Sex-specific pruning of neuronal synapses in Caenorhabditis elegans

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Whether and how neurons that are present in both sexes of the same species can differentiate in a sexually dimorphic manner is not well understood. A comparison of the connectomes of the Caenorhabditis elegans hermaphrodite and male nervous systems reveals the existence of sexually dimorphic synaptic connections between neurons present in both sexes. Here we demonstrate sex-specific functions of these sex-shared neurons and show that many neurons initially form synapses in a hybrid manner in both the male and hermaphrodite pattern before sexual maturation. Sex-specific synapse pruning then results in the sex-specific maintenance of subsets of these connections. Reversal of the sexual identity of either the pre- or postsynaptic neuron alone transforms the patterns of synaptic connectivity to that of the opposite sex. A dimorphically expressed and phylogenetically conserved transcription factor is both necessary and sufficient to determine sex-specific connectivity patterns. Our studies reveal new insights into sex-specific circuit development.

Like other invertebrate or vertebrate nervous systems, the nervous system of C. elegans contains a number of sex-specific neurons, most of which are generated during the process of sexual maturation in late larval stages1–3. Apart from these sex-specific neurons (8 in hermaphrodites, 91 in males), there are 294 neurons shared by both sexes, most of which are embryonically generated3,4. The hermaphrodite and male versions of these shared neurons display the same lineage history, the same cell body position, share molecular features (for example, neurotransmitter identity) and display similar neurite projection patterns5–7. Intriguingly, the recent reconstruction of the posterior nervous system of the C. elegans adult male3 and its comparison to the connectome of the hermaphrodite (a derived female)8 show that several of the sex-shared neurons are strongly sexually dimorphic in their synaptic wiring patterns. These anatomical observations provide a fascinating opportunity to study how seemingly similar sex-shared neurons develop sexually dimorphic characteristics.

Synaptic target choices between sex-shared neurons

Here we focus on a group of sex-shared—but dimorphically connected—sensory, inter- and motorneurons5 (Fig. 1a). The sexually dimorphic connectivity differences do not simply reflect sex-specific modifications of similar neuronal circuits, but rather sex-shared neurites wire into completely distinct circuits5 (Fig. 1a). For example, the PHB phasmid sensory neuron synapses onto three different sex-shared command interneurons only in hermaphrodites (AVA, AVD and PVC). In males, PHB connects to a sex-shared interneuron, AVG, which in turn connects to downstream tail motor neurons only in males (Fig. 1a). To examine whether dimorphic connections are due to dimorphic synaptic partner choice or a reflection of sex-specific neuron or process placement, we analysed serial electron micrographs and found that the phasmid neuron processes are directly adjacent to the AVG process in both sexes (Extended Data Fig. 1). Dimorphic connections between these neurons are therefore a consequence of sex-specific synaptic partner choice.

Although electron microscopy provides a powerful tool to identify synaptic partners, the presently available electron microscopy analysis relies on one or two animals at a single stage (adult). To confirm the electron microscopy results, and to visualize the reproducibility as well as the developmental aspects of dimorphic synapses, we used two distinct trans-synaptic labelling techniques (Fig. 1b), ‘GRASP’ (GFP reconstitution across synaptic partners)9 and ‘iBLINC’ (in vivo biotin labelling of intercellular contact)10. We generated transgenic lines in which seven distinct synaptically connected neuron pairs are labelled with GRASP and/or iBLINC. Using cytosolic mCherry to label neurites, we examined synaptic puncta along these mCherry-labelled adjacent processes. For all seven synaptic connections examined, we reproducibly identified discrete synaptic puncta that appear in the sex-dimorphically predicted by the electron microscopy analysis (Fig. 1a, b, Extended Data Figs 1–3).

Sexually dimorphic functions of sex-shared neurons

To assess whether sexually dimorphic wiring is an indication of dimorphic neuronal function, we either surgically removed individual dimorphically connected neurons or genetically silenced them using ectopic expression of a histamine-gated chloride channel11. Silencing of the PHB neurons (using a driver that is strongly and consistently expressed only in PHB; Extended Data Fig. 4) affected forward locomotion of hermaphrodites, but not of males (Extended Data Fig. 5a). Another previously described PHB function also displays notable sex-specificity. Specifically, it has been shown that the PHB sensory neuron modulates chemorepulsive behaviour of hermaphrodites in response to the noxious chemical sodium dodecyl sulfate (SDS)12. The modulatory effect of PHB is observed upon functionally disabling subsets of head sensory neurons12. Such head-neuron-defective animals fail to avoid SDS because PHB provides an antagonistic input to command interneurons12. This antagonistic input to the command circuit is revealed through ablation of PHB, which restores the ability of head-sensory-neuron-defective animals to avoid SDS12. We corroborated this antagonistic input through silencing of PHB with a histamine-gated chloride channel, by examining ceh-14 mutant animals, in which all three phasmid neurons fail to adopt their glutamatergic identity13, and by silencing both ASH and PHB neurons (Fig. 2a, Extended Data Fig. 5d, e). Importantly, male PHB does not connect to command interneurons and no antagonistic input to the command circuit is expected. One would therefore predict that the disabling of head-sensory-neuron function in males (ASH silenced and...
ablated; or *tax-4* mutants) should not have the impact on SDS avoidance that is observed in hermaphrodites. We indeed found this to be the case (Fig. 2a, Extended Data Fig. 5).

As adult male PHB neurons are not involved in the avoidance of noxious chemicals, we next investigated the function they adopt in males. We noted that the male-specific innervation target of PHB, the interneuron AVG, becomes innervated by male-specific hook sensory neurons (HOA and HOB), known to be involved in sensing a hermaphrodite-derived signal that induces males to stop at the vulva during mating behaviour. Through genetic silencing, genetic ablation and microsurgical laser ablations, we found that PHB and AVG are involved in response to vulva stop signal in males, and automated worm-tracking analysis revealed that ablation of LUA results in pausing defects in hermaphrodites but not in males (Extended Data Fig. 6).

**Sexually dimorphic synapse patterning**

We next asked how sexually dimorphic connectivity patterns are established. With the exception of the VD13 neuron (which is born at the end of the first larval stage), the shared neurons that we analysed are born and project their neurites during embryogenesis. Sex-specific neurons in the tail are born in the last larval stage, and male-specific behaviours emerge soon after. One could therefore envision that dimorphic connections between the embryonically born neurons could form during sexual maturation, that is, long after the respective neurons and axons have been established in the animal (‘late maturation’ model; Fig. 3a). Alternatively, a ‘default connectivity’ could be established by both sexes early in development, followed by a sex-specific process.

**Figure 1 | Visualizing sexually dimorphic synapses.** a. Connectivity of selected neurons at the adult stage, as inferred from serial section reconstructions of electron micrographs. Chemical synapses between sensory (triangles), inter- (hexagons) and motor (circles) neurons are depicted as arrows. Thickness of arrows correlates with degree of connectivity (number of sections over which en passant synapses are observed). The inset indicates where synaptic connections are formed. Black/grey, shared-sex neurons; white, male-specific neurons. Arrows indicate hermaphrodite-specific (red) and male-specific (blue) chemical synapse between shared neurons. b. Visualizing sexually dimorphic synapses. Fluorescent micrographs of GRASP GFP signal in preanal ganglion region outlined in the inset in Fig. 1a. Neuronal processes are labelled with cytoplasmic codon-optimized Cherry markers. GRASP data are shown, additional iBLINC data are shown in Extended Data Fig. 3. Expression pattern of the promoters used in this study to drive cell-specific expression can be found in Extended Data Fig. 4. Quantification of data are shown in Fig. 3b; the number of fluorescent puncta (outlined with white boxes) is similar to those observed in the electron microscopy analysis. Gut auto-fluorescence gut granules. Scale bars, 10 μm. In all images anterior is left, and dorsal is up. Blue and red indicate male and hermaphrodite, respectively, in all figures.

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**Figure 2 | Functional repurposing of dimorphic neurons.**

(a) Chemosensory repulsion assays (see Methods for full description of behavioural assays). Scatter diagrams plotting the avoidance index of single animals. Each black dot represents the fraction of reversal responses (scored as reversing or not reversing) in 10 or more assays of a single animal. Magenta vertical bars represent the median. The left column indicates predictions of reversal behaviour, based on previously published data that demonstrated a strong reversal drive from head neurons (thick arrows) that is counteracted by a forward drive mediated by the PHB neurons in the tail. Control experiments for silencing using histamine and additional SDS assays can be found in Extended Data Fig. 5. *sra-6p::HisCl1* and *gpa-6p::HisCl1* were used for ASH and PHB silencing, respectively. Summary of dimorphic behaviours induced by PHB sensory neurons can be found in Extended Data Fig. 6c. b, Changes in male movement and posture triggered by mate contact. c, Mutant or laser-operated animals tested for the male vulva location efficiency. In *lin-11* and *ceh-14* mutants, the AVG and phasmid neurons, respectively, fail to differentiate. Error bars, s.e.m. d, Initiation of backward movement in response to hermaphrodite contact is dependent on PHB activity, measured as contact response efficiency. Each dot represents one animal. We performed the nonparametric Mann–Whitney test (Wilcoxon rank sum test) with Bonferroni correction for multiple comparisons (a, c) and Fisher’s exact test (d). ****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05; NS, not significant.

The sex of neurons controls synaptic patterning

We next investigated whether sexually dimorphic wiring patterns are determined by the sex of both the pre- and postsynaptic cell, or by non-cell-autonomous processes. We addressed this question by generating sexually mosaic animals through cell-type specific, ectopic expression of the *fem-3* gene, which downregulates the global hermaphroditic identity determinant TRA-1 (a G-like zinc finger transcription factor) thereby imposing a male identity on the specific cell-type in an otherwise hermaphroditic animal. Conversely, ectopic cell-type-specific expression of the intracellular domain of the TRA-2 receptor (“TRA-2NC”) feminizes cells via stabilization of the TRA-1 transcription factor in otherwise male animals. We found that identity transformations of single neurons transformed synaptic wiring patterns to that of the opposite sex (Fig. 4, Extended Data Fig. 8). For example, masculinization of PHB results in a loss of the PHB–AVG connection, demonstrating that the sex of PHB dictates which connection is pruned or maintained. Non-dimorphic PHB connectivity at the L1 stage is not affected by sex-reversal (Extended Data Fig. 8). Notably, masculinization of PHB also restores the sexual differences of head-sensory-neuron-disabled hermaphrodites (Fig. 2a). Thus, the behavioural differences between males and hermaphrodites in the noxious chemical response can be linked specifically to the sex of an individual neuron.

For example, PHB neurons connect to AVA and AVG in hermaphrodites and males at early larval stages (Fig. 3b, c). Therefore, male-specific pruning of the PHB–AVA synapses results in an adult male-specific PHB–AVG connection, and conversely, hermaphrodite-specific pruning of the PHB–AVG synapses results in the hermaphrodite-specific PHB–AVA synapses. We observed these synaptic pruning events to occur during sexual maturation in the fourth larval stage (L4) [Extended Data Fig. 7].

As the PHB–AVA connection exists in both sexes at the L1 stage, we asked whether at this stage both hermaphrodites and males display a PHB-dependent modulation of the repulsive response to noxious chemicals. We indeed found this to be the case (Fig. 2a). The repulsive response was restored in both sexes upon silencing of PHB (Extended Data Fig. 5f). These observations demonstrate that PHB does not merely acquire dimorphic functions in the adult, but that PHB neurons in males undergo a repurposing of function, from initially being involved in sensing noxious environmental cues to processing sex-specific cues.

Sexually dimorphic pruning of synaptic connections can also be observed in a number of additional neuronal contexts (Fig. 3, Extended Data Fig. 2). For example, PHA connects to AVG in both sexes at the L1 stage, but this connection is selectively lost in males (Fig. 3b, c). Similarly, the AVG interneuron connects to the cholinergic DA9 motor neuron in both sexes at the L1 stage, but the synaptic connection persists only in males, not hermaphrodites [Figs 1b, 3b, c].

One other sexually dimorphic synaptic connection arises by a fundamentally different principle: the AVG to VD13 connection is only ever observed in males, and never in hermaphrodites [Figs 1b and 3b, c]. Some prepatterning is also already evident in the pruned AVG–DA9 synapses; these are present in both sexes in the L1 stage, but are stronger in the male (Fig. 3b, c). Analysis of ~1,000 serial electron microscopy sections shows that the axons of the AVG and VD13 (synaptically connected in a male-specific manner) are more adjacent in males compared to hermaphrodites (Extended Data Fig. 1). Even though we cannot exclude the possibility that dimorphic adjacency is merely a secondary consequence of failure to establish synaptic contact, we propose that the prepatterning of dimorphic synapse may be a consequence of dimorphic axon placement. Taken together, we identified two types of synaptic maturation events: pruning events that coincide with sexual maturation and dimorphic prepatterning events that precede sexual maturation (summarized in Fig. 3d).
the PHB–AVG synapses are stabilized (Fig. 4). These results suggest a masculinized AV A, the PHB–AV A synapses are not only pruned, but synapse. Indeed, in animals in which affects the maintenance of the hermaphrodite-specific PHB–AV A synapses in hermaphrodites, through masculinization of AVG, sex-specific synapses by asking whether the maintenance of the PHB–AVG connection. Similarly, masculinization of AV A disrupts maintenance of the PHB–AV A synapses in males with feminized PHBs reverse less and can antagonize the VD13 connection. With feminization of the PHB neurons in male animals is sufficient to maintain the normally hermaphrodite-specific PHB–AV A connection. Similarly, masculinization of AV A disrupts maintenance of the PHB–AV A synapses in hermaphrodites (Fig. 4).

We further probed the non-autonomous nature of maintaining sex-specific synapses by asking whether the maintenance of the PHB–AVG synapses in hermaphrodites, through masculinization of AVG, affects the maintenance of the hermaphrodite-specific PHB–AV A synapse. Indeed, in animals in which fem-3 is driven in AVG (resulting in stabilization of the PHB–AVG synapse), PHB–AV A synapse number is significantly reduced. Conversely, in hermaphrodites in which we masculinized AV A, the PHB–AV A synapses are not only pruned, but the PHB–AVG synapses are stabilized (Fig. 4). These results suggest a competition mechanism in which one synaptic wiring configuration is maintained at the expense of the ‘alternative’ wiring pattern.

**Doublesex-like transcription factors control dimorphic connectivity**

We next sought to determine the link between the globally acting sex determination system, mediated by TRA-1, and synaptic pruning. A recently framed hypothesis posits that globally acting hormonal signals in vertebrates operate through region-specific modular effector system. In C. elegans, the global TRA-1 regulator may operate in a cell-type-specific manner through one of the 11 members of the DMD (Doublesex/MAB-3 domain) family of transcription factors, which are conserved regulators of sexual identity in various organisms. Three of these family members (mab-3, mab-23, dmd-3) have previously been implicated in somatic sex differences, but the other eight have remained uncharacterized. We found that dmd-5 and dmd-11 are dimorphically expressed in the dimorphically connected AVG neuron described above; expression is observed in male AVG, but not in hermaphrodite AVG (Fig. 5a). This dimorphic expression is controlled cell-autonomously via the canonical sex-determination pathway, which is controlled cell-autonomously via the canonical sex-determination

Figure 3 | Synaptic patterning during development. a, Models of how sexually dimorphic connectivity patterns may arise during development. b, c, Quantification and fluorescent micrographs of synaptic pruning measured by the number of synaptic puncta observed using GRASP GFP (PHB–AVA, PHB–AVG, AVG–VD13, AVG–DA9) and iBLINC GFP (PHA–AVG) in L1 and adult hermaphrodites and in males. As VD neurons are born at the end of the L1 stage, juvenile VD13 puncta were quantified at the L2 stage. We performed nonparametric Mann–Whitney test (Wilcoxon rank sum test) with Bonferroni correction for multiple comparisons. **P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05; NS, not significant. Magenta horizontal bars represent the median. Region of neurite overlap and observed synaptic puncta marked with white boxes. Gut, auto-fluorescence gut granules. Scale bars, 5 µm. Note that the roughly twofold increase in synapse number from L1 to L4 in the hermaphroditic PHB–AVA connection and the male PHB–AVG is in line with an overall increase in total synapse numbers seen between neurons between L1 and adult stage as deduced by recent reconstruction of an L1 stage animal (M. Zhen, personal communication). M, merge; N, neurite; P, puncta.

d, Summary of synaptic connection differences between juvenile, pre-L4 animals and adults.
pathway and TRA-1, as expression of FEM-3—the negative regulator of hermaphroditic TRA-1 protein—in the AVG neurons derepresses dmd-5 and dmd-11 expression in hermaphrodites (Fig. 5a, Extended Data Fig. 9a).

*dmd-5* and *dmd-11* single mutants show defects in the male-specific function of AVG in mating behaviour (Fig. 5b, Extended Data Fig. 9f–h) and *dmd-5; dmd-11* double mutant animals show even

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**Figure 4 | Autonomy and non-autonomy of sex-specific synapse pruning.** Either the presynaptic cell (PHB) or the postsynaptic cells (AVA, AVG) were masculinized (by expression of FEM-3) and feminized (by expression of TRA-2)\(^\text{8c}\), and the number of synaptic puncta were quantified in hermaphrodites and males. We performed nonparametric Mann–Whitney test (Wilcoxon rank sum test) with Bonferroni correction for multiple comparisons. \(***P < 0.0001\), \(**P < 0.001\), \(*P < 0.01\). Magenta horizontal bars represent the median. Results are summarized in Extended Data Fig. 8a. Note that a general trend in the sex-reversal experiments is that the change of the sex of the presynaptic neuron (PHB) appears to have a stronger effect than changing the sex of either of the postsynaptic cells. Changing the sex of both postsynaptic cells simultaneously did not enhance the effects (Extended Data Fig. 8c).

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**Figure 5 | Sexually dimorphic expression and function of dmd-5 and dmd-11.** a, Sex-specific expression of *dmd-5* and *dmd-11* reporter genes in AVG. There are no additional dimorphisms in the retrovascular ganglion, apparent differences are due to differences in z-planes incorporated into the final Z-stack projection. Masculinization of AVG derepresses *dmd-5* and *dmd-11* expression in hermaphrodites AVG. Quantified in Extended Data Fig. 9a. b, Vulva location efficiency is affected in *dmd-5* \((n = 11)\), *dmd-11* \((n = 20)\) and *dmd-5; dmd-11* double mutant males \((n = 29)\) compared with wild-type males \((n = 15)\). Expression of either *dmd-5* \((n = 10)\) or *dmd-11* \((n = 10)\) in AVG (using the *inx-18* promoter) of double mutant males rescues behaviour defects. Error bars, s.e.m. c, *dmd-5* and *dmd-11* are required for maintenance of AVG synapses. Fluorescent micrographs \((c)\) and quantification of synaptic puncta marked with white boxes. Quantification of DMD effect on LUA–AVG puncta are found in Extended Data Fig. 9i. We performed nonparametric Mann–Whitney test (Wilcoxon rank sum test) with Bonferroni correction for multiple comparisons. \(****P < 0.0001\), \(***P < 0.001\), \(**P < 0.01\), \(*P < 0.05\). Magenta horizontal bars represent the median. Scale bars, 10\(\mu\)m.
stronger defects (Fig. 5b). These defects can be rescued by AVG-specific expression of either dmd-5 or dmd-11 (Fig. 5b). Moreover, dmd-5 single mutants and dmd-5; dmd-11 double mutants display the alterations in AVG synaptic wiring that one would expect from factors that control the sexually dimorphic nature of AVG wiring (Fig. 5c, d, Extended Data Fig. 9). Male-specific PHB–AVG synapses fail to be maintained in dmd-5; dmd-11 mutant males. Synaptic defects can be rescued through AVG-specific expression of dmd-5 (Fig. 5c, d). There are no synaptic defects observed in the L1 stage of dmd-5; dmd-11 mutants when no synaptic dimorphism is yet apparent (Extended Data Fig. 9b), indicating that dmd-5 and dmd-11 are not required for synapse formation per se, but are specifically involved in controlling sex-specific synapse maintenance by preventing synaptic pruning, as predicted by their expression pattern. Furthermore, the PHB–AVA hermaphroditic connection is non-autonomously stabilized in dmd-5; dmd-11 mutant males, supporting the competition model discussed above (Extended Data Fig. 9e).

dmd-5 is not only required but also sufficient to prevent synaptic pruning, as deduced by ectopic dmd-5 expression in the AVG neurons of hermaphrodites. In these animals the PHB–AVG synapses are maintained (Fig. 5d). As DMD proteins are generally thought to work as repressors, we propose that dmd-5, in conjunction with dmd-11, represses the expression of gene(s) in male AVG neurons that are involved in the pruning of AVG connections to PHB and that repression of these pruning factor(s) in hermaphroditic AVG, via ectopic dmd-5 expression, inhibits pruning (summarized in Extended Data Fig. 9j). Notably, although AVG-masculinized dmd-5(+−) hermaphrodites (masculinized through fem-3-driven degradation of TRA-1) do maintain PHB–AVG synapses, AVG-masculinized dmd-5(−) hermaphrodites do not (Fig. 5d, Extended Data Fig. 9d). This demonstrates that dmd-5 functions downstream of tra-1, as already suggested by the observation of ectopic dmd-5 expression in animals in which we degraded TRA-1 cell-autonomously in AVG (see above; Fig. 5a, Extended Data Fig. 9a).

In conclusion, our studies show how sex-shared neurons adopt sex-specific synaptic wiring patterns. Sex-specific wiring patterns arise in a neuron-type specific manner. In most cases, we observe sexual maturation–coupled sex-specific pruning of synaptic connections that were indiscriminately generated in both sexes. In at least one case, sex-specific synapses are prepatterned before overt sexual maturation of the animal. We observed notable patterns of cellular autonomy of the synaptic pruning events and we found pruning to be regulated by sex-specifically expressed transcription factors.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Supplementary Information is available in the online version of the paper.

Author Contributions M.O.-S. and O.H. designed the experiments. M.O.-S. and E.A.B. performed most experiments. E.A.B. quantified the data for PHA-AVG synapses and all iBLINC transgenes, tracked silenced PHB animals and generated driver lines for expression analysis of gap-6 and fpl-18. M.O.-S. and O.H. wrote the paper with input from E.A.B.

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Methods

Data reporting. No statistical methods were used to predetermine sample size. The experiments were not randomized and the investigators were not blinded to allocation during outcome assessment.

C. elegans strains. Wild-type C. elegans were C. elegans variety Bristol, strain N2. Worms were maintained according to standard methods24. Worms were grown at 20°C on nematode growth media (NGM) plates seeded with E.coli OP50 as a food source. Mutant strains used in this study include: CB1489 hpc-4[ts2(k974)]; CB4066 him-5(e1490) V; MT333 lin-11(n3684); lin-1(hc1467) V; RB1295 F10C1.5(k13944) II; F1X0176 dmd-5(m17620) II; PR678 ttxp-3::gfp(∆678) III, DAS09 unc-31(c932) IV, TB528 ceh-14(ch3) X, VC30074 dmd-5(gk408945) II, VC1193 dmd-11(∆g525) V.

All transgenic strains used in this study are listed in Supplementary Table 1, ordered by figures and extended data figures.

Cloning and constructs. To generate inx-18p::wCherry (pMO10), inx-18 second intron was amplified from pPH260 (ref. 25) and restriction sites were added to ends (5/-SpeI, NheI and the fragment containing riboTAATGTACCTGCAGAA-3). This fragment was digested and ligated into pPD59.75 vector in which the GFP was replaced with codon-optimized mCherry (‘worm Cherry’). To restrict expression to AVG, AYI motif (5/-ATTAGTTTCGTTAA-3) was deleted from the 2nd intron of inx-18 using site directed mutagenesis (Forward primer: 5AATTTTTTTGTGTGTATTATTTTTTTTTTTTTATTGCTGATAT3′, Reverse 5AATCTATGACGCTAATAGAAAAATAAGTACGACATGAAAAAATT3′). inx-18 2nd intron is also dimly and variably expressed in URX.

To generate PHB histamine-induced silencing construct, a 2.6 kb gpa-6 promoter from MVC6 was cloned into pNP403 (tag-168p::HisCl2; a gift from N. Pokala and C. Bargmann) to replace the tag-168 promoter using RF cloning. To generate dmd-5 and dmd-11 genomic rescue constructs, dmd-5 and dmd-11 genomic sequences including 500 bp 3′-UTR were amplified from genomic DNA and cloned into pMO10 to replace wCherry using RF cloning. The resulting constructs are pMO31, inx-18p::dmd-5_genomic-3′-UTR and pMO38, inx-18p::dmd-11_genomic-3′-UTR.

Microscopy. Worms were anaesthetized using 100 mM of sodium azide (NaN3) and mounted on 5% agar on glass slides. Worms were analysed by Nomarski optics and fluorescence microscopy, using a Zeiss 780 confocal laser-scanning microscope. When using GFP, we estimated the resolution of our confocal to be ~250 nm. Multidimensional data were reconstructed as maximum intensity projections using Zeiss Zen software. Puncta were quantified by scanning the original full Z-stack for distinct dots in the area where the processes of the two neurons overlap. Figures were prepared using Adobe Photoshop CS6 and Adobe Illustrator CS6.

Cell ablation. We performed laser ablations using a MicroPoint Laser System Basic Unit (N2 pulsed laser (dye pump), ANDOR Technology) attached to a Zeiss Axioplan 2E widefield microscope (objective EC Plan-Neofluar 100×/1.3 Oil M13). The laser delivers 120 J Joules of 337 nm energy with a 3-nsec pulse length.

Ablations were performed as previously described27, with pulse repetition rates of ~15 Hz. Cell identification was performed with GFP or Cherry markers. Ablations were performed at the L4 stage, and worms were analysed 24–48 h later. Mock animals were placed on same slide under microscope but were not ablated, and were allowed to recover in a similar manner. After relevant assays were performed (tracking or mating assays), worms were mounted again on glass slides and analysed under microscope to validate that cell-ablation was successful.

Mating behaviour assays. Mating assays were based on procedures described previously12,29. Males were picked at an early L4 stage and kept apart from hermaphrodites for 24 h. One male was transferred to a plate covered with a thin fresh OP50 lawn containing 10–15 adult unc-31(e928) hermaphrodites. These hermaphrodites move very little, allowing for an easy recording of male behaviour. Hermaphrodites were also isolated from opposite sex at the L4 stage and used 24 h later. Animals were monitored and sequence of events was recorded within a 15 min 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 efficiency30. The number of passes or hesitations at the vulva until the male first stops at the vulva were counted: location efficiency = number of encounters to stop. PhR-silenced males were digitally recorded using the Exo Labs model 1 camera mounted on Nikon Eclipse E400 compound microscope with long-distance X20 lens. These videos were analysed for vulva location efficiency and percentage of successful contact responses, which requires tail apposition and initiation of backward locomotion.

Percentage response to contact = 100 × (the number of times a male exhibited contact response/the number of times the male makes contact with a hermaphrodite via the rays)31.

SDS-avoidance behaviour. SDS-avoidance assay was based on procedures described previously32. A small drop of solution containing either the repellent (0.1% SDS in M13 buffer) or buffer (M13 buffer: 30 mM Tris-HCl (pH 7.0), 100 mM NaCl, 10 mM KCl) is delivered near the tail of an animal while it moves forward. Once in contact with a hermaphrodite via the rays)15. The male makes contact with the female and moves forward. The tail surrounds the entire animal by 3-dimensional space. The animal makes contact with a hermaphrodite via the rays15. The male makes contact with the female.
and ASH::HisCl1; PHBp::HisCl1 transgenic animals were picked at the L4 stage and placed on NGM plates containing 10 mM histamine with OP50 bacteria as food source. As a control, animals were placed on NGM plates containing OP50 bacteria but no histamine. Chemorepulsion behaviour assays were performed 24h later. Histamine plates were prepared as previously described. As additional controls, panneuronal HisCl1 transgenic worms (CX14373 kyEx4571 (pNP403 (tag-168::HisCl1::SL2::gfp), myo-3::mCherry); a gift from C. Bargmann) were placed on histamine plates prior to use. Histamine plates on which tag-168::HisCl1 animals were paralyzed under a minute were used for behavioral assays. There was no difference in the avoidance index of wild-type worms grown on histamine plates and on plates without histamine.

Automatic worm tracking. Hermaphrodite and male transgenic otlIs462 animals were ablated at the L4 stage and left to recover for 24 h before tracking. PHB-silenced hermaphrodites and males were transferred into NGM plates containing 10 mM histamine for 24 h before tracking. At the adult stage, animals were placed on an NGM plate seeded with diluted 20 μl of OP50 bacteria in the centre. As a control, mock ablated transgenic otlIs462 animals were used. Automatic tracking was performed at ~22°C (room temperature) with Worm Tracker 2.0 (WT2), which uses a mobile camera to track and record individual worms and 5 min videos were generated. Analysis of the tracking videos was performed as previously described.

To assess the effect of LUA ablation, we first conducted a pilot tracking study of LUA-ablated hermaphrodites worms versus controls and LUA-ablated males versus controls. Owing to the extensive analysis of the tracking system, 702 features were initially measured. After correction for multiple testing, pausing and pausing-related features emerged as the main, significant difference between LUA-ablated males and hermaphrodites. Therefore, to obviate further heavy corrections for multiple testing, we chose to re-run these experiments, this time only measuring pausing, permitting us to use of the uncorrected P values. Within this subset of new experiments, wherein only one feature was measured (that is, pausing), we found highly significant P values. Given the necessity of four tests to make the proof, we chose the most conservative correction possible for multiple testing, Bonferroni, to illustrate that the proof, we chose the most conservative correction possible for multiple testing, Bonferroni, to illustrate that the

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Extended Data Figure 1 | Adjacency of neuronal processes in hermaphrodites and males. Four transmission electron microscopy prints from wild-type adult hermaphrodite 'JSE' and four from adult male 'N2Y', showing adjacency of neuronal processes. These images were collected at MRC/LMB for ref. 8 and ref. 2, and the annotated images are available online at http://www.wormimage.org, courtesy of D. Hall. The set of processes directly adjacent to one another has been defined as the ‘neighbourhood’ of that process, and the placement of processes into specific neighbourhoods is a major determinant of connectivity. Connections form only in one sex, although processes are adjacent in both sexes. a, Print 385, JSE series (JSE_122283; http://wormimage.org/image.php?id=122283&page=2) and print 620, N2Y series (PAG620; http://wormimage.org/image.php?id=103528&page=18) shows PHB–AVG adjacent processes, pseudo labelled in green (AVG) and red (PHB). b, Print 359, JSE series (JSE_122257; http://wormimage.org/image.php?id=122257&page=2) and print 500, N2Y series (PAG500; http://wormimage.org/image.php?id=103408&page=20) shows AVG–VD13 adjacent processes, pseudo labelled in green (AVG) and pink (VD13). c, Print 377, JSE series (JSE_122275; http://wormimage.org/image.php?id=122275&page=2) and print 800, N2Y series (PAG800; http://wormimage.org/image.php?id=103706&page=16) shows PHA–AVG adjacent processes, pseudo labelled in green (AVG) and orange (PHA). d, A table summarizing the number of electron microscopy sections in which direct adjacency of processes was observed. Over a 1000 PAG (preanal ganglion) serial sections were analysed for each sex.
Extended Data Figure 2 | Dimorphic connections of LUA. a, The connectivity diagram shown in Fig. 1a, including dimorphic connections of the LUA and PHC connections. The GRASP data that we show in this figure as well as the pruning data, sexual reversal data and mutant data shown in Extended Figs 6, 8 and 9, supports the original LUA connectivity data reported in ref. 5 and summarized in this schematic. However, a recent reassessment of the tracing of electron micrographs suggests that the connectivity assignments of the PHC and LUA neurons may have been swapped with each other (S. Emmons, personal communication). b, Overview of LUA synaptic connections labelled in this paper. c, Visualizing LUA sexually dimorphic synapses. Quantification and fluorescent micrographs of GRASP trans-synaptically labelled puncta between LUA→AVG and LUA→AVA, in L1 and adult hermaphrodites and in males. M, merge; N, neurite; P, puncta. Region of neurite overlap and observed synaptic puncta are marked with white boxes. Gut, auto-fluorescence gut granules. Scale bars, 10 μm (adult) and 5 μm (L1). d, Fluorescent micrographs of the preanal ganglion region of transgenic animals expressing the presynaptic BirA::nrx-1 fusion in LUA (using the eat-4p9 promoter), and postsynaptic acceptor peptide::nlg-1 fusion in AVG. For more details see Extended Data Fig. 3. Scale bars, 10 μm. We performed nonparametric Mann–Whitney test (Wilcoxon rank sum test) with Bonferroni correction for multiple comparisons. ****P < 0.0001, ***P < 0.001, **P < 0.01; NS, not significant. Magenta horizontal bars represent the median.
Extended Data Figure 3 | Trans-synaptic labelling of dimorphic connections using iBLINC. a, Overview of synaptic connections labelled in this paper (Fig. 1 and this figure). Some connections were labelled with both GRASP and iBLINC, yielding similar results. We generally note that the number of synapses is roughly reproducible from animal to animal and the number of the fluorescent dots is roughly comparable to the number of synapses identified by the electron microscopy analysis. However, there is also some variance from animal to animal (quantified in Fig. 3), consistent with previous analysis.  

b, Labelling data not shown in Fig. 1. Fluorescent micrographs of the preanal ganglion region of transgenic animals expressing the presynaptic BirA::nrx-1 fusion in PHB (using the gpa-6 promoter), AVG (using the inx-18 promoter) and PHA (using the srg-13 promoter), and postsynaptic acceptor peptide::nlg-1 fusion in AVG and DA9 (using the acr-2 promoter). Transgenic worms also express the streptavidin detector fused to 2× sfGFP from the coelomocytes (unc-122 promoter). Neuronal processes are labelled with cytoplasmic Cherry markers of the iBLINC pairs. Region of neurite overlap and observed synaptic puncta are marked with white boxes. Scale bars, 10 μM. Anterior is left and dorsal is up.

### Table: GRASP and/or iBLINC

| Pre | Post | Sex | Presynaptic marker | Postsynaptic marker |
|-----|------|-----|---------------------|---------------------|
| AVG | VD13 | ♀   | inx-18p::nlg-1::spGFP | unc-25p::nlg-1::spGFP |
|     | DA9  | ♀   | inx-18p::nlg-1::spGFP | unc-25p::nlg-1::spGFP |
|     | AVG  | ♀   | gpa-6p::nlg-1::spGFP1-10 | inx-18p::AP::nlg-1 |
| PHB | AVG  | ♀   | gpa-6p::BirA::nrx-1 | inx-18p::nlg-1::spGFP1-10 |
| PHA | AVG  | ♀   | srg-13p::BirA::nrx-1 | inx-18p::AP::nlg-1 |
Extended Data Figure 4 | Specificity of driver lines. a, A 2.6 kb gpa-6 promoter fragment fused to GFP is expressed consistently in PHB at all stages. There is also faint and variable expression in AWA. b, The 3.1 kb flp-18 promoter fused to GFP is expressed consistently in AVA, and dimly and variably in AIY (85% of animals) and RIM (15% of animals), which were identified based on comparison to published flp-18 expression patterns. c, The 1.8 kb inx-18 2nd intron fused to codon-optimized Cherry is expressed brightly and consistently in AVG, and dimly and variably in URXs. The A1Y motif present in this fragment was deleted (see Methods). d, The 170 bp eat-4 promoter (eat-4p9) fused to GFP is expressed in LUAs and PVR.
Additional SDS avoidance assays. a. PHB silenced hermaphrodites move slower forward (quantified as absolute mid-body speed) and as a result cover less of the plate (quantified as forward path range) compared to control hermaphrodites, whereas PHB-silenced males do not show any difference compared to control males. In addition, the tail-bending wave is affected in PHB-silenced hermaphrodites, but not in males (quantified as tail crawling frequency). Statistics were computed using Wilcoxon rank-sum test, and correction for multiple testing (q values) was computed across all measures (approximately 1404 tests) using the Benjamini–Hochberg procedure. b. Silencing of ASH neuronal activity using the histamine chloride channel 1 (HisCl1) affects the animals’ chemosensory avoidance response. Males and hermaphrodites were assayed for effects of histamine on SDS avoidance behaviour. We used the him-5 mutant background (which gives a high incidence of male progeny) as wild type. There is no difference between worms assayed in the presence and absence of histamine (Fig. 2a). The avoidance index of single animals was calculated as the fraction of reversal responses in 10 or more assays, depicted as black dots. Magenta vertical bars represent the median. L4 animals carrying the kyEx5104 (pNP424 [sra-6::HisCl1::SL2::mCherry])11 transgene were grown on 10 mM histamine-containing NGM plates for 24 h. As a control, kyEx5104 animals were grown on NGM plates without histamine. ASH silencing reduces the head sensory response to SDS, thus in hermaphrodites the antagonizing activity of the PHBs inhibits the backward movement and the worms do not reverse. In males, no such antagonizing activity occurs and the worms reverse, albeit with reduced ability. c. Ablation of ASH neurons affects the animals’ chemosensory avoidance response in a similar manner to histamine-induced silencing. sra-6::gfp was used to identify the ASH neurons. d. Behavioural differences stem from dimorphic connectivity differences and not from amphid/phasmid sensory function. tax-4; ceh-14 double mutants behave in a similar manner in both sexes. PHB silencing (gpa-6p::HisCl1::SL2::gfp) does not affect behaviour in either sex. Silencing both the ASHs and PHBs in males showed no difference compared to ASH-silenced males. However, silencing ASHs and PHBs in hermaphrodites showed a significant difference compared to ASH silenced hermaphrodites, where we expect the PHBs to function in an antagonistic manner; thus in its absence, ASH-silenced hermaphrodites showed an increased ability to respond to SDS by reversing. e. PHB silencing in tax-4 mutant background. tax-4 is a subunit of a cyclic nucleotide gated channel expressed in chemosensory and thermosensory neurons39, see g. tax-4 animals show a strongly reduced avoidance response to SDS31. Silencing of PHBs in tax-4 hermaphrodites eliminated the antagonizing effect and animals were able to avoid SDS by backing. f. PHB silencing in tax-4 mutant background at the L1 stage. Lack of avoidance seen in tax-4 L1 males and hermaphrodites depends on PHB function. For all panels, we performed the nonparametric Mann–Whitney test with Bonferroni correction for multiple comparisons. ****P < 0.0001, **P < 0.01, *P < 0.05; NS, not significant. g. tax-4 expression pattern is identical in hermaphrodites and males. kyEx744 (tax-4p::TAX-4::gfp)36, was analysed in adult male and hermaphrodites. Amphid neurons in the head (ADL, ASH, ASI, ASJ, ASK, AFD, AWC, URX ASK ASI, ABL) were stained using DiD to facilitate cell identification. Neurons identified, shown in the ‘Merge’ panel are identical in both sexes and match published data. All neurons are bilaterally symmetric left–right pairs, and for simplicity only left cells are shown. Scale bars, 10 μM.
Extended Data Figure 6 | Additional behavioural analysis.

a, Hermaphrodites in which LUAs have been ablated pause more frequently than mock-ablated hermaphrodites and LUA-ablated males.

Error bars, s.e.m. (b), LUA laser-ablated animals tested for the male’s vulva location efficiency. The behavioural data shown in a and b supports the reported connectivity data shown in Extended Data Fig. 2. We performed the nonparametric Mann–Whitney test (Wilcoxon rank sum test) with Bonferroni correction for multiple comparisons (a, b). Error bars, s.e.m. (b). ****P < 0.0001, ***P < 0.001, *P < 0.05; NS, not significant.

b, LUA-laser-ablated animals tested for the male’s vulva location efficiency. Error bars, s.e.m. (b).

Extended Data Figure 6 - Additional behavioural analysis.

**Hermaphrodite**

SEXUAL CUE (NO RESPONSE)  NOXIOUS CUE (ELICITS ESCAPE)

ESCAPE RESPONSE  PAUSING FREQUENCY

**Male**

SEXUAL CUE (NO RESPONSE)

MATING BEHAVIORS
Extended Data Figure 7 | Time-course analysis of synapse pruning and development. Hermaphrodites and males were analysed at the L1, L3, L4, young adult and gravid adult stages, and the number of synaptic puncta observed at each stage was plotted against developmental time points. Synaptic puncta in hermaphrodites are plotted in red, synaptic puncta in males are plotted in blue. a, PHB–AVG synapses are pruned in hermaphrodites at the L3 stage. b, PHB–AVA synapses are pruned in males at the L3 stage. c, LUA–AVG synapses are pruned earlier, starting at the L1 stage in hermaphrodites. Error bars, s.e.m.; for each time point depicted in graphs, at least 15 animals were analysed.
Extended Data Figure 8 | Autonomy and non-autonomy of sex-specific synapse pruning. a, Cartoon summarizing sex-change effects on synapses. b, L1-stage connectivity is not affected by sex reversal. c, Simultaneous sex reversal of both AV A and AVG. d, Masculinization of the postsynaptic cell AV A is sufficient to induce LUA–AV A synaptic puncta in hermaphrodites. Masculinization of AVG was not sufficient to induce synapses between LUA and AV A in hermaphrodites. Feminizing LUA, AV A and AVG by expression of TRA-2IC was sufficient to induce ectopic LUA–AVA synaptic puncta in males. We performed the nonparametric Mann–Whitney test with Bonferroni correction for multiple comparisons. ****P < 0.0001, *P < 0.01; NS, not significant. Magenta horizontal bars represent the median.
Extended Data Figure 9 | See next page for caption.
Extended Data Figure 9 | dmd-5 and dmd-11 expression, sequence and function. a, Quantification of dimorphic expression of dmd-5 and dmd-11 in AVG. Expression in hermaphrodites was off or extremely faint. Expression of inx-18p::FEM-3 derepressed dmd-5 and dmd-11 gene expression in hermaphrodite AVGs. Statistics calculated using Fischer's exact test. 
b, Quantification of the number of PHB–AVG synaptic puncta in L1 dmd-5(gk408945); dmd-11(gk552) double mutants, compared with wild-type L1 animals. At the L1 stage, dmd-5 and dmd-11 do not affect PHB–AVG synapses, suggesting they are required for maintenance of mature synapses. 
c, dmd-5 single mutants and dmd-5; dmd-11 double mutants display similar alterations in AVG synaptic wiring. 
d, dmd-5 mutation suppresses the ectopic PHB–AVG synapses in AVG-masculinized animals. 
e, The PHB–AVA connection is non-autonomously partially stabilized in dmd-5; dmd-11 mutants. 
f, dmd-11 genomic locus and gk552 deletion location. 
g, dmd-5 genomic locus and mutation description. ok1394 location was not curated, to determine location we used the following primers: forward primer: 5′-CAGAATGCCTGTTTCTCCGTC-3′; and reverse: 5′-CACTGCTTTTCCCGTTCAAAC-3′. ok1394 and tm1760 were both found to have an embryonic lethal phenotype that could not be rescued by the genomic locus (data not shown); thus we searched for single-point mutations of the ‘million mutation project’37. gk408945 is a missense substitution mutation of W54 to R, located in the second exon. Genomic analysis revealed that this mutation lies within the conserved DM domain (h.), with perfect conservation across evolution. 
h, DM-domain sequence conservation and location of gk408945 mutation. Conservation and multiple sequence alignment were performed using UCSC Genome Browser (http://genome.ucsc.edu) and ClustalW. The DM domain is an intertwined zinc-containing DNA binding module. The DM domain binds DNA as a dimer, allowing the recognition of pseudopalindromic sequences38. i, dmd-5 and dmd-11 are required for maintenance of AVG synapses. Fluorescent micrographs and quantification of synaptic puncta of LUA–AVG. Region of neurite overlap and observed synaptic puncta marked with white boxes. M, merge; N, neurite; P, puncta. Statistics were calculated using the nonparametric Mann–Whitney test (b, d, e, i) or Kruskal–Wallis test with Dunn's multiple comparison test (c). ****P < 0.0001, ***P < 0.001, *P < 0.05; NS; not significant. Magenta horizontal bars represent the median. When using a parametric t-test, there is also a significant difference for the LUA–AVG synapse between dmd-5;dmd-11 mutant hermaphrodites and dmd-5;dmd-11 mutant hermaphrodites that overexpress DMD-5 (*P < 0.05). 
j, Summary of data. TRA-1 and DMD proteins are commonly thought to work as transcriptional repressors22. As dmd-5 and dmd-11 are already dimorphically expressed in AVG in embryos and L1 stage animals (not shown in this schematic), there must be other timer mechanisms that control the onset of pruning. For example, DMD-5 and DMD-11 may work together with a regulatory factor of the stage-specifically acting heterochronic pathway. Furthermore, we hypothesize that other neurons, such as the AVA neuron, may have its own complement of sex-specific dmd genes that control pruning.