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Sexually dimorphic differentiation of a *C. elegans* hub neuron is cell-autonomously controlled by a conserved transcription factor

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SUMMARY

Functional and anatomical sexual dimorphisms in the brain are either the result of cells that are generated only in one sex, or a manifestation of sex-specific differentiation of neurons present in both sexes. The PHC neurons of the nematode *C. elegans* differentiate in a strikingly sex-specific manner. While in hermaphrodites the PHC neurons display a canonical pattern of synaptic connectivity similar to that of other sensory neurons, PHC differentiates into a densely connected hub sensory/interneuron in males, integrating a large number of male-specific synaptic inputs and conveying them to both male-specific and sex-shared circuitry. We show that the differentiation into such a hub neuron involves the sex-specific scaling of several components of the synaptic vesicle machinery, including the vesicular glutamate transporter *eat-4/VGLUT,* induction of neuropeptide expression, changes in axonal projection morphology and a switch in neuronal function. We demonstrate that these molecular and anatomical remodeling events are controlled cell-autonomously by the phylogenetically conserved Doublesex homolog *dmd-3,* which is both required and sufficient for sex-specific PHC differentiation. Cellular specificity of *dmd-3* action is ensured by its collaboration with non-sex specific terminal selector-type transcription factors whereas sex-specificity of *dmd-3* action is ensured by the hermaphrodite-specific, transcriptional master regulator of hermaphroditic cell identity, *tra-1*, which represses transcription of *dmd-3* in hermaphrodite PHC. Taken together, our studies provide mechanistic insights into how neurons are specified in a sexually dimorphic manner.

INTRODUCTION

Male and female brains display sexual dimorphisms on the level of function, anatomy and gene expression. In theory, such dimorphisms can be a reflection of two distinct scenarios, one in which dimorphisms are the result of the presence of neurons exclusively in one sex but not the other, and another in which neurons are present in both sexes (“sex-shared neurons”) but acquire sex-specific features [1, 2]. These scenarios are hard to untangle in

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E.S.-S. performed all experiments except the behavioral experiments which were performed by M.O.-S. (mating) and E.B. (touch). E.S.-S. and O.H. wrote the paper.

vertebrate nervous systems, since their cellular complexity confounds the visualization and systematic comparison of individual neuron types between the two sexes. In contrast, clear evidence exists for both scenarios in the *Drosophila* nervous system, that is, both sex-specific neurons, as well as sex-specific anatomical features of sex-shared neurons have been unambiguously mapped [1–5]. However, dimorphic features of shared neurons have so far been only studied on a relatively coarse level and, aside from the well-characterized sex-specific regulatory factors Fruitless and Doublesex [2], there are relatively few molecular and functional features that have been mapped onto sex-shared, but sexually dimorphic neurons.

In the nematode *C. elegans,* sexually dimorphic features have been analyzed with unprecedented anatomical and molecular resolution. Like in other organisms, the *C. elegans* nervous system contains sex-specific neurons found exclusively in either the male or the hermaphrodite (a somatic female)[6, 7]. Moreover, there are neurons present in both sexes that share an identical lineage history, position, molecular features and overall appearance but that display sexually dimorphic synaptic connectivity patterns [8, 9]. In one set of striking examples, the sex-shared phasmid neurons PHA and PHB connect in a sexually dimorphic manner to distinct downstream interneurons which are also sex-shared [8, 9].

Apart from sexual dimorphisms of synaptic connectivity between sex-shared neurons, there are also sexually dimorphic synaptic connections between sex-shared neurons and sex-specific neurons [8]. Relatively few sex-shared neurons receive synapses from sex-specific neurons and these neurons can be considered “hub neurons” since they connect sex-specific network modules to the shared and non-sex specific “core nervous system” [8]. The most remarkable examples of such hub neurons are the PHC sensory neuron, and the DD6 and PDB motorneurons. All three neurons are sex-shared and receive a tremendous amount of additional synapses from male-specific neurons [8]. PHC stands out among those, because unlike DD6 and PDB, it is not a motor neuron that directly connects male-specific sensory inputs into muscle. As schematically diagrammed in Fig. 1A, PHC rather connects in a sexually dimorphic manner to a diverse set of inter- and motorneurons and of all neurons in the animal, it is the neuron that receives the most male-specific synaptic inputs from a variety of different sensory neurons. This is in notable contrast to the synaptic connectivity of PHC in hermaphrodites, where PHC appears to be a canonical sensory neuron receiving few synaptic inputs and generating modest outputs on a number of interneurons (Fig. 1A) [9]. Taken together, PHC undergoes a sexual differentiation process in males that transforms this neuron from a conventional sensory neuron to a hub neuron with sensory and interneuron properties. In this manuscript we assign sex-specific functions to PHC, describe a number of distinct molecular features of the PHC neurons, which parallel its differentiation into a hub neuron and identify a transcription factor required and sufficient for male-specific differentiation of PHC into a hub neuron.

RESULTS

Sex-specific differences in PHC neuron function

We first set out to define a behavioral consequence of the sex-specific wiring of the PHC neuron. We generated transgenic animals in which the PHC neurons can be silenced in an

inducible manner with a histamine-gated chloride channel [10]. Based on its direct innervation of command interneurons, a unique feature of several, previously described nociceptive neurons [9, 11], we tested the response of these transgenic animals to nociceptive stimuli and found that silencing of PHC results in defects in the response to harsh touch applied to the tail of the hermaphrodite (Fig. 1B). In contrast, silencing of PHC in males has no effect on the tail harsh touch response, suggesting that another neuron is now responsible for this response in the male tail and consistent with the absence of PHC innervation of command interneurons in males (Fig. 1A).

We found that male PHC becomes repurposed for male mating behavior since PHC silenced males show profound defects in vulva location behavior. Instead of stopping when the male tail has reached the vulva, PHC-silenced animals continue to search for the vulva (Fig. 1C). This phenotypic defect is consistent with the male-specific PHC wiring pattern: two sensory neurons that innervate PHC (PHB and HOA), as well as an interneuron (AVG) innervated by all three of these sensory neurons were previously shown to be involved in the same vulval stop behavior [12, 13]. Similar defects can be observed upon genetically disrupting the neurotransmitter system, glutamate, used by all these neurons (PHB, PHC, HOA) (Fig. 1C).

Sex-specific, cell-autonomous morphological differentiation of the PHC neurons

Having established a sex-specific function of PHC, we set out to define the sex-specific differentiation program of PHC in more detail. The electron micrographic analysis of two adult hermaphrodite tails and one adult male tail indicates that apart from their notably distinct synaptic connectivity patterns, the PHC neurons display different axon and dendrite lengths [8, 9, 14]. We used a reporter transgene to visualize the sexually dimorphic morphology of PHC in a larger cohort of animals, and we examined its previously undocumented dynamic differentiation during larval stages (Fig. 2A). We find that before sexual maturation, up until the L3 stage, the PHC dendrite extends into the tail tip of both sexes (Fig. 2A, purple arrow). During retraction and remodeling of the male tail hypodermis, the PHC dendrite then retracts and meanders (Fig. 2A). The PHC axons extend initially only into the pre-anal ganglion of both sexes. However, beginning at L4 stage, when male-specific neurons are generated and start to differentiate, the axon of male but not hermaphrodite PHC extends significantly beyond the pre-anal ganglion into the ventral nerve cord (Fig. 2A, B), to gather synaptic inputs from male-specific neurons and innervate newly generated male-specific motor neurons as well as sex-shared neurons (Fig. 1A)[8].

We asked whether this sex-specific extension of the PHC axons depends on male-specific synaptic targets which may secrete attractive cue(s) or on any other male-specific structure or whether sex-specific PHC axon extension is controlled in a cell-autonomous manner. To this end, we altered the sex of PHC in a cell-autonomous manner through manipulation of the activity of the Gli transcription factor TRA-1, the master regulator of sexual differentiation [15, 16]. TRA-1 is expressed in all hermaphroditic cells and required autonomously to promote hermaphroditic cellular identities and repress male identities [17]. In males, TRA-1 is downregulated via protein degradation (Fig. 2C)[18, 19]. We feminized male PHC through PHC-specific expression (*eat-4prom11Δ11* driver, from now on called *PHCp*) of the intracellular domain of TRA-2, *tra-2ic*, which constitutively signals to prevent

TRA-1 downregulation by FEM-3 and other factors [20, 21]. We find that in otherwise male animals this cell-autonomous feminization results in a failure of the PHC axon to undergo its characteristic extension through the pre-anal ganglion (Fig. 2C). Conversely, masculinization of PHC via *fem-3*-mediated degradation of TRA-1 protein in otherwise hermaphroditic animals results in a male-like extension of the PHC axon (Fig. 2C). These sex-reversal experiments confirm cell autonomy but also suggest that (1) the TRA-1 transcription factor represses a male morphological differentiation program in PHC and (2) that TRA-1 is both required and sufficient to control the male-specific features of PHC.

Transcriptional scaling of glutamatergic neurotransmission in the male PHC neuron

We find that sex-specific morphological changes of the PHC neurons are paralleled by a change in a number of molecular features. Specifically, the expression of the vesicular glutamate transporter *eat-4/VGLUT* is strongly upregulated in PHC, as assessed with a fosmid reporter construct (Fig. 3A). We quantified this upregulation through normalization of *eat-4/VGLUT* expression relative to *eat-4/VGLUT* expression in other neurons, and also relative to a ubiquitously expressed nuclear envelope protein-encoding gene, *mel-28* (Fig. 3B). We independently validated this upregulation using quantitative single mRNA fluorescence in situ hybridization (smFISH)(Fig. 3D). Both reporter gene and smFISH analysis indicate a three-fold upregulation in *eat-4/VGLUT* expression in male PHCs (Fig. 3). This upregulation begins at the late L4/young adult stage (Fig. 3C).

The increase in *eat-4/VGLUT* expression and synaptic output is accompanied by the male-specific transcriptional upregulation of other synaptic proteins including synaptotagmin (*snt-1*), synaptobrevin (*snb-1*) and Rab-3 (*rab-3*) (Fig. 3E,F). *ric-19*/ICA69, a gene involved in dense core vesicle mediated secretion, is not upregulated (Fig. 3E). Taken together, we define here a previously unrecognized transcriptional scaling phenomenon in which the generation of novel synaptic contact is mirrored by a neuron-type specific transcriptional upregulation of vesicular proteins.

We again used cell-specific sex reversal experiments to test whether the scaling of synaptic vesicle machinery requires the presence of the entire male-specific circuitry that PHC becomes wired into or whether it is controlled cell autonomously, like PHC axon extension. We find that masculinization of PHC via *fem-3*-mediated degradation of TRA-1 in otherwise hermaphroditic animals results in scaling of *eat-4/VGLUT* expression (Fig. 3G). Conversely, feminization of PHC in otherwise male animals using the intracellular domain of TRA-2, *tra-2ic*, expressed specifically in PHC, results in a failure to upregulate *eat-4/VGLUT* expression (Fig. 3G).

Sexually dimorphic neuropeptide expression in PHC

Fast synaptic transmission machinery is not the only neurotransmission-related feature that becomes modulated in male PHC. We found that a FMRFamide neuropeptide-encoding gene, *flp-11* (which encodes at least four different phylogenetically conserved peptides [22]) is expressed exclusively in PHC in adult males (Fig. 4A). *flp-11* is not expressed in either sex in early larval stages (L2, L3), after the birth of PHC in the L1 stage and before sexual maturation, but it becomes activated specifically in PHC in L4 males (Fig. 4B).

Like PHC axon extension and scaling of synaptic vesicle machinery, the male-specific induction of *flp-11* expression is controlled cell-autonomously. Masculinization of PHC via *fem-3*-mediated degradation of TRA-1 in otherwise hermaphroditic animals results in induction of *flp-11* expression (Fig. 4A) and, conversely, feminization of PHC in otherwise male animals using the intracellular domain of TRA-2, *tra-2ic*, expressed specifically in PHC, results in a failure to induce *flp-11* expression (Fig. 4A).

flp-11 mutants do not display defects in the vulva location behavior controlled by PHC (Fig. 1C), suggesting either redundancy of *flp-11* function with other neuropeptides or *flp-11*-dependent PHC involvement in the regulation of as yet unknown male-specific behaviors.

The *eat-4/VGLUT* locus contains *cis*-regulatory modules that confer sexually dimorphic expression

As a starting point to dissect the mechanistic basis of PHC remodeling, we turned to the *eat-4/VGLUT* locus and asked how its sex-specific scaling is controlled. The *eat-4/VGLUT* locus contains modular *cis*-regulatory elements that drive *eat-4/VGLUT* expression, and hence glutamatergic identity, in distinct glutamatergic neuron types of the hermaphrodite nervous system [23]. One module (*"prom5")* drives expression in PHC as well as multiple other glutamatergic neurons [23](Fig. 5A). We narrowed down this module to a 494bp region ("*prom11*") that not only recapitulates the sex-specific scaling of the endogenous *eat-4/VGLUT* locus in PHC during sexual maturation, but also shows sexually dimorphic expression in a single pair of head interneurons, the AIM neurons (Fig. 5C). In males, these neurons switch their neurotransmitter usage from glutamate (*eat-4/VGLUT* expression) to acetylcholine [24].

How is the expression of this *prom11* module controlled? Terminal differentiation of both the AIM and PHC neurons in hermaphrodites requires the LIM homeobox gene *ceh-14*, the *C. elegans* ortholog of vertebrate Lhx3/4 and its presumptive partner, the Brn3-like POU homeobox gene *unc-86* [23]. *ceh-14* and *unc-86* not only control *eat-4/VGLUT* expression in hermaphrodite PHC [24] but are also required for *eat-4/VGLUT* expression and scaling in males (Fig. S1). Predicted binding sites for UNC-86 and CEH-14 in the *eat-4prom11* element are necessary for expression of the *prom11* module in both AIM and PHC (Fig. 5B). The authenticity of the CEH-14 binding site is corroborated by the ModEncode consortium, which mapped a CEH-14 binding peak to the location of the *prom11* element [25].

To decipher the *cis*-regulatory logic of sex-specific modulation of *eat-4/VGLUT* expression in PHC, we further dissected the *prom11* module. We expected to either define sex-specific repressor elements that may counteract the non-sex specific activity of *unc-86* and *ceh-14* (deletion of such repressor elements should result in depression of *eat-4prom11* expression in hermaphrodites) or to define sex-specific activator element(s) that may assist *unc-86* and *ceh-14* to provide sex-specific upregulation of *eat-4/VGLUT* expression (deletion of such elements should result in a failure to scale reporter gene expression). Through the introduction of deletions in the *prom11* module we found evidence for both mechanisms; beside the presumptive UNC-86 and CEH-14 binding sites, we mapped a 15bp repressor element whose deletion resulted in derepression of reporter expression in the PHC neurons of hermaphrodites (*prom11Δ18*; Fig. 5B,C). The hermaphrodite-specific TRA-1 protein

mentioned above, commonly thought to be a repressor protein [26], may act through this *cis*-regulatory element (however, we could not find any canonical TRA-1 binding site contained in *prom11*). Notably, the *Δ18* deletion in *prom11* also results in derepression of reporter gene expression in the AIM head neuron, but with opposite sex-specificity. While the *prom11* construct is only expressed in AIM in hermaphrodites, the *Δ18* deletion results in derepression in males (Fig. 5B,C).

In addition to the negative regulatory element that prevents the *prom11* module from being expressed in hermaphrodite PHC, we also identified an element that is required, in addition to the CEH-14 and UNC-86 sites, for the full extent of *eat-4/VGLUT* scaling in PHC in males (Fig. 5B,C; *Δ20*). Like the repressor element *Δ18* described above, the *Δ20* activator element also has an activating effect in AIM, but again with the opposite sexual specificity since the *Δ20* deletion results in a failure to activate reporter gene expression in hermaphrodite AIM (Fig. 5B,C).

In conclusion, these findings suggest that sex-specificity of *eat-4/VGLUT* scaling in PHC is achieved by combination of (a) non-sex specific activation, (b) specific repression in hermaphrodites (possibly via TRA-1) and (c) sex-specific activation in males (Fig. 5D). A similar activator/repressor logic operates in the AIM neurons, but with opposite sexual specificity (Fig. 5D).

dmd-3 is required and sufficient to cell-autonomously control male-specific PHC differentiation

To identify *trans*-acting factors that may control the male-specific upregulation of the *cis*-regulatory module of the *eat-4/VGLUT* locus mentioned above, we turned to the phylogenetically conserved family of Doublesex/DMRT transcription factors, of which there are 11 homologs encoded in the *C. elegans* genome. Sexually dimorphic, male-specific functions have been identified for five of these genes so far (*mab-3, mab-23, dmd-3, dmd-5, dmd-11*)[12, 27]. We analyzed mutants of most *dmd* genes and found that loss of *dmd-3* results in a failure to scale *eat-4/VGLUT* expression in PHC (Fig. 3B,D). This effect matches the *Δ20* deletion within the *prom11* module, suggesting that DMD-3 may act directly or indirectly through this module.

We next tested whether the activity of *dmd-3* is restricted to scaling *eat-4/VGLUT* expression, or whether *dmd-3* may be a “master regulator” of all PHC remodeling events that we described above. We find that in *dmd-3* mutants, the male-specific axon extension of PHC neurons fails to occur (Fig. 2B, Fig. 6B). Moreover, expression of the FMRFamides encoded by the *flp-11* locus does not become induced in PHC during sexual maturation of the male (Fig. 4A). We conclude that *dmd-3* affects all currently measurable aspects of male-specific PHC differentiation.

Upon sexual maturation, male tail hypodermis undergoes remodeling to form a copulatory structure and this remodeling requires hypodermally expressed *dmd-3* [28–30]. To examine the focus of *dmd-3* action, we first examined the previously uncharacterized neuronal expression pattern of *dmd-3.* Single molecule *in situ* hybridization demonstrates that *dmd-3* is expressed in a sex-specific manner not only in the male hypodermis, as previously

reported [29, 30], but also in the PHC neurons. PHC expression is only observed in males, but not hermaphrodites (Fig. 6A). Male-specificity is controlled cell-autonomously by *tra-1*, because masculinization of PHC using *fem-3* expression results in the induction of *dmd-3* expression in the PHC of hermaphrodites (Fig. 6A). This derepression is functionally relevant, since both the axon extension and *eat-4/VGLUT* scaling observed in transgenic hermaphrodites in which we masculinized PHC via *fem-3* expression requires *dmd-3* (Fig. 6C,D).

To corroborate that *dmd-3* acts in PHC, we generated transgenic animals in which *dmd-3* is expressed under control of the PHC (and AIM)-specific fragment of the *eat-4/VGLUT* locus. This construct rescues the axon extension defect of the PHC neurons in *dmd-3* mutant males (Fig. 6B) as well as the *eat-4/VGLUT* scaling defects of *dmd-3* mutants (Fig. 6D). Since the rescuing driver is active in both sexes, we could also assess whether ectopic expression of *dmd-3* in hermaphrodites is sufficient for axon extension of PHC and scaling of *eat-4/VGLUT* expression in a hermaphrodite. We indeed find that *dmd-3* is sufficient to induce these PHC features in hermaphrodites (Fig. 6C,D). We conclude that *dmd-3* is both required and sufficient to autonomously specify the male-specific identity differentiation program of PHC and that its sex-specificity is controlled by sex-specific repression via TRA-1.

DISCUSSION

While sexually dimorphic neuronal projection patterns have been observed in many different species from flies to humans [3, 4, 31], the simplicity and well-described nature of the *C. elegans* nervous system reveals neuronal sexual dimorphisms with unrivaled anatomical, cellular and molecular resolution. The case of the PHC neurons particularly stands out because among sex-shared neurons, PHC displays the largest extent of sexually dimorphic connectivity [8]. Other sensory neurons, like PHA or PHB, also switch synaptic partners between the different sexes [8], but they do not differentiate into a densely connected hub neuron as PHC does. Differentiation into a sex-specific hub neuron is not only characterized by changes of *en passant* synaptic target choices, but apparently requires the coordination of a multitude of anatomical and molecular processes. These include the sex-specific extension of the PHC axon, changes in neuropeptide usage and a transcriptional scaling phenomenon that affects the synaptic vesicle machinery. Cellular differentiation is often accompanied by the scaling of specific subcellular structures and their molecular components in order to adapt to the specialized needs of a cell [32], but such scaling phenomena have not previously been described in the context of the synaptic vesicle machinery. To the contrary, the expression levels of synaptic vesicle components are particularly resilient to genetic perturbations, a robustness that is ensured by their control through multiple redundant *cis*-regulatory elements [33].

Two remarkable aspects of the sex-specific differentiation process of PHC into a hub neuron are (a) its cellular autonomy and hence independence from other cellular network components and (b) the apparent coordination of different aspects of this sex-specific differentiation process by a single transcription factor, *dmd-3. dmd-3* is required and sufficient to trigger the male-specific program of this neuron, including the scaling of the synaptic machinery. *dmd-3* operates in a highly context-dependent manner. As previously

shown, *dmd-3* is required in skin cells for their remodeling during male tail morphogenesis [29, 30], in male-specific cholinergic ray neurons for their differentiation [34] and, as we have shown here, is independently required in a sex-shared neuron to remodel specific anatomical and molecular features. The specificity of action of *dmd-3* is determined by cell type-specific combinations of cofactors. In the case of the hypodermis and male-specific cholinergic neurons, DMD-3 interacts with another DMD factor (MAB-23)[29, 34], while in PHC *dmd-3* cooperates with the *unc-86* and *ceh-14* homeobox genes. For *dmd-3* to exert its effect on *eat-4/VGLUT* scaling, *unc-86* and *ceh-14* are required as permissive co-factors. One unanticipated conclusion from the *cis*-regulatory analysis of the synaptic scaling process is that the sex-specificity of this transcriptional regulatory phenomenon is not merely assured by one sex-specific regulatory factor that operates in one sex to either activate or repress a specific gene (Fig. 5D). Rather, sex-specific *eat-4/VGLUT* expression requires sex-specific repression in one sex (mediated by a specific *cis*-regulatory element that may be directly or indirectly controlled by TRA-1) and sex-specific activation in the opposite sex (mediated by a specific *cis*-regulatory element that may be directly or indirectly controlled by DMD-3).

The *C. elegans* genome encodes eleven predicted DM domain proteins, most of them uncharacterized. We hypothesize that these DM proteins may also intersect with other non-sex-specific differentiation programs to control sex-specific features in the nervous system, such as dimorphisms in synaptic wiring or the hermaphrodite-specific neurotransmitter switch of the AIM interneurons (Fig. 5D). Vertebrate genomes also encode multiple DM domain proteins [27] but their function in sexually dimorphic brain development awaits characterization. In the context of gonad development, DM domain proteins represent the only unifying theme of the otherwise very divergent regulatory mechanisms of sex determination and sexual differentiation throughout the animal kingdom [27].

EXPERIMENTAL PROCEDURES

Transgenes and DNA

A list of transgenes and information on DNA constructs used to generate transgenic lines can be found in the Supplementary Information.

Male mating assay

Mating assays were done as described previously [12, 13, 35]. Early L4-stage males were transferred to a fresh plates and kept apart from hermaphrodites until they reach sexual maturation (24 hours). Single virgin males were assayed for their mating behavior in the presence of 10–15 adult *unc-31(e928)* hermaphrodites on a plate covered with a thin fresh OP50 lawn. *unc-31* hermaphrodites move very little, allowing for an easy recording of male behavior. Mating behavior was scored within a 15 min time window or until the male ejaculated, whichever occurred first. Males were tested for their ability to locate vulva in a mating assay, calculated as location efficiency (L.E.)[36]. The number of passes or hesitations at the vulva until the male first stops at the vulva were counted. Location Efficiency = 1 / # encounters to stop. *PHCp::HisCl1* transgenic animals were transferred to NGM plates containing 10mM histamine a day prior to the mating assay [10, 12].

Harsh touch assay

Harsh touch assays were done as previously described [37]. L4 animals were separated by sex and transferred to either NGM plates or NGM plates containing 10mM histamine (both seeded with OP50) and allowed to mature to adulthood (24 hours). For the assay, adults were transferred to NGM or NGM plus histamine plates seeded with a thin fresh OP50 lawn. Harsh touch stimulus was administered to the anus of the worm using a platinum wire pick while the worm was stationary. An animal that initiated abrupt forward locomotion in response to harsh touch was considered "responding." Each animal was only assayed one time.

Single molecule FISH

smFISH was done as previously described [38]. Young adult animals were incubated over night at 37°C during the hybridization step. *dmd-3* and *eat-4* probes were designed by using the Stellaris RNA FISH probe designer and were obtained already conjugated with Quasar 670 and purified from Biosearch Technologies. Both probes were used at a final concentration of 0.25 μM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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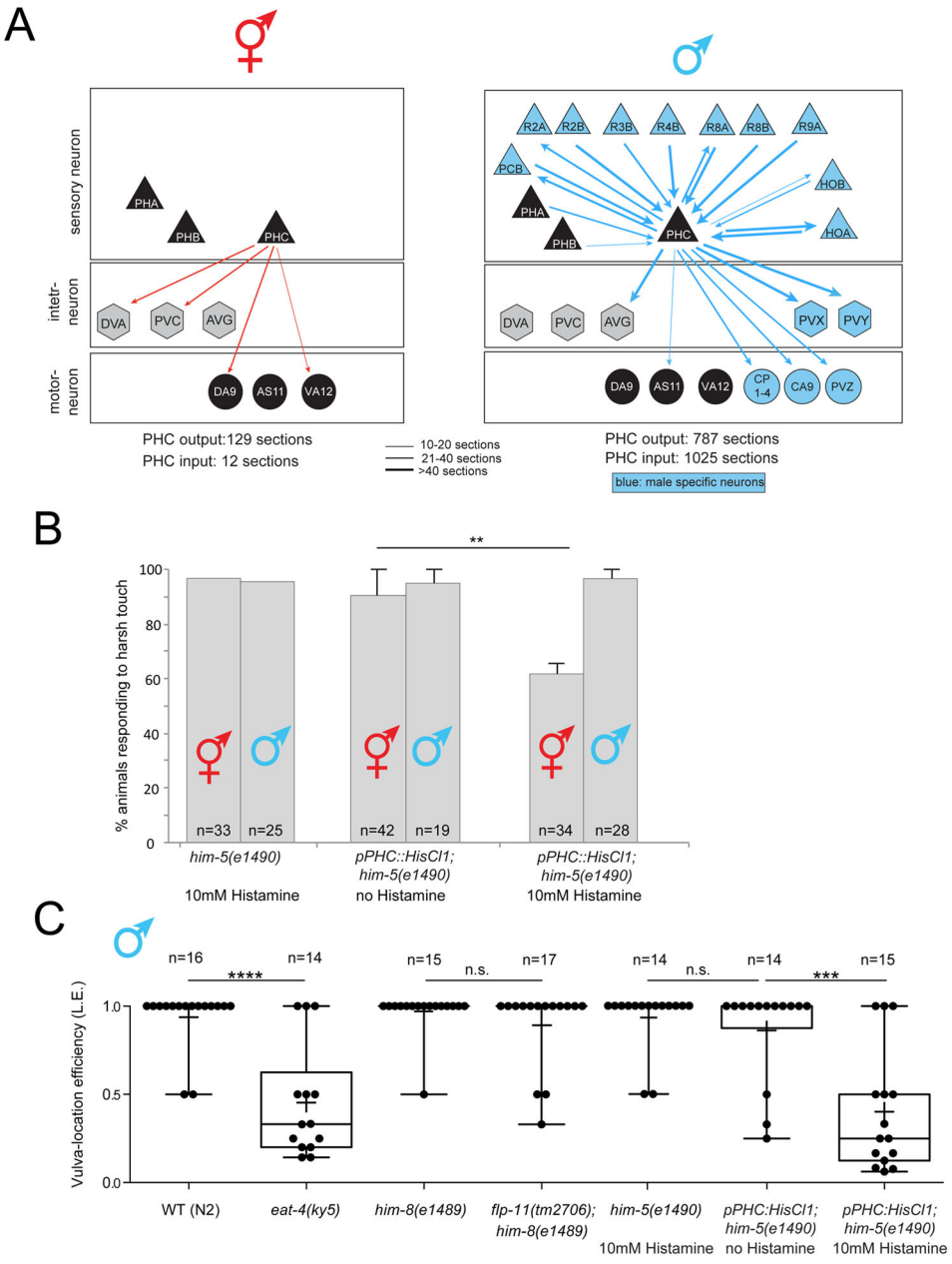


Fig. 1. Dimorphic synaptic connectivity and function of PHC

A: Schematic synaptic connectivity. Edge weights were collected from www.wormwiring.org [8]. Only connections that directly involve PHC are shown.

B: PHC is required in hermaphrodites for response to harsh (picking-force) touch to the tail. Bar graph indicates the mean percentage of animals responding to harsh touch, with error bars indicating the minimum and maximum from two experimental replicates. Significance was calculated using Fisher's exact test. ** $p<0.01$, n.s. $p>0.05$. "n" in each column indicates the total number of animals assayed.

C: PHC is required for a specific step of the male mating behavior, vulva search behavior. Mutant and Histamine-silenced animals tested for the male's vulva location efficiency. Box

plot representation of the data is shown, with whiskers pointing from min to max. Median and mean values are indicated by horizontal line and "+", respectively. Statistics was calculated using Kruskal-Wallis test. ****$P < 0.0001$, ***$P < 0.0005$. "n" above graphs indicates the total number of animals assayed.

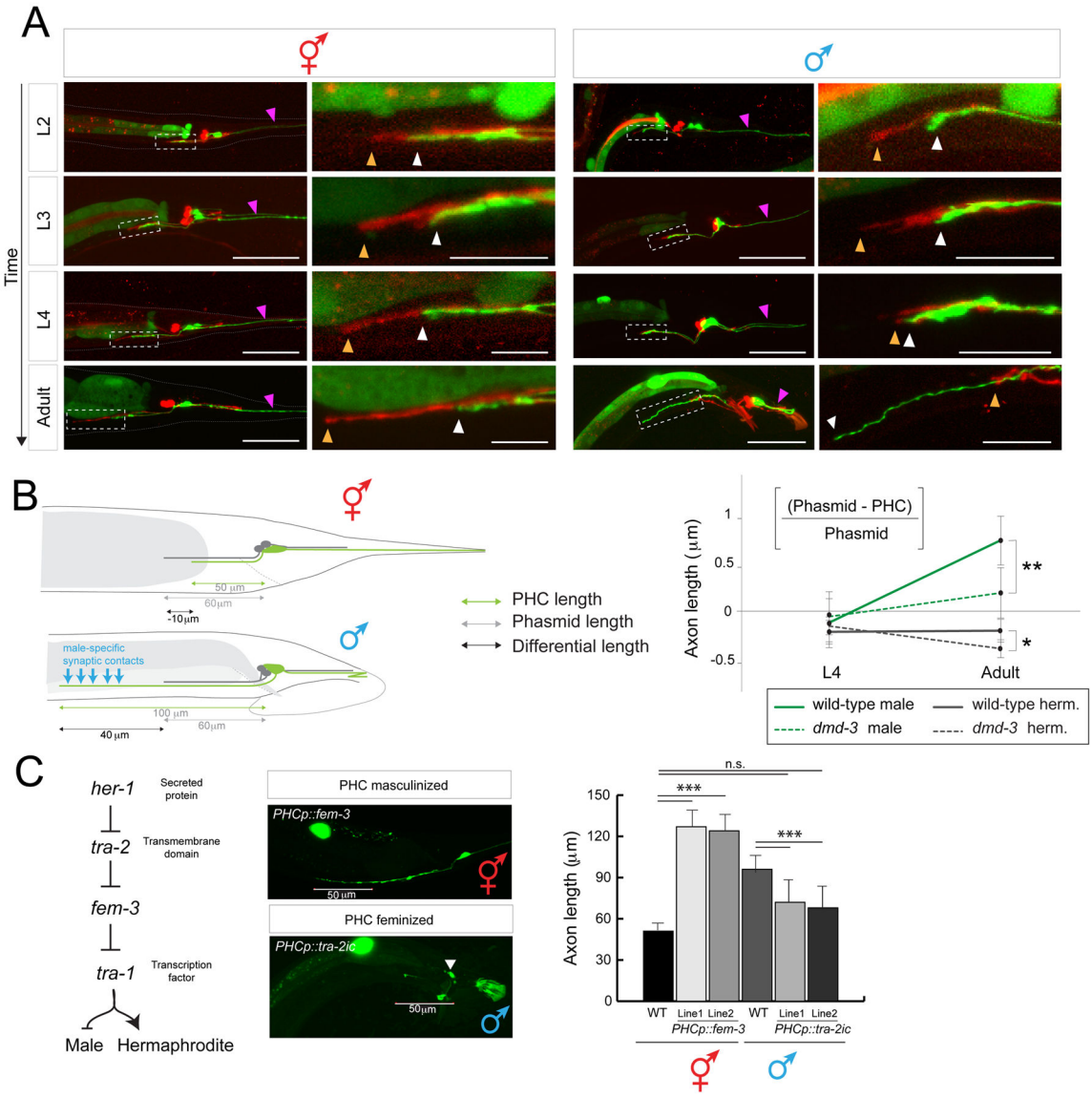


Fig. 2. Sexually dimorphic extension of the PHC axon

A: PHC axon and dendrite morphology at different developmental stages. PHC was visualized with the transgene *otEx6776*, which expresses *gfp* under the control of the *eat-4prom11Δ12* driver, which expresses in PHC of both males and hermaphrodites (described in more detail in Fig. 5). As landmark, the PHA and PHB neurons, which do not noticeably change morphology between the sexes, are filled with DiD (termination point of PHA/B marked with orange arrowhead, PHC with white arrowhead). PHC dendrites are marked with purple arrowhead. Scale bar: 50 µm. Dashed box indicates the area that is magnified in the right column. Scale bar in magnified panels: 10 µm.

B: Summary of PHC axon extension. Male specific synaptic contacts (referring to both inputs and outputs) are schematically indicated with blue arrows.

C: Male-specific axon extension is controlled cell-autonomously as determined by cell-specific sex change via manipulation of the sex-determination pathway. Transgenic array

names: *otIs520* (*eat-4prom11::gfp*) was crossed with *PHCp::fem-3* (lines *otEx6879* and *otEx6980*) and *PHCp::tra-2ic (lines otEx6881 and oEx6882)* strains. PHCp = *eat-4prom11Δ11* driver (Fig. 5B). Significance was calculated using student t-test, ***$P <$ 0.0005, **$P < 0.005$ and *$P < 0.01$. n.s. >0.1

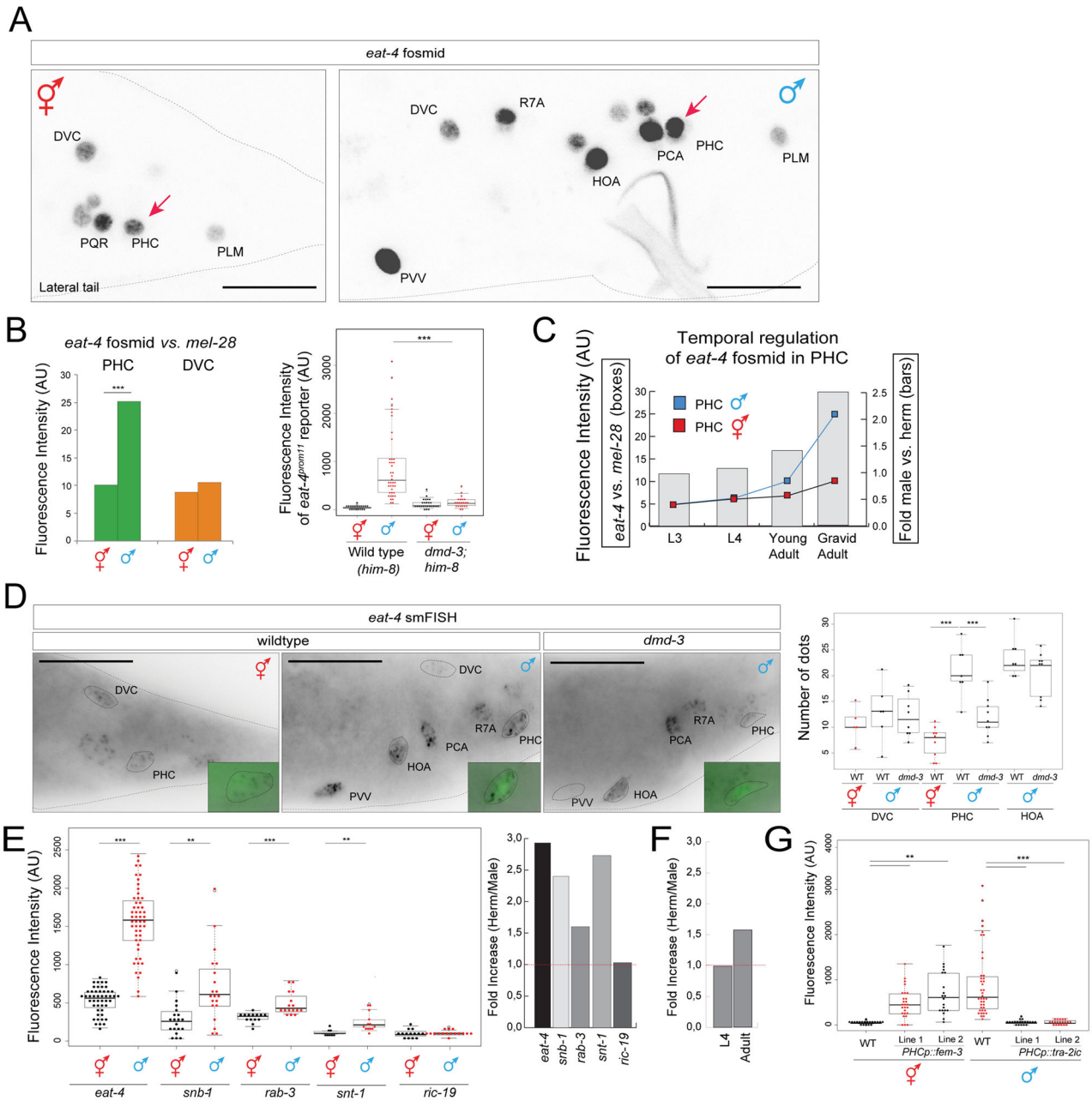


Fig. 3. Scaling of synaptic vesicle components in PHC

A: *eat-4/VGLUT* fosmid reporter expression (*otIs518*) in the adult male tail. The complete set of all male-specific, *eat-4/VGLUT*-expressing neurons will be reported elsewhere. Scale bar: 20μm.

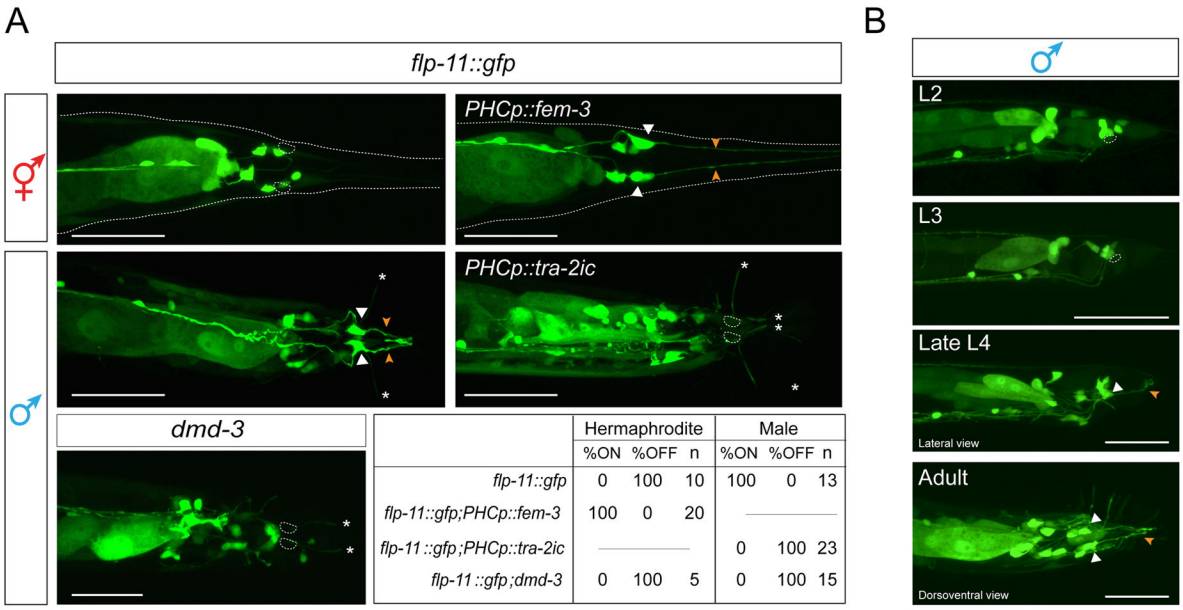
B: *eat-4/VGLUT* fosmid (*otIs518*) reporter expression measured by absolute fluorescence (right panel) and normalized to expression of the *mel-28* nuclear envelope protein (*bq5). otIs520* reporter expression measured by absolute fluorescence in *him-8(e1489)* background and *dmd-3(tm2863);him-8(e1489)* (left panel).

C: Temporal dynamics of scaling of *eat-4/VGLUT* fosmid (*otIs518*) reporter expression.

D: smFISH analysis of endogenous *eat-4/VGLUT* expression in young adult animals. PHC was marked with *otIs520* (green). See Fig. S2 for *eat-4* smFISH probe specificity. Scale bar: 20μm.

E,F: Scaling of transcription of other vesicular markers, as assessed by SL2-based fosmid reporter expression [33]. Temporal dynamic of *rab-3* fosmid (*otIs498*) expression in L4 vs. adult animals (**F**).

G: *eat-4/VGLUT* scaling is controlled cell-autonomously as shown by masculinization and feminization experiments. *otIs520* (*eat-4prom11::gfp*) was crossed with *PHCp::fem-3* (lines *otEx6879* and *otEx6980*) and with *PHCp::tra-2ic (lines otEx6881 and oEx6882)* strains. PHCp (*eat-4prom11Δ11* driver). Wildtype data showing in this plot is same as in panel B (left panel), genotypes were scored in parallel. Significance was calculated using student t-test, $***P < 0.001$, $**P < 0.01$. See Fig. S1 for more regulators of *eat-4* expression.



	Hermaphrodite			Male		
	%ON	%OFF	n	%ON	%OFF	n
flp-11::gfp	0	100	10	100	0	13
flp-11::gfp;PHCp::fem-3	100	0	20			
flp-11 ::gfp ;PHCp::tra-2ic				0	100	23
flp-11 ::gfp ;dmd-3	0	100	5	0	100	15

Fig. 4. Sexually dimorphic neuropeptide expression in PHC

A: *flp-11* reporter expression (*ynIs40)* in wildtype hermaphrodites and males, in animals in which the sex of PHC was changed via *fem-3* or *tra-2ic* expression and in *dmd-3(tm2863)* mutants. The table indicates the percentage of neurons that show expression of the *flp-11* reporter in the different conditions assayed. N=number of animals. Scale bar: 50 μm.

B: Temporal dynamics of *flp-11* reporter gene expression in larval and adult male stages. Cell bodies are labeled with white arrow, dendritic projections with orange arrow. White asterisks indicate ray projections. Scale bar: 50 μm.

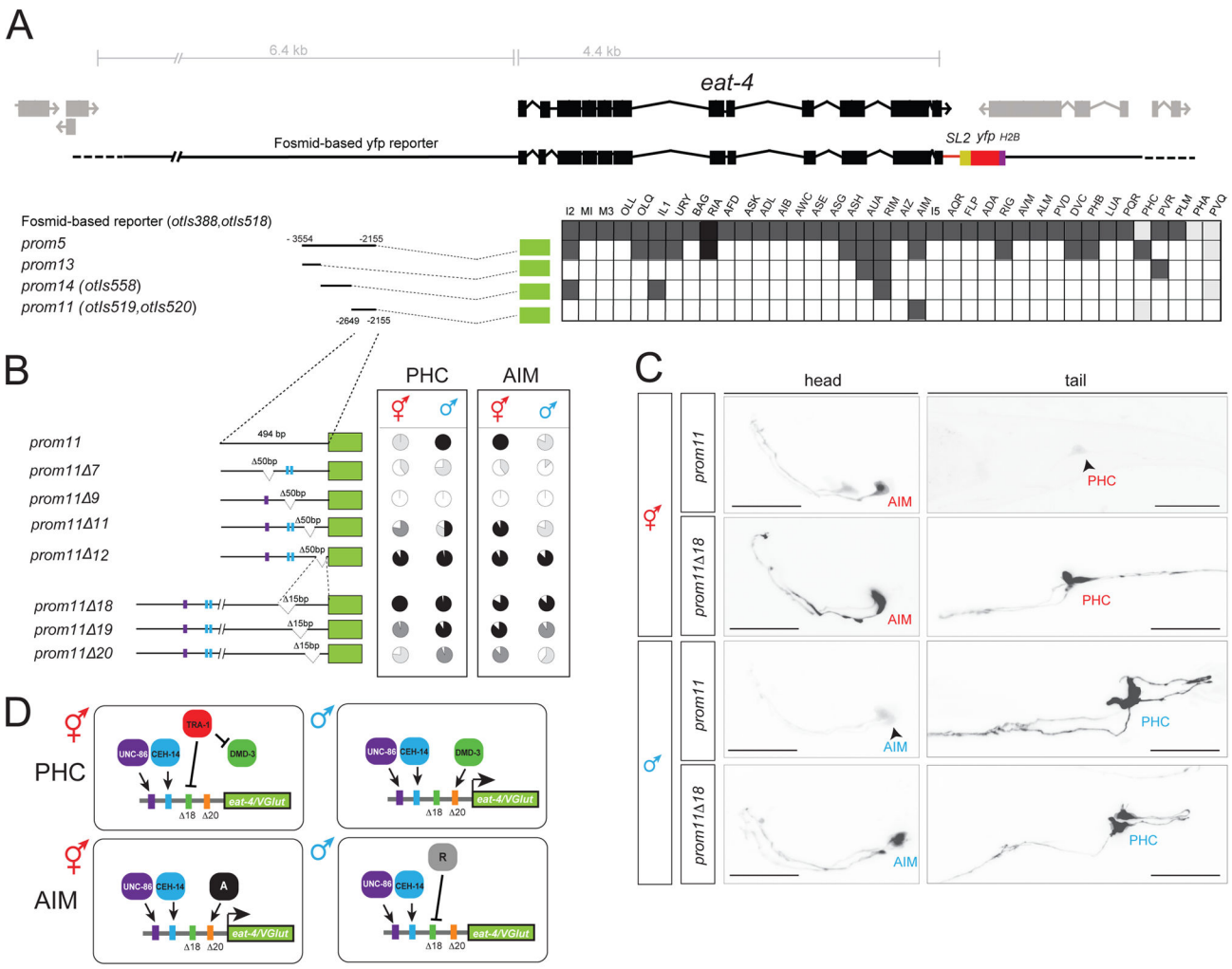


Fig. 5. Analysis of the *cis*-regulatory control elements of the *eat-4* locus

A: Dissection of the *eat-4prom5* promoter. In the analysis of expression between two and three lines (n > 10) were scored for expression. The different shades of gray indicate the relative fluorescence intensity.

B: Deletion analysis of the *eat-4prom11* promoter. The pie charts indicate the percentage of neurons that express the array with the four different shades denoting the overall fluorescence intensity in AIM and PHC in both sexes (white: no expression; black: strongest expression observed in any construct; light and dark grey: intermediate levels that are clearly distinct from each other and from maximum or no expression). At least two lines were analyzed for each deletion construct. Color bars in the promoters refer to putative homeodomain binding sites: blue bar indicates the CEH-14 binding site according to the ModEncode consortium; purple bar indicates the putative UNC-86 binding site. See Methods for sequences. See Fig. S1 for effect of CEH-14 and UNC-86 on *eat-4* expression.

C: *eat-4prom11* and *eat-4prom11Δ18* reporter expression in the head and tail of young adults. Scale bar: 25 μm.

D: Summary schematic of *eat-4/VGLUT* regulatory logic. The same elements are required to achieve activator and repressor effects in the PHC and AIM neurons, but with opposite

sexual specificity. In the AIM neurons, unknown factors “A” (for activator) and “R” (for repressor) may be different DMD transcription factors.

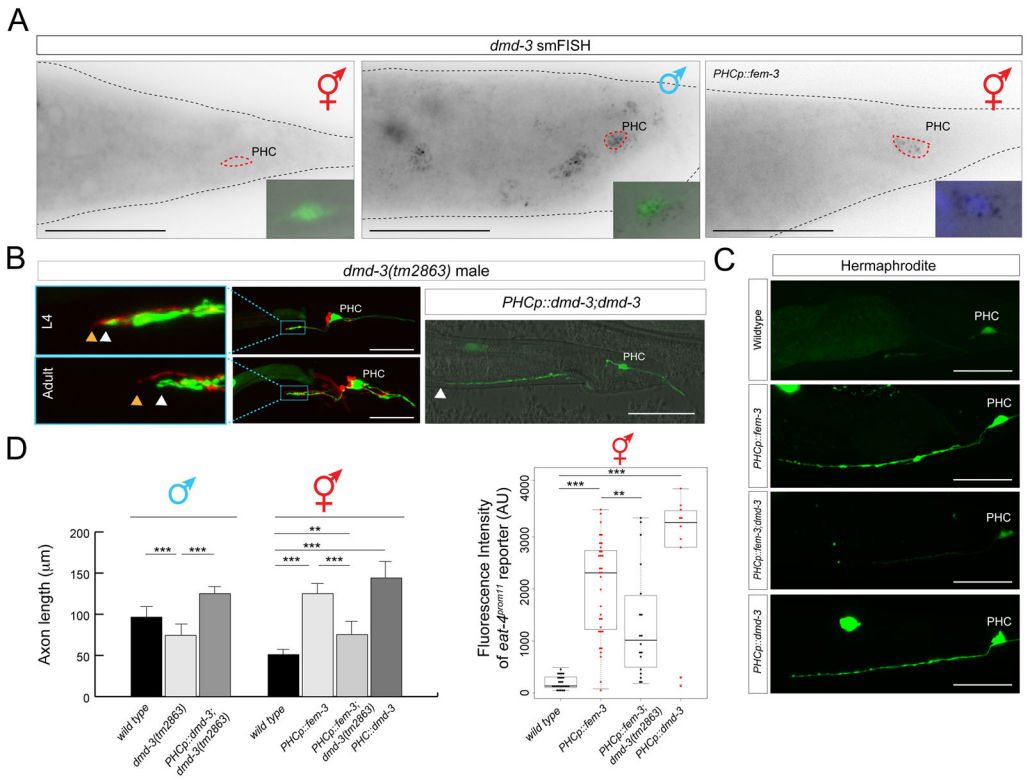


Fig. 6. *dmd-3* expression and function

A: smFISH analysis of endogenous *dmd-3* expression in young adult animals. No expression is observed in hermaphrodites; in males, expression is observed in multiple cells including PHC, marked with the transgenic array *otIs520*. Masculinization of PHC (via *PHCp::fem-3*) in otherwise hermaphroditic animals activates *dmd-3* transcription. The inset shows the PHC nucleus stained with DAPI. More than 10 animals were scored for presence of dots in each condition and all animals showed similar staining patterns relative to one another. See Fig. S2 for *dmd-3* smFISH probe specificity. Scale bar: 50 µm.

B: PHC axons fail to extend in *dmd-3(tm2863)* mutant males and these defects are rescued by PHC-specific expression of *dmd-3* [*oExt6908 (eat-4p11Δ11::dmd-3)*]. Scale bars: 50 µm. Wildtype and *PHCp::fem-3* data showing in this plot is the same as in Fig. 2B for the adult stage. See panel D for quantification.

C: Ectopic expression of *dmd-3* in PHC of hermaphrodites is sufficient to scale *eat-4/VGLUT* expression (4th panel) and *dmd-3* is required in hermaphrodites for the axon extension conferred by masculinization of PHC (2nd and 3rd panel). Transgenic array names: *otEx6879, otEx6880 (eat-4p11Δ11::fem-3); otEx6908(eat-4p11Δ11::dmd-3).* See panel D for quantification.

D: Quantification of the axon extension and *eat-4/VGLUT* scaling in the different conditions showed in previous panels. Significance was calculated using student t-test, ***$P < 0.005$, **$P < 0.05$.