression patterns have been mapped in the male tail. We reasoned that our neurotransmitter reporter strains can serve as invaluable landmarks to facilitate cell identification of unknown reporter genes. As proof of principle, we show in Figure 7 the identification of the cellular sites of expression of the *oig-1* gene. *oig-1* encodes a immunoglobulin domain-containing synaptic organizer protein (Howell *et al.* 2015) whose expression in the male nervous system had not been reported before. We crossed transgenic animals carrying an *oig-1* fosmid-based reporter with mCherry-nuclear localized *cho-1/CHT*, *eat-4/VGLUT* and *unc-47/VGAT* fosmid-based reporter transgenes. We identified *oig-1* sites of expression based on the overlap of green and red fluorescent proteins: with *cho-1/CHT* in the sex-shared interneuron DVA and in the male-specific neurons PVX, PVY, and PVZ [*cho-1/CHT*(+) and also *unc-47/VGAT*(+)] (Figure 7, A and C); with *eat-4/VGLUT* in the sex-shared interneuron PVR and in the male-specific neurons HOA, R5A, R9A, and PCA [*eat-4/VGLUT*(+); R5A and R9A are also *unc-47/VGAT*(+)] (Figure 7, B and C); and with *unc-47/VGAT* in the sex-shared VD-type motorneurons and in the male-specific R8A (Figure 7C). Finally, expression in the sex-shared interneurons PVW and the male-specific DX1/2 and DX3/4 neuron class was identified because of lack of colocalization with any of the three neurotransmitter landmarks (Figure 7C).

Discussion

Frequency and distribution of neurotransmitter usage in the male nervous system

We discuss the findings described here in conjunction with the previously reported maps of GABA and ACh usage in the male nervous system (Pereira *et al.* 2015; Gendrel *et al.* 2016). The key themes already observed in the analysis of the sex-shared

in the male express fivefold higher levels of *unc-17/VACHT* fosmid compared to hermaphrodites. Quantification is provided on the right. (B) ADF neurons express *unc-47/VGAT* in adult male. Anti-5HT and anti-mCherry staining in *unc-47/VGAT* fosmid-based reporter (*otIs564*) is shown for the hermaphrodite and male head. *unc-47/VGAT* is not detected in larval stages before sexual maturation (L3 male is shown in upper panel). 5HT staining is detected in ADF in both sexes. (C) PVN interneurons acquire a glutamatergic identity in the adult male. PVN was identified by *ceh-14* (*wgIs73*) fosmid reporter expression (*ceh-14* expression is not sexually dimorphically regulated). At larval stages *eat-4/VGLUT* (*otIs518*) expression is not detected in PVN (upper panel). *unc-17/VACHT* (*otIs576*) expression is detected in both sexes (lower panel). Male-specific neurons are labeled in blue. (D) PVW interneurons become serotonergic in adult males. Anti-5HT (in red) and anti-GFP staining in *ceh-14* fosmid reporter (*wgIs73*). While *ceh-14* fosmid expression is maintained between male and hermaphrodite in PVW, this neuron becomes serotonergic after sexual maturation. 5HT staining (in green) is not detected at larval stages in the male tail (upper panel). (E) *unc-47/VGAT* fosmid reporter (*otIs564*) expression in the tail of hermaphrodite and male. *unc-47/VGAT* is expressed in PDB, PVN, PHC, AS11, and AS10/DA7 (we could not disambiguate the identity between AS10 and DA7) only in the male and in PQR only in the hermaphrodite. *unc-47/VGAT* expression induction occurs during sexual maturation. At earlier larval states (L3 male is shown in upper panel), *unc-47/VGAT* fosmid expression is present in PQR and is not detected in PDB, PVN, PHC, AS11, and AS10/DA07. Only sex-shared neurons are labeled. For the adult male, maximum projections were generated using the same file as for Figure 4B, lateral view. Sex-shared neurons are labeled in white. White arrowheads indicate to sexually dimorphic neurons. Bar, 10 μm. 5HT, 5-hydroxytryptamine; VACHT, vesicular ACh transporter; VGAT, vesicular GABA transporter; VGLUT, vesicular glutamate transporter.

Table 4 Comparison of neurotransmitter usage in the male-specific, hermaphrodite-specific, and sex-shared nervous system

Attributes	Shared Nervous System (294 Neurons)		Male-specific (93 Neurons, %)	Hermaphrodite-Specific (8 Neurons, %)
	Hermaphrodite (%)	Male (%)		
Acetylcholine	151 (51)	153 (52)	48 (52)	8 (100)
Glutamate	78 (27)	78 (27)	16 (17)	0 (0)
GABA[a]	33 (11)	33 (11)	11 (11)	0 (0)
Aminergic[a] (5HT, DA, OA, Tyr)	17 (6)	19 (6)	20 (22)	2 (25)
Orphan VGAT(+)[b]	5 (2)	5 (2)	4 (4)	0 (0)
None of the above[c]	22 (7)	20 (7)	10 (11)	0 (0)
Cotransmission[d]	12 (4)	14 (5)	16 (16)	2 (25)

Percentages in parentheses indicate the respective neuron number relative to the total number of neurons in the shared or sex-specific nervous system. Note that numbers do not simply add up to the total number of neurons because some neurons use multiple transmitters. GABA, γ-aminobutyric acid; 5HT, 5-hydroxytryptamine; DA, dopamine; OA, octopamine; Tyr, tyramine; VGAT, vesicular GABA transporter; Glu, glutamate.

[a] Only those that antibody-stain for GABA or 5HT and express the vesicular transporter to release GABA or 5HT. This excludes a number of neurons that only take up the respective neurotransmitter and/or release it by unknown means, if they release it all.

[b] These neurons do not contain GABA and must transport some other transmitter, possibly glycine (see *Discussion*). Only neurons that exclusively express *unc-47/VGAT* are included in this count, many others express this gene as well (see text).

[c] Refers to neurons that do not express any known neurotransmitter transporter: ASI, AVF, AVH, AVJ, AWA, BDU, PVM, PVW only in hermaphrodites, RIP, RMG, and I4. Note we have also included the CAN in this category since no synthesizing enzymes for monoamines are expressed in this neuron class.

[d] Refers to the following combinations: ACh + Glu, ACh + GABA; ACh, Glu, or GABA neurotransmitters + aminergic transmitters [only cat-1(+)] (ACh/5HT; Glu/5HT, Glu/DA, Glu/Tyr; GABA/5HT).

nervous system also apply to the male-specific nervous system; ACh is the most commonly used neurotransmitter systems and GABA the most infrequently used (Table 4, Figure 8, and Table S1). Compared to the sex-shared nervous system, Glu is less frequently used and, in contrast to the sex-shared system, is almost exclusively used in sensory neurons (Table 1 and Table 4).

There is no apparent correlation of neurotransmitter usage with lineal history of the neuron (Table S1), meaning that lineally-related neurons can use different neurotransmitters (example: P10.pp-derived glutamatergic HOA *vs.* cholinergic HOB) and neurons using the same neurotransmitter can be lineally unrelated (example: Glu neurons derive from the Y, T, V, and P-lineage). There is also no clear correlation of neurotransmitter usage with function or connectivity. For example, ACh is used by sensory, inter-, and motorneurons that operate in different male circuit contexts.

Cotransmission in the male nervous system

Dale's principle ("one neuron - one neurotransmitter") has long been overturned in vertebrate nervous systems (Vaaga *et al.* 2014) and in *C. elegans* as well. Here, we add a number of new cases of cotransmission (summarized in Table 4 and Table S1); we find that the PVV interneuron and the sex-shared PVN interneuron cotransmit ACh and Glu, the only two cases of such cotransmission in the male and hermaphrodite nervous system. ACh and GABA are cotransmitted by CP09, R2A, and R6A. We also identified instances of Glu cotransmission with monoamines, namely with dopamine (R5A and R9A), 5HT (CP05 and CP06 neurons), and tyramine (HOA; interestingly, tyramine synthesis is lost in older animals). Cholinergic neurons can also cotransmit with monoamines, but in the male nervous system there is only a single clear example, the PGA interneuron, that coexpresses the vesicular ACh transporter *unc-17/VACHT* and the monoamine vesicular transporter *cat-1/VMAT* and stains for 5HT (R8A/B neurons express *tdc-1*, required to synthetize tyramine but no *cat-1/VMAT* is detected). Similarly, we only identified one example of GABA/5HT cotransmission in the R9B neuron. Overall, the percentage of sex-specific neurons that use more than one transmitter system is four times higher than in the sex-shared neurons (Table 4). Different combinations of neurotransmitters are observed, mostly entailing one aminergic transmitter.

Monoaminergic uptake neurons

Our analysis of monoaminergic neurons, which included improved (fosmid-based) reporter transgenes (for *cat-1/VMAT* and *tph-1*) and an improved 5HT antibody-staining protocol, revealed a few interesting discordances (summarized in Table 2). The majority of neurons that contain a monoamine also express the biosynthetic enzyme and a cognate vesicular transporter. However, several neurons (half of them male-specific) contain 5HT, as assessed by antibody staining, and express the vesicular transporter *cat-1/VMAT* but do not express the 5HT-synthesizing enzyme *tph-1* (Table 2), suggesting that these neurons take up 5HT (likely via a dedicated 5HT reuptake transporter, *mod-5/SERT* (Jafari *et al.* 2011), for ensuing release. Other 5HT antibody-staining neurons express neither the 5HT biosynthetic enzyme nor the vesicular transporter, suggesting that these neurons constitute clearance neurons that remove 5HT without ensuing reusage as a neurotransmitter. Such clearance cells exist for other transmitter systems as well (Gendrel *et al.* 2016) and may serve to limit the diffusion of neurotransmitters.

Neuronal subclasses express distinct neurotransmitters

Previous studies of the A-type and B-type ray sensory neurons have already underscored their molecular diversity: distinct subtypes of A-type and of B-type motorneurons can be defined through the subtype-specific patterns of usage of monoamines and neuropeptides (Lints *et al.* 2004), a notion now further supported by additional neurotransmitter systems (ACh, GABA, and Glu, as well as expression of *unc-47/VGAT* in non-GABAergic neurons). This

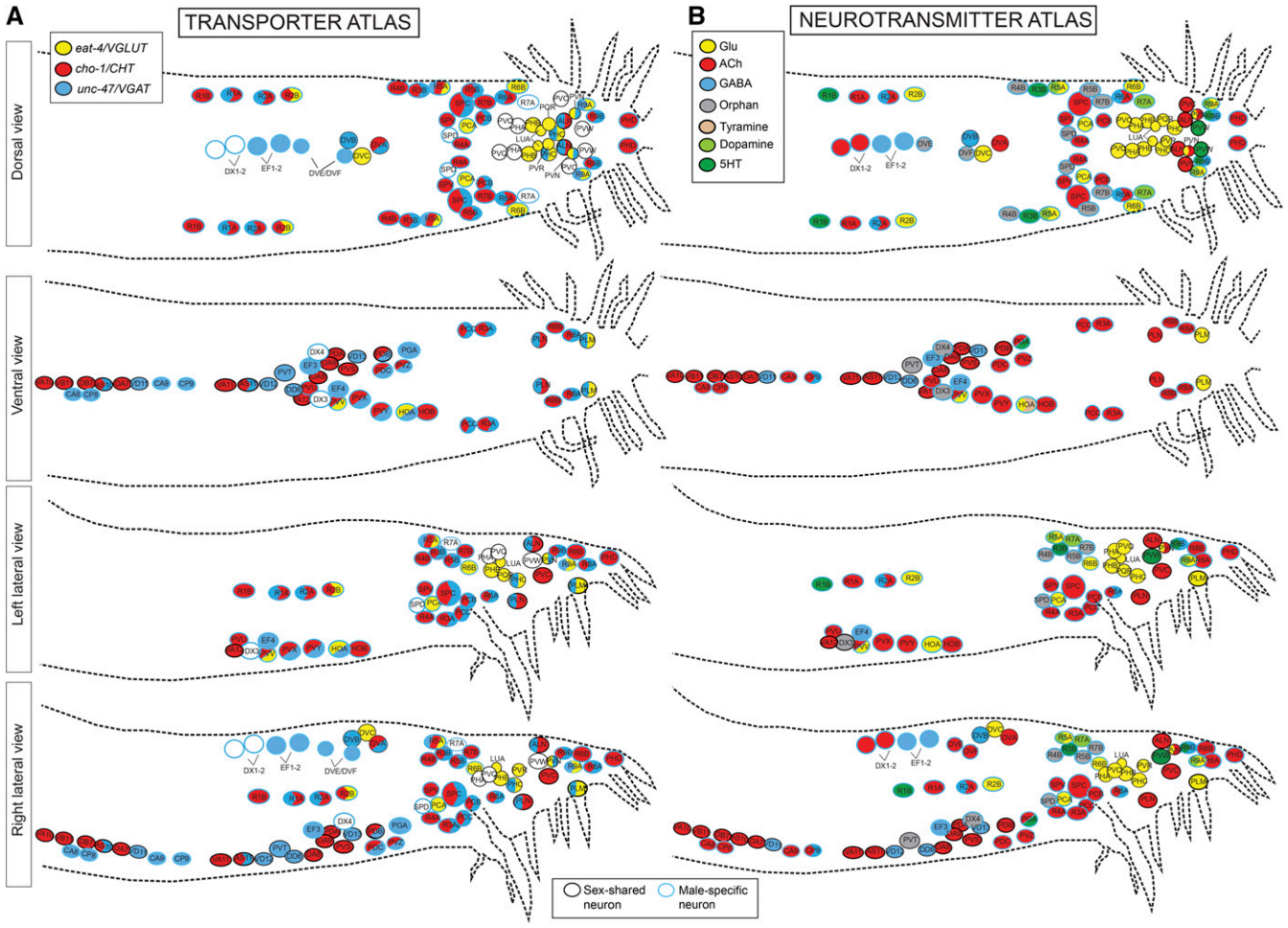

Figure 8 Atlas of the adult male tail. (A) Landmarks for cell identification in *C. elegans* adult male tail using fosmid reporter transgenes for the vesicular neurotransmitter transporters *eat-4/VGLUT* and *unc-47/VGAT*, and the reuptake transporter *cho-1/CHT*. The schematics show the reporter transgene expression pattern in the male tail. Black circle: sex-shared neurons. Blue circle: male-specific neurons. (B) Neurotransmitter atlas of the *C. elegans* male tail. The current status of the *C. elegans* neurotransmitter atlas in the male tail is shown presenting the sex-shared (black circle) and the male-specific (blue circle) neurons. 5HT, 5-hydroxytryptamine; ACh, acetylcholine; GABA, γ-aminobutyric acid; Glu, glutamate; VGAT, vesicular GABA transporter; VGluT, vesicular glutamate transporter; CHT, choline transporter.

subclassification does not correlate with the connectivity-based classification of "response rays" and "PVV rays" (Jarrell *et al.* 2012). Neurotransmitter diversity is also seen in the CP ventral cord neuron class, which was reported to be distinct based on connectivity and patterns of serotonin and neuropeptide usage (Loer and Kenyon 1993; Jarrell *et al.* 2012; Kalis *et al.* 2014). The subclass-specific usage of Glu and GABA as well as the differential expression of *unc-47/VGAT* and *unc-17/VACHT* in subsets of CP neurons that we report here further corroborates a subclass diversification into at least six distinct subclasses (CP0, CP01-04, CP05-06, CP07, CP08, and CP09; Table S1).

Sexual dimorphisms in neurotransmitter usage in sex-shared neurons

While examining the male-specific nervous system, we also assessed whether sex-shared neurons display dimorphisms in neurotransmitter usage. In the context of our analysis of ACh neurons, a switch in neurotransmitter usage in the sex-shared AIM head interneuron was previously noted (Pereira *et al.* 2015). These neurons switch from a glutamatergic to a cholinergic identity upon sexual differentiation of males, but not hermaphrodites. A sexually dimorphic scaling of *eat-4/VGLUT* expression in the sex-shared PHC neurons has also been reported (Serrano-Saiz *et al.* 2017). In this article, we have uncovered other cases of sexually dimorphic neurotransmitter usage differences in sex-shared neurons. The interneuron AVG showed a fivefold upregulation of the expression of *unc-17/VACHT* in males compared to hermaphrodite adult animals. The PVW interneurons become serotonergic only in the male where we have detected 5HT staining and *cat-1/VMAT* expression. The PVN interneurons acquire a glutamatergic identity during sexual maturation and start cotransmitting ACh and Glu in the adult male. In other cases, there is an induction of *unc-47/VGAT* expression in cholinergic neurons (PDB, AS11, and AS10/DA07). In most of the cases, upregulation or addition of a neurotransmitter system

correlates with large increases in synaptic connections (Table 4). All the neurotransmitter sexual dimorphisms described previously and in this study have a similar temporal onset of induction during sexual maturation at the fourth larval stage.

Neurons with no presently assigned neurotransmitter

Thirteen neurons in the male-specific nervous system have not yet been assigned a neurotransmitter identity (MCML/R, DVE, DVF, DX3/4, R4BL/R, R5BL/R, R7BL/R, and SPDL/R) (Table 4). Similarly, ~8% of sex-shared neurons also do not yet have a neurotransmitter assigned (ASI, AVF, AVH, AVJ, AVK, AWA, BDU, CAN, PVM, PVT, PVW only in hermaphrodites, RID, RIP, RMG, I4, and I6) (Pereira *et al.* 2015; Gendrel *et al.* 2016). A number of reasons may account for this: first, two more *eat-4*-like VGLUT-encoding genes are present in the *C. elegans* genome. We have not been able to reliably detect robust expression of these two genes in the nervous system, but this may be for technical reasons. Second, other neurotransmitter systems may await identification in *C. elegans* (Hobert 2013). Third, those neurons might be releasing only neuropeptides [based on the expression of neuropeptide-processing enzymes (Stefanakis *et al.* 2015), all neurons in *C. elegans* are predicted to contain neuropeptides]. Fourth, neurons may induce a known neurotransmitter system only when subjected to specific stresses. As a precedent, the sensory neuron ASG acquires a serotonergic phenotype under hypoxic conditions (Pocock and Hobert 2010). Lastly, it is also possible that our mostly fosmid-based reporters lack regulatory elements. We consider this unlikely given the congruence of the fosmid reporters with either reporter-tagged endogenous alleles and/or antibody staining.

Additional neurotransmitter usage suggested by UNC-47/VGAT expression in non-GABAergic neurons

C. elegans UNC-47/VGAT is the sole vesicular GABA transporter, but it is also expressed in 76 non-GABAergic neurons. Five of them (AVKL/R, I6, RID, and PVT) are orphan neurons, *i.e.*, neurons with currently unknown neurotransmitter identity. In addition, 48 male-specific neurons expressed *unc-47/VGAT* and are GABA-negative, including four orphan neurons (R5BL/R, DVE, and DVF). Nine sex-shared neurons expressed *unc-47/VGAT* and are GABA-negative exclusively in males (PDB, PHCL/R, PVNL/R, ADFL/R, AS11, AS10, or DA7) and one sex-shared neuron (PQR) exclusively expressed *unc-47/VGAT* and is GABA-negative in hermaphrodites. Apart from GABA, UNC-47/VGAT can transport glycine (Aubrey *et al.* 2007), but the *C. elegans* genome encodes no obvious sequence orthologs to glycine receptors. However, it is conceivable that one of the many orphan ligand-gated channel families in the worm genome may be gated by glycine. β-alanine is also transported by VGAT in vertebrates (Juge *et al.* 2013) and is thought to act via $GABA_A$ receptors (Tiedje *et al.* 2010). At this point, it remains unclear whether *unc-47/VGAT*-positive neurons utilize glycine, β-alanine, or yet another small molecular neurotransmitter.

Neurotransmitter atlas as a tool for cell identification and cell identity

One immediate and practical utility of our neurotransmitter maps lies in its use as a tool for neuron identification. In *C. elegans*, gene expression is characterized mostly through the analysis of reporter gene expression patterns. The most definitive strategy to identify the sites of expression of a novel reporter transgene is to assess its colocalization with a well-characterized reporter "landmark" transgene. The availability of such landmark reporters has been very sparse for the male tail, but is particularly relevant for the male tail in which cell body position is variable. As we have shown here, our red fluorescent *eat-4/VGLUT*, *cho-1/CHT*, and *unc-47/VGAT* reporter transgenes can provide a set of unique landmarks that can help to unambiguously identify the expression pattern of novel reporter transgenes. This set of landmarks is schematized in Figure 8.

Other usages of the neurotransmitter atlas

In addition, as amply demonstrated in the past, neurotransmitter identity serves as a useful entry point to study mechanisms of neuronal identity control (*e.g.*, Lints and Emmons 1999; Sze *et al.* 2002; Flames and Hobert 2009; Serrano-Saiz *et al.* 2013; Kalis *et al.* 2014; Zhang *et al.* 2014; Pereira *et al.* 2015; Gendrel *et al.* 2016). We anticipate that our neurotransmitter atlas, summarized in Figure 8, will provide further entry points into studying neuron identity control. Lastly, knowledge of neurotransmitter identity will be instrumental in manipulating neuronal circuit activity.

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