Neurexin controls plasticity of a mature, sexually dimorphic neuron

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Experience modifies the structure and function of neurons and circuits in the brain through multiple mechanisms of neuronal plasticity1,2. Plasticity in adult brains refines circuits in response to experience in order to mediate adaptation and homeostasis, and as a cellular correlate of learning and memory1,3–4; this type of plasticity includes extension and retraction of dendrites and axons5–7. The molecular mechanisms that underlie morphological plasticity in adult neurons are not well understood. Similarly, though the sexual identity of an organism influences the function and plasticity of its nervous system, the molecular and cellular bases of such sexual dimorphism are also not fully understood.

Morphological plasticity in adult male DVB neuron

The GABAergic motor neuron/interneuron DVB is located in the tail of *C. elegans* and projects anteriorly in the ventral nerve cord in both sexes (Fig. 1a). We used fluorescent reporter gene technology to visualize DVB and found that it displays extensive post-developmental morphological plasticity exclusively in males, characterized by the progressive extension of new neurites posteriorly into the tail (Fig. 1b; Extended Data Fig. 1). The total neurite length and the number of neurite junctions increase significantly (P < 0.001) from day 1 to day 5 of adult life (Fig. 1c, d). The branching pattern of male DVB neurites lacks any overt stereotypy (Extended Data Fig. 2a, b). The generation of new DVB neurites in males is accompanied by the addition of presynaptic boutons containing the synaptic marker RAB-3, suggesting that these neurites are axon-like (Fig. 1b, Extended Data Fig. 1); electron microscopy analysis supports this conclusion8,9. We have not identified other neurites that undergo comparable neurite outgrowth in adulthood (Fig. 1b, Extended Data Fig. 2c–h).

Dimorphic DVB connectivity influences behaviour

In hermaphrodite worms, DVB controls defecation behaviour10; in males it also contributes to protraction of the male-specific spicule structures, which are inserted into the hermaphrodite vulva during copulation11 (Fig. 1e–g). Consistent with a sexually dimorphic function, the synaptic wiring pattern of DVB is also notably sexually dimorphic8,9 (Fig. 1g). To test for functional roles of DVB neurite outgrowth, we examined DVB function over the period of DVB neurite outgrowth.
spicule protraction at day 5 (aldicarb assay described below, Extended Data Fig. 4a–d), suggesting that GABA contributes to restriction of spicule protraction in later adulthood.

To further characterize the role of DVB in active spicule protraction, we used the acetylcholine esterase inhibitor aldicarb, which induces spicule protraction through the accumulation of acetylcholine at neuromuscular synapses onto spicule protractor muscles (Fig. 2d). Aldicarb-induced spicule protraction took longer as males aged from day 1 to day 5 (Fig. 2e), during the same period as DVB neurite outgrowth. To directly test whether DVB is involved in this behavioural change, we combined laser ablation of DVB with aldicarb-induced spicule protraction. DVB ablation at day 1 resulted in slower spicule protraction in response to aldicarb than in control and mock-ablated males, demonstrating a functional switch for DVB from an excitatory to an inhibitory input on spicule protraction (Fig. 2f). These results were confirmed using genetic ‘ablation’ of DVB (lim-6 transcription factor mutant14; Fig. 2f). Together, our results confirm that DVB switches function in adulthood, and implicate DVB as the main contributor to the temporal change observed in spicule protraction and defecation behaviour.

To investigate how the switch of DVB function during DVB neurite outgrowth relates to changes in synaptic connectivity, we used trans-synaptic labelling (GRASP15) to visualize synapses between DVB and the spicule protraction neurons and muscles (Fig. 2g). The number of these specific synaptic connections increased from day 1 to day 5 (Fig. 2h, i). We also visualized synapses between DVB and the spicule protractor muscles (Fig. 2j; Extended Data Fig. 4h); the number of these synapses decreased from day 1 to 5 (Fig. 2k, l). These results provide evidence that structural remodelling of axons and dendrites in control and mock-ablated males, demonstrating a functional switch for DVB from an excitatory to an inhibitory input on spicule protraction (Fig. 2f). These results were confirmed using genetic ‘ablation’ of DVB (lim-6 transcription factor mutant14; Fig. 2f). Together, our results confirm that DVB switches function in adulthood, and implicate DVB as the main contributor to the temporal change observed in spicule protraction and defecation behaviour.

**Figure 1 | Progressive neurite outgrowth of the GABAergic DVB neuron in adult males.** a, DVB neuron schematic. b, DVB visualized with lim-6int4::wCherry in adult males and hermaphrodites (asterisk, PVT neuron; arrowheads, DVB neurites, n as indicated in c). Presynaptic boutons visualized with presynaptic marker lim-6int4::gfp::rab-3. DIC, differential interference contrast. c, d, DVB neurite outgrowth in males quantified by total neurite length (c) and number of neurite junctions (d). Dot represents one worm; magenta bar, median; boxes, quartiles. Comparison using one-way ANOVA and post-hoc Tukey HSD, P values shown above plots, bold shows significance (P < 0.05). e, Schematic of DVB and postsynaptic spicule-associated neurons and muscles in male tail. f, Sample images of males with non-protracted or protracted spicules (red triangles indicate base and tip of spicules; tail and male fan are outlined, demonstrating protracted spicules extending underneath male fan; n > 10). g, Connectivity of DVB at adult stage inferred from electron micrographs (sections indicate number of EM sections over which en passant synapses were observed)8,11. Behavioural output indicated for each sex. Scale bars, 10 µm.
adulthood can rewire specific synaptic targets, supporting the notion that this remodelling can markedly alter connectivity within circuits and alter downstream behaviour.

Male spicule protraction into the hermaphrodite vulva is the most complex step of the male mating behaviour, involving coordination of cholinergic and GABAergic signalling. The balance of excitatory and inhibitory signalling is crucial for successful spicule insertion, which must be further coordinated with changes in sex muscle excitability in early adulthood. Day 1 and day 3 males are proficient at most steps of mating, however, in five-minute timed mating assays, day 3 males were significantly more likely than day 1 males to successfully complete mating with sperm transfer (P = 0.003; Extended Data Fig. 5a). We scored the spicule-related steps of mating (spicule prodding and spicule protraction) and found that day 1 males showed more spicule prodding attempts overall and a lower ratio of protraction to prodding attempts compared with day 3 males (Extended Data Fig. 5b, c), indicating that day 1 males are less capable than day 3 males of transitioning from spicule prodding to spicule protraction. This suggests that the morphological and functional plasticity of DVB in males may fine-tune and coordinate the defecation and spicule protraction circuits to increase mating success.

DVB neurites are experience- and activity-dependent

To determine whether DVB plasticity occurs in response to experience, we tested whether the act of mating itself altered DVB neuron morphology by exposing males to hermaphrodites for the first 48 h of adulthood. Single males housed with hermaphrodites showed significant increases in DVB neurite length and junctions compared to males housed alone (P < 0.001; Fig. 3a–c). C. elegans males housed with other males or in isolation can engage in mating-like behaviours, which may include spicule protraction. To minimize mating sensory input and self-mating behaviour, we analysed DVB neurite outgrowth in pkd-2 (carnation channel) mutant males and in genetically paralysed mutant males (unc-97); pkd-2 mutant males have reduced DVB neurite outgrowth at day 3, whereas unc-97 mutant males have almost no DVB neurites at day 3 (Extended Data Fig. 4e–g); however, they can protract spicules in response to aldicarb (data not shown) and their neurites can be ectopically induced (Extended Data Fig. 5d–f). Paralysed males also show no change in neurite outgrowth when housed with hermaphrodites for 48 h (Fig. 3a–c). These results demonstrate that DVB neurite outgrowth is experience-dependent and is potentially driven by spicule protraction and activity of the postsynaptic spicule protraction circuit.

We next investigated whether activity of the postsynaptic targets of DVB contributes to DVB neurite outgrowth. Channelrhodopsin
mediated activation of postsynaptic DVB targets (spicule neurons and muscle) resulted in immediate protraction of spicules16 (Fig. 2b, Supplementary Video 2). Repeated activation of the spicule protraction circuit caused a significant increase in DVB neurite length and junctions (P = 0.002 and P < 0.001, respectively; Fig. 3d–f, day 1), independent of GABA signalling (Extended Data Fig. 5d–f). Males exposed to repeated activation, but subsequently allowed to recover, had DVB neurites that were indistinguishable from those of controls, suggesting that neurite growth is dynamic and potentially reversible (Fig. 3d–f). Repeated activation of either spicule neurons or muscles separately demonstrated that activity in either can induce DVB neurite growth (Extended Data Fig. 5g–i).

We next tested whether activity-induced DVB neurites influence DVB neuron function and worm behaviour. We activated and recovered males in the same manner as above, and then used the aldicarb assay to analyse spicule protraction behaviour. Males at day 1 following repeated activation of the spicule protraction circuit showed a significant delay in the time to aldicarb-induced protraction (P < 0.001; Fig. 3g, day 1), implying that activity-induced neurites have a direct and immediate effect on DVB spicule function. Males that were exposed to repeated activation of the spicule protraction circuit but allowed to recover had spicule protraction indistinguishable from that of day 2 controls (Fig. 3g, day 2), indicating that induced behavioural changes are dynamic and repeated activation does not result in lasting protraction defects.

To test whether a reduction in circuit activity affects DVB neurites, we exposed males to exogenous GABA, expecting to silence the targets of GABAergic DVB signalling. This resulted in a reduction in DVB neurites (Extended Data Fig. 6a–c). To implicate spicule circuit inhibition more specifically, we silenced spicule protraction neurons and muscles with a histamine-gated chloride channel in day 5 males; this also reduced DVB neurites (Extended Data Fig. 6d–f). In summary, DVB neurites extend in response to the activity levels of the spicule protraction circuit, including postsynaptic targets of DVB.

**Neurexin and neuroligin control DVB plasticity**

DVB neurite outgrowth appears to be a form of morphological and functional plasticity that fine-tunes the excitatory and inhibitory balance for coordinated spicule protraction. Several synaptic molecules have been implicated in excitatory and inhibitory balance, including the synaptic adhesion molecule neurexin and its trans-synaptic binding partner neuroligin24–27. Males with a deletion allele of the single neuroligin 24–27. Males with a deletion allele of the single
DVB neuron, the SPC, PCA and PCB neurons, or the SPC neuron and spicule muscles did not rescue the nlg-1 mutant phenotype, whereas expression in the spicule protractor and anal depressor muscles or in the spicule retractor muscles did rescue the phenotype (Extended Data Fig. 7d, e), indicating that NLG-1 contributes to DVB neurite outgrowth by functioning in multiple postsynaptic DVB muscles. Silencing the spicule protraction circuit in nlg-1 mutant males at day 5 with gar-3b::HisCl1 or overnight exposure to exogenous GABA resulted in no significant reduction in DVB neurite branching (Extended Data Fig. 7f, g). These results suggest that the nlg-1 mutant phenotype cannot be explained by indirect alteration of the spicule circuit or more global perturbations in activity as a result of loss of NLG-1.

Unexpectedly, males with a deletion allele of nrx-1 (which encodes the C. elegans orthologue of neurexin) displayed a significant reduction in neurite outgrowth at days 3 and 5, a phenotype opposite to the nlg-1 mutant phenotype (P = 0.006 and P < 0.001, respectively; Fig. 4d–g). nrx-1 mutants showed a corresponding decrease in time to aldicarb-induced spicule protraction (Fig. 4h). The nrx-1 locus produces both a long and short isoform, and two long isoform-specific mutant alleles recapitulated the null phenotype (Extended Data Fig. 9a–c). Repeated channelrhodopsin-mediated activation of the spicule protraction circuit failed to induce DVB neurites in nrx-1 mutants (Extended Data Fig. 5d–f), indicating that the nrx-1 phenotype is not explained solely by reduced circuit activity that could be envisioned to result from loss of NRX-1.

NRX-1 is broadly expressed throughout the C. elegans nervous system. Expression of the long isoform of NRX-1 in DVB using the lim-6'::gfp promoter resulted in rescue of the nrx-1(wy778) neurite outgrowth defect (Extended Data Fig. 9d, e). The long NRX-1 isoform still rescued the mutant phenotype even after deletion of the C-terminal PDZ binding motif, whereas the short NRX-1 isoform did not (Extended Data Fig. 9d, e). Overexpression of the long isoform of NRX-1 in wild-type male DVB neurons significantly increased DVB neurite length (P = 0.047) (Extended Data Fig. 9d, e), and when tagged with GFP, localized diffusely on the soma and neurites of DVB (Extended Data Fig. 9g). The reduction in time to aldicarb-induced spicule protraction in nrx-1 mutants was rescued by expression of the long isoform of NRX-1 in DVB, but overexpression of NRX-1 in wild-type worms did not change time to spicule protraction compared with control wild-type males (Extended Data Fig. 9f). These results indicate that the long isoform of NRX-1 is required in DVB for neurite outgrowth, which may extend the gene’s role beyond its canonical function at synapses. Varying the levels of NRX-1 in DVB directly alters the neurite branching observed upon NRX-1 overexpression is not further enhanced by loss of NLG-1 (Extended Data Fig. 9g–i). Furthermore,
nrx-1(wo778);nlg-1(ok259) double null mutant males with NRX-1 expressed in DVB showed an increase in neurites, similar to nlg-1 mutants (Extended Data Fig. 9g–i). Hence, restoration of NRX-1 expression in DVB with otherwise global loss of NRX-1 and NLG-1 recapitulates NLG-1 loss alone, suggesting that the nlg-1 phenotype requires NRX-1 in DVB. GFP-tagged NRX-1 localized diffusely onto the membranes of soma and processes and did not appear to change between days 1 and 3 (Extended Data Fig. 9j). By contrast, expression of GFP-tagged NLG-1 decreased from days 1 to 3 in DVB-targeted muscles and neurons (Extended Data Fig. 8). Hence, NRX-1 appears to function cell-autonomously in DVB to promote DVB neurite outgrowth, whereas NLG-1 operates in postsynaptic partners of DVB to antagonize NRX-1-dependent growth. Decreases in NLG-1 expression may result in a reduction in the antagonistic relationship, thereby permitting more NRX-1-dependent neurite elaboration. Our demonstration of an antagonistic neurexin–neuroligin relationship that influences neurite outgrowth hints at a signalling process downstream of neurexin that is antagonized by neurolin and is independent of neurolin's PDZ domain.

Finally, we tested whether manipulations that induce DVB neurites in males can also induce neurites in hermaphrodite DVB neurons. Activation of the anal depressor muscle (gar-3b::ChR2::yfp), loss of NLG-1, loss of NRX-1, or overexpression of NRX-1 in DVB had no effect on the axon morphology of hermaphrodite DVB neurons (Extended Data Fig. 10). Cell-autonomous sexual identity changes of either DVB or postsynaptic muscles using genetic manipulations of the sex-determination pathway also did not alter DVB morphology (see Methods). Thus, sexually dimorphic morphology and plasticity of the sex-shared DVB neuron seems to be non-autonomously instructed by male-specific circuit components.

Experience-dependent neuronal plasticity in the adult brain can include remodelling of dendrites and axons for behavioural adaptation or homeostatic maintenance of circuits. Our findings regarding male-specific DVB neurite outgrowth in C. elegans reveal the functional effect of morphological remodelling on circuits and behaviour. Through neurite outgrowth and rewiring of specific synapses, the DVB neuron undergoes a functional change that is likely to serve as an adaptive mechanism, perhaps translating experience into finer coordination of circuit activity and subsequent muscle contraction. These findings may have implications for the normal functions of neurexin and neurolin in plasticity, and for the many human diseases associated with them.

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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1. Fu, M. & Zuo, Y. Experience-dependent structural plasticity in the cortex. Trends Neurosci. 34, 177–187 (2011).
2. Holtmaat, A. & Svoboda, K. Experience-dependent structural synaptic plasticity in the mammalian brain. Nat. Rev. Neurosci. 10, 647–658 (2009).
3. Wutera, N. & Goda, Y. Cell biology in neuroscience: the interplay between Hebbian and homeostatic synaptic plasticity. J. Cell Biol. 203, 175–186 (2013).
4. Yin, J. & Yuan, Q. Structural homeostasis in the nervous system: a balancing act for wiring plasticity and stability. Front. Cell. Neurosci. 8, 439 (2015).
5. Lee, W. C. et al. Dynamic remodeling of dendritic arbors in GABAergic interneurons of adult visual cortex. PLoS Biol. 4, e29 (2006).
6. Mark, S. A., Yamahachi, H., Meyer zum Asten Borgloh, S. & Gilbert, C. D. Large-scale axonal reorganization in inhibitory neurons following retinal lesions. J. Neurosci. 34, 1623–1632 (2014).
7. Keck, T. et al. Loss of sensory input causes rapid structural changes of inhibitory neurons in adult mouse visual cortex. Neuron 71, 869–882 (2011).
8. Jarrell, T. A. et al. The connectome of a decision-making neural network. Science 337, 437–444 (2012).
9. White, J. G., Southgate, E., Thomson, J. N. & Brenner, S. The structure of the nervous system of the nematode Caenorhabditis elegans. Phil. Trans. R. Soc. Lond. B 314, 1–340 (1986).
10. Reiner, D. J. & Thomas, J. H. Reversal of a muscle response to GABA during male development. J. Neurosci. 15, 6094–6102 (1995).
11. LeBoeuf, B. & Garcia, L. R. Caenorhabditis elegans male copulation circuitry incorporates sex-shared detection components to promote intromission and sperm transfer. G3 7, 647–662 (2017).
12. Pokela, N., Liu, Q., Gordus, A. & Bargmann, C. I. Inducible and titratable silencing of Caenorhabditis elegans neurons in vivo with histamine-gated chloride channels. Proc. Natl Acad. Sci. USA 111, 2770–2775 (2014).
13. Garcia, L. R., Mehta, P. & Sternberg, P. W. Regulation of distinct muscle behaviors controls the C. elegans male’s copulatory spicules during mating. Cell 107, 777–788 (2001).
14. Hobert, O., Tessmar, K. & Ruvkun, G. The Caenorhabditis elegans im-5-LIM homeobox gene regulates neurite outgrowth and function of particular GABAergic neurons. Development 126, 1547–1562 (1999).
15. Feinberg, E. H. et al. GFP reconstitution across synaptic partners (GRASP) defines cell contacts and synapses in living nervous systems. Neuron 57, 353–363 (2008).
16. Liu, Y. et al. A cholinergic-regulated circuit coordinates the maintenance and bi-stable states of a sensory-motor behavior during Caenorhabditis elegans male copulation. PLoS Genet. 7, e1001326 (2011).
17. Liu, Y., LeBoeuf, B. & Garcia, L. R. Gn-coupled muscarinic acetylcholine receptors enhance nicotinic acetylcholine receptor signaling in Caenorhabditis elegans mating behavior. J. Neurosci. 27, 1411–1421 (2007).
18. Jobson, M. A. et al. Spillover transmission is mediated by the excitatory GABA receptor LQC-35 in C. elegans. J. Neurosci. 35, 2093–2016 (2015).
19. Garcia, L. R. & Sternberg, P. W. Caenorhabditis elegans UNC-103 ERG-like potassium channel regulates contractile behaviors of sex muscles in males before and during mating. J. Neurosci. 23, 2696–2705 (2003).
20. Guo, X., Navetta, A., Gualberto, D. G. & Garcia, L. R. Behavioral decay in aging male C. elegans correlates with increased cell excitability. Neurobiol. Aging 33, 1483.e5–1483.e23 (2012).
21. LeBoeuf, B. & Garcia, L. R. Cell excitability necessary for male mating behavior in C. elegans is coordinated by interactions between bic current and ether-a-go-go family K+ channels. Genetics 190, 1029–1041 (2012).
22. Barr, M. M. et al. The Caenorhabditis elegans autosomal dominant polycystic kidney disease gene homologs lov-1 and plc-2 act in the same pathway. J. Cell Biol. 117, 1341–1346 (2001).
23. Hobert, O., Moerman, D. G., Clark, K. A., Beckerle, M. C. & Ruvkun, G. A conserved LIM protein that affects muscular adherens junction integrity and mechanosensory function in Caenorhabditis elegans. J. Cell Biol. 144, 45–57 (1999).
24. Bang, M. L. & Owczarek, S. A matter of balance: role of neurexin and neurolin at the synapse. Neurochem. Res. 38, 1174–1189 (2013).
25. Mackowiak, M., Mordalska, P. & Wetzony, K. Neurelin, synapse balance and neuropsychiatric disorders. Pharmacol. Rep. 66, 830–835 (2014).
26. Pizzarelli, R. & Cherubini, E. Alterations of GABAergic signaling in autism spectrum disorders. Neurol. Plast. 2011, 297153 (2011).
27. Chinn, B., Engelman, H. & Scheiffele, P. Control of excitatory and inhibitory synapse formation by neurelins. Science 307, 1324–1328 (2005).
28. Maro, G. S. et al. MADD-4/punctin and neurexin organize C. elegans GABAergic postsynapses through neurexin. Neuron 86, 1420–1432 (2015).
29. Haklai-Topper, L. et al. The neurexin superfamily of Caenorhabditis elegans. Gene Expr. Patterns 11, 144–150 (2011).

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METHODS

C. elegans strains. Wild-type strains were C. elegans variety Bristol, strain N2. Worms were grown at 23 °C on nematode growth medium (NGM) plates seeded with bacteria (Escherichia coli OP50) as a food source. All males contained either him-8(e1489) IV or him-5(e1490) V as indicated by strain. Male worms were picked at the fourth larval stage onto plates with ten other males (unless otherwise indicated), and allowed to moult into adults and age to the day indicated for each analysis or experiment.

Mutant alleles used in this study include: him-8(e1489) IV, him-5(e1490) V, unc-31(e928) IV, nrl-1(ok2599) X, nr-1(ok1649) V, unc-113(ed3) III, nr-1(e7078)(unc-113(+)) V, and unc-97(su10) X, unc-25(e156) III, unc-94(e070) III, unc-1(ok1649) V, and nr-1(xk24627).

All transgenic strains used in this study are listed in Supplementary Table 1 ordered by Figures and Extended Data Figures. All plasmids were injected at 25 ng μl⁻¹ with coinjection marker ttx-3::gfp or ttx-3::wCherry also at 25 ng μl⁻¹ to generate extrachromosomal arrays (unless otherwise noted).

Cloning and constructs. To generate lim-6::wCherry (pM198) and lim-6::eGFP (pM141), a 291-bp fragment of the lim-6 fourth intron was amplified with primers adding BamHI to forward (CCCCGGATCTTGGCAATCCCGGGGATGATTAATAAATGTGCAGGAGGAGTA) and reverse (CTTTGATTTG, and cloned into DACR10 (a gift from D. Colon-Ramos) to replace the gar-3b(e124) III into a μGTCCTTTGGCCAATCCCGGGGATGATTAATAAATGTGCAGGAGGAGTA, and cloned into pNP471 to replace the gar-3b(e124) III into a

To generate lim-6::eGFP::ChR2-β1/2 (pM177), lim-6::eGFP was PCR-amplified from pm11, using primers forward CTATGATCATAAAAGGGCTGATGTT and reverse TTTTGATTTG, and cloned into pLR183 (gfp-13::ChR2·β1/2, a gift from L. R. Garcia) to replace the gfp-13 promoter using restriction free cloning.

To generate lim-6::eGFP::BirA::nrx-1SHORT (pM201), lim-6::eGFP was PCR-amplified from pm199 using primers forward GATGGATCATCAAAACATCTTCGAAAT and reverse CTTTTGGCCAATCCCGGGGATGATTAATAAATGTGCAGGAGGAGTA, and cloned into DARC10 (a gift from D. Colon-Ramos) to replace the ttx-3 promoter using restriction free cloning. The resulting plasmid was injected at 45 ng μl⁻¹ with coinjection marker ttx-3::gfp also at 45 ng μl⁻¹. An extrachromosomal array was integrated to yield ots154 and ots155. lim-6::eGFP was found to express brightly in both Avi and AV, and dimly in about 70% of worms in PVT.

To generate lim-6::eGFP::BirA::nrx-1LONG (pM235), lim-6::eGFP was PCR-amplified from pm11 using primers forward CTATGATCATAAAAGGGCTGATGTT and reverse TTTTGATTTG, and cloned into pLR183 (gfp-13::ChR2·β1/2, a gift from L. R. Garcia) to replace the gfp-13 promoter using restriction free cloning.

To generate lim-6::eGFP::BirA::nrx-1LONG (pM235), lim-6::eGFP was PCR-amplified from pm11 using primers forward CTATGATCATAAAAGGGCTGATGTT and reverse TTTTGATTTG, and cloned into pLR183 (gfp-13::ChR2·β1/2, a gift from L. R. Garcia) to replace the gfp-13 promoter using restriction free cloning.

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mated plates were checked for fluorescent progeny to ensure successful mating had occurred, and then mated and non-mated (individually housed) males were subjected to confocal microscopy. *C. elegans* males housed with other males or in isolation can engage in mating-like behaviours, which may include spicule protraction. To minimize mating sensory input and self-mating behaviour, we also analysed DVB neurite outgrowth in males with mutation in *pkd-2* and in males genetically paralysed by a mutation in unc-97/lim51 (affects body wall muscle ultrastructure).33

**Mating behaviour assay.** Mating assays were based on procedures described previously.34–35. Males were picked at the L4 stage and kept apart from hermaphrodites. One male was transferred to a plate covered with a fresh OP50 lawn containing 15 adult unc-31(e928) hermaphrodites. Day 1 males were counted as less than 18h after L4 moult. Males were observed for 5 min from the time of first contact with a hermaphrodite or until they ejaculated, whichever came first. Males were scored for their ability to prod the vulva, protract spicules, and transfer sperm. Mating success was calculated as 100× the number of males that transferred sperm successfully divided by the total number of males tested. The number of attempts at prodding was calculated by summing attempts at prodding for each male. The protraction/prodding ratio was calculated by dividing the number of spicule protractions by the number of attempts at prodding for each male.

**Synapse visualization.** GRASP plasmid construction is described above. For visualization of synaptic connections between DVB and neurons and muscles downstream of DVB that form the spicule protraction circuit, we injected *lim-6* promoter:: *nlg-1::gfp* 1-10 (pMH18) to label the presynaptic DVB, together with *lim-6* promoter:: *nlg-1::gfp* 1-10 (pMH20) to label the postsynaptic SPC and spicule protractor muscles. Plasmids were injected together at 25 ng μl−1 with the coinjection marker *ttx-3:gfp* (also at 25 ng μl−1) to generate extrachromosomal arrays. For visualization of synaptic connections between DVB and neurons and muscles downstream of DVB that form the spicule protraction circuit, the *lim-6* promoter also labels DVB neurites in males, which has few synapses with DVB that were located in the electron microcopy reconstruction anterior to the DVB neurites, and the branched and divided axons of the DVB and CP6 appear not to make contact (Extended Data Fig. 4b).

**Spicule activation assay with channelrhodopsin.** All-trans retinal was added to LB/OP50 medium and coated over the entire plate at a final concentration of 0.1 mM. We obtained strains expressing channelrhodopsin under the *gar-3b* promoter17,30,31,32 labelling spicule protraction neurons and muscles, and under the *unc-103E* promoter labelling spicule protractors and anal depressor muscles, and under the *unc-103F* promoter labelling spicule neurons SPC, PCA, and PCB44 (gifts from L. R. Garcia). Worms were incubated overnight on retinal plates before all assays involving channelrhodopsin-containing strains. For the spicule protraction assay, male worms on retinal plates were rinsed in distilled water and placed on a Nikon eclipse E400 microscope. Obvious spicule muscle contraction for any of the three trials was recorded as a response. Videos were recorded using a mounted Eco Labs Focus camera. For the activation protocol, male worms on retinal plates were subjected to alternating 488-nm light three times (15s light/15s dark) on a Leica M165 FC dissecting scope, repeated every 45 min for 4.5h. Worms were then subjected to confocal microscopy or aldicarb behavioural assay. Controls for neurite outgrowth and aldicarb behaviour were performed on males under the same conditions but not exposed to the channelrhodopsin cofactor all-trans retinal (Extended Data Fig. 5j–l). For recovery experiments in the dark, males were kept under the same illumination after they were subjected to the same analysis. A small number of individual males subjected to confocal imaging before and after activation, or after activation and following recovery, demonstrated addition of neurites following activation, and removal of neurites following recovery; however, the difficulty of this analysis precluded quantification.

**Neuronal silencing with histamine chloride channel (HisCl1).** Control or transgenic worms were picked onto normal NGM plates seeded with OP50 at the L4 stage, then picked the evening before the indicated day of analysis onto 10 mM histamine or control plates with OP50 bacteria as a food source. For *gar-3b*: *HisCl1* silencing assays, males were left on histamine or control plates overnight then subjected to confocal microscopy the following morning. For *lim-6* promoter:: *nlg-1::gfp* 1-10 males, males were picked onto histamine plates, allowed to adjust for 5 min and then analysed for defecation behaviour. Histamine plates were prepared as previously described.32

**Defecation assay.** Males were placed on control or 10 mM histamine plates with food on the day of analysis, allowed to explore for 5 min, and then observed for 10–12 min on a low magnification Leica MZ8 light dissecting microscope.

Expulsion steps were recorded for the time between consecutive expulsions, and the presence of spicule protrusion within 3s before or after expulsion. The percentage of expulsion steps associated with spicule protraction was calculated for each male. The time between consecutive expulsion steps was calculated by averaging all times recorded between consecutive expulsions for each male.

**Exogenous GABA exposure.** Males were picked onto normal NGM plates seeded with OP50 at the L4 stage, then picked before the day of analysis onto 30 mM GABA39 or control plates seeded with OP50 and left overnight, and then subjected to confocal microscopy. For 1-day GABA exposure, males were picked onto 30 mM GABA or control plates seeded with OP50, left for 3 days and then subjected to confocal microscopy.**Measurement of fluorescence intensity.** To quantify the fluorescence intensity of *nlg-1::gfp*, a stack of images was acquired using confocal microscopy with the same acquisition parameters between samples (objective, pixel size, laser intensity, pinhole size, and PMT settings). The fluorescence intensity mean was obtained using ZEN Black software. For the dorsal spicule muscles, the muscles were outlined and the cross-section with the highest mean was recorded. Dorsal spicule muscles include the gubernacular retractor, gubernacular ector, anterior oblique, and anal depressor, which could be outlined easily, whereas the spicule protractor could not always be observed in males after day 1. For the pre-anal gondlion and DVB or background, a pre-defined circle was used to outline the region of interest, and the cross section with the highest mean was recorded. The ratio of fluorescence intensity was calculated by dividing the mean of the dorsal spicule muscles (arbitrary units) by the mean of the DVB or background (arbitrary units) or by dividing the mean of the pre-anal gondlion by the mean of the DVB or background (arbitrary units).

**Cell-autonomous changes in sexual identity.** We tested cell-autonomous changes in the sexual identity of DVB (*lim-6* promoter) and muscles (myo-3 promoter) by expressing either the cDNA of *fen-3* in hermaphrodites to masculinize each tissue39–40 or to feminize each tissue.44 In males with feminized DVB or muscles, we observed no suppression of DVB neurites, and in hermaphrodites with masculinized DVB or muscle, we observed no induction of DVB neurites.

**Statistics and reproducibility.** We performed two-tailed Student's t-test or one-way ANOVA with post-hoc Tukey HSD test using *R* and *RStudio*. Values are shown on each graph. No statistical methods were used to predetermine sample size, and the experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment. Number of independent biological replicates: Fig. 1b–d; 7; Fig. 2a–c, h–k, 3 or more; Fig. 2e, f, 2 or more; Figs 3a–g, 3 or more; Figs 4a–h, 3 or more; Extended Data Fig. 1a–c, 4 or more; Extended Data Fig. 1d, 1 or more; Extended Data Fig. 2a, b, 4 or more; Extended Data Fig. 2c–h, 2 or more; Extended Data Fig. 3a–c, 3 or more; Extended Data Fig. 3d–f, 2 or more; Extended Data Fig. 4a–c, h, 2 or more; Extended Data Fig. 4d–g, 3 or more; Extended Data Fig. 5a–f, 4 or more; Extended Data Fig. 5g–l, 2 or more; Extended Data Fig. 6a–f, 2 or more; Extended Data Fig. 7a–h, 3 or more; Extended Data Fig. 8a–c, 3 or more; Extended Data Fig. 8d–f, 2 or more; Extended Data Fig. 9b–i, 3 or more; Extended Data Fig. 9j, 2 or more; Extended Data Fig. 10a–c, 3 or more; Data availability. The data that support the findings of this study are available from the corresponding author upon reasonable request.

30. LeBoeuf, B., Correa, P., Jee, C. & Garcia, L. R. Caenorhabditis elegans male sensory-motor neurons and dopaminergic support cells couple ejaculation and post-ejaculatory behaviours. *eLife* 3, e02398 (2014).
31. Oren-Suissa, M., Bayer, E. A. & Hobert, O. Sex-specific pruning of neuronal synapses in Caenorhabditis elegans. *Nature* 533, 206–211 (2016).
32. Longair, M. H., Baker, D. A. & Armstrong, J. D. Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics* 27, 2453–2454 (2011).
33. Fang-Yen, C., Gabel, C. V., Samuel, A. D., Bargmann, C. I. & Avery, L. Laser ablation in Caenorhabditis. *Methods Cell Biol.* 107, 177–206 (2012).
34. Garcia, L. R., LeBoeuf, B. & Koo, P. Diversity in mating behavior of hermaphroditic and male-female Caenorhabditis nematodes. *Genetics* 175, 1761–1771 (2007).
35. Hilliard, M. A., Bargmann, C. I. & Bazzicalupo, P. C. elegans responds to chemical repellents by integrating sensory inputs from the head and the tail. *Curr. Biol.* 12, 730–734 (2002).
36. Sulston, J. E., Albertson, D. G. & Thomson, J. N. The Caenorhabditis elegans hermaphrodite: postembryonic development of gonadal structures. *Dev. Biol.* 38, 2453–2454 (2011).
37. Mowrey, W. R., Bennett, J. R. & Portman, D. S. Distributed effects of biological sex define sex-typical motor behavior in Caenorhabditis elegans. *J. Neurosci.* 34, 1579–1591 (2014).
Extended Data Figure 1 | Progressive neurite outgrowth in DVB in adulthood. a–c, DVB neuron visualized with lim-6\textsuperscript{pos-2}::gfp at days 1, 3, and 5 in adult males (a) and quantification of total neurite length (b) and number of neurite junctions (c) (dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD, \(P\) values shown above plots, bold shows significance (\(P<0.05\))).

d, DVB neurite outgrowth visualized with flp-10::gfp in males at days 1, 3, and 5 of adulthood (\(n>10\), scale bars, 10\(\mu m\)).

e, Tracing reconstruction of male DVB from electron micrograph sections compiled by http://wormwiring.org showing DVB neurites. f, Inset of DVB neurites showing presynaptic specializations identified in electron micrograph sections shown in pink. g, h, Electron micrograph section showing DVB pseudo-coloured yellow with presynaptic specialization indicated with red x with SPCR (Image Right1200, Section 14871) (g) and spicule sheath (Image N2YDRG1175, Section 14816) (h), shown in white in inset panel. Scale bars, 1\(\mu m\).
Extended Data Figure 2 | DVB neurite outgrowth in adult male
*C. elegans* is stochastic and other neurons in the male tail do not show progressive neurite outgrowth in adulthood. a, b, DVB neurites at day 5 visualized with *lim-6*::wCherry (a) or *lim-6*::gfp (b) (*n* > 10 for each). DVB posterior neurites were traced through confocal stacks using Simple Neurite Tracer4 plugin. c, DVA neuron visualized with *ser-2*(prom-2)::gfp (*n* = 5) (red dashed line indicates axon of relevant neuron). d, DVC neuron visualized with *inx-18p::gfp* (*n* = 5). e, CP6 neuron visualized with *flp-13p::gfp* (cell soma not shown) (*n* = 5). f, Ray neurons visualized with *dat-1p::gfp* (ventral view) (*n* = 5). g, h, PVT neuron visualized with *srz-102p::gfp* (*n* = 5) (g) and *srg-4p::gfp* (*n* = 5) (h) at day 1 and day 5. Axons of indicated neurons highlighted by red dashed lines. Scale bars, 10 μm.
Extended Data Figure 3 | DVB inhibits expulsion-associated spicule protraction at day 3. Laser ablation of DVB and channelrhodopsin expression in DVB and spicule protraction circuit. a, Confocal images of male worm with lim-6<sup>int4::wCherry</sup> and lim-6<sup>int4::HisCl1::gfp</sup> at day 3. b, Quantification of the percentage of expulsion steps with spicule protraction for day 1 control, day 3 control, day 3 control + histamine, and day 3 lim-6<sup>int4::HisCl1::gfp + histamine</sup> males. c, Time between consecutive expulsion steps for day 1 control, day 3 control, day 3 control + histamine, and day 3 lim-6<sup>int4::HisCl1::gfp + histamine</sup> males (+ histamine is on 10 mM histamine plates; dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD. P values shown above plots, bold shows significance (P < 0.05)). d, Confocal images of male worms with or without laser ablation of DVB at day 1 or 2, visualized with lim-6<sup>int4::gfp</sup>. e, Confocal images of DVB (lim-6<sup>int4::wCherry</sup>) expressing channelrhodopsin at day 1 and 5, Ex[lim-6<sup>int4::ChR2::yfp</sup>]. f, Confocal images of DVB (lim-6<sup>int4::wCherry</sup> and spicule circuit expressing channelrhodopsin at day 1 and 5, Ex[gar-3b::ChR2::yfp], n > 10 for d-f. Scale bars, 10 μm.
Extended Data Figure 4 | DVB neurite outgrowth in unc-49, pkd-2 and unc-97 mutant males. flp-13p::gfp labels CP6 and spicule retractor muscles. a–c, Confocal images (a) and quantification of total neurite outgrowth (b) and number of neurite junctions (c) in control and unc-49(e407) males at days 3 and 5. d, Time to spicule protraction on aldicarb at day 5 for control and unc-49(e407) males. e–g, Confocal images (e) and quantification of total neurite outgrowth (f) and number of neurite junctions (g) in control, pkd-2(pt8), and unc-97(su110) males at day 3. h, Confocal images of male worms with lim-6int4::wCherry, flp-10p::gfp, and differential interference contrast at day 1 in ventral and lateral views. Inset showing DVB and CP6 axons, with schematic of axons demonstrating lack of contact (red is DVB axon, green is CP6 axon, blue dashed lines are spicule retractor muscles). Asterisks in flp-13::gfp panel mark spicule retractor muscles. Dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD, P values shown above plots, bold shows significance (P < 0.05), scale bars, 10 µm.
Extended Data Figure 5 | See next page for caption.
Extended Data Figure 5 | Day 1 male mating defects involving spicule coordination, spicule circuit activation in unc-25, unc-97, and nrx-1 mutant males, and spicule neuron or muscle activation induces DVB neurite outgrowth. a, Per cent average mating success (sperm transfer) for day 1 and 3 males during 5-min timed mating assays with 15 unc-31(e928) hermaphrodites (n is number of worms; data points represent average percentage for each replicate of multiple males). b, Quantification of attempts at spicule prodding during 5-min timed mating assay for day 1 and 3 males. c, Ratio of protraction:prodding attempts during 5-min timed mating assay for males at days 1 and 3. d–f, Confocal images of lim-6(e46):wCherry (d), total neurite length (e), and number or neurite junctions (f) of unc-25(e156), unc-25(e156);Ex[gar-3b::ChR2::yfp], unc-97(su110), unc-97(su110);Ex[gar-3b::ChR2::yfp], nrx-1(wy778), and nrx-1(wy778);Ex[gar-3b::ChR2::yfp] males following activation at day 1 (488-nm light for 3 × 15 s every 45 min for 4.5 h). g–i, Confocal images (g) and quantification of total neurite outgrowth (h) and number of neurite junctions (i) in control, Ex[unc-103E::ChR2::yfp], and Ex[unc-103F::ChR2::yfp] worms after activation at day 1 with retinal (488-nm light for 3 × 5 s every 45 min for 4.5 h). j, k, Quantification of total neurite outgrowth (j) and number of neurite junctions (k) at day 1 in control, Ex[lim-6(e46):ChR2::yfp] (DVB), Ex[unc-103E::ChR2::yfp] (neuron-specific), and Ex[unc-103F::ChR2::yfp] (muscle-specific) males after activation but in the absence of retinal. l, Time to protraction of control and Ex[lim-6(e46):ChR2::yfp] males after day 1 activation in the absence of retinal. Dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD, P values shown above plots, bold shows significance (P < 0.05), scale bars, 10 μm.
Extended Data Figure 6 | Exposure to exogenous GABA or silencing of spicule protraction circuit activity overnight reduces DVB neurites on day 5. a–c, Confocal images of lim-6int4::wCherry (a), total neurite length (b), and number of neurite junctions (c) of males exposed overnight to 30 mM GABA at days 3 and 5. d–f, Confocal images of lim-6int4::wCherry (d), total neurite length (e), and number of neurite junctions (f) at day 5 of control worms with or without overnight 10 mM histamine, and gar-3b::HisCl1::gfp worms with or without overnight 10 mM histamine. Dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD, *P* values shown above plots, bold shows significance (*P* < 0.05), scale bars, 10 μm.
Extended Data Figure 7 | NLG-1 expression in multiple male sex muscles rescues nlg-1 mutant DVB neurite phenotype. Silencing spicule circuit or exposure to exogenous GABA does not reduce DVB neurites in nlg-1 mutant males. a–c, Confocal images of DVB (lim-6int4::wCherry) (a), and quantification of total neurite outgrowth (b) and number of neurite junctions (c) in control, nlg-1(ok259), nlg-1(ok259);nlg-1p::nlg-1::gfp, and nlg-1p::nlg-1::gfp day 3 males. d, e, Quantification of total neurite outgrowth (d) and number of neurite junctions (e) in control or nlg-1(ok259) mutant males with or without tissue-specific NLG-1 expression. Expression patterns for rescue promoters: lim-6 in DVB; gar-3b in SPC and spicule protractor muscles; unc-103F in SPC, PCA, PCB and other neurons; unc-103E in male sex muscles; flp-13 in spicule retractor muscles and CP6. f–h, Confocal images (f) of lim-6int4::wCherry and Ex[gar-3b::HisCl1::gfp] in day 5 male worms, with total neurite length (g) and number of neurite junctions (h) of nlg-1(ok259) worms with or without 10 mM histamine overnight, nlg-1(ok259); gar-3b::HisCl1::gfp worms with or without 10 mM histamine overnight, and nlg-1(ok259) worms with 30 mM GABA overnight. Dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD, P values shown above plots, bold shows significance (P < 0.05), scale bars, 10 μm.
Extended Data Figure 8 | NLG-1 expression decreases from day 1 to day 3. a, Confocal images of nlg-1p::nlg-1::gfp in males at days 1, 3, and 5. Example regions of interest for measurements taken from single planes: blue, dorsal spicule muscles; red, pre-anal ganglion; magenta, DVB. b, c, Quantification of fluorescence intensity of nlg-1p::nlg-1::gfp in males at days 1, 3, and 5 reported as a ratio of mean fluorescence in dorsal spicule muscles (b) or pre-anal ganglion (c) normalized to background of DVB, which has little-to-undetectable expression. Dorsal spicule muscles refer to the gubernacular retractor, gubernacular erector, anterior oblique, and anal depressor. d, Confocal images of nlg-1p::nlg-1::gfp in day 3 males as follows: control, nlg-1(ok259), nrx-1(wy778). e, f, Quantification of fluorescence intensity of nlg-1p::nlg-1::gfp in day 1 and 3 male worms as follows: control, nlg-1(ok259), nrx-1(wy778), day 3 nlg-1(ok259) with overnight GABA exposure, and nlg-1(ok259) with 3-day GABA exposure, as a ratio of mean fluorescence in dorsal spicule muscles (e) or pre-anal ganglion (f) normalized to background of DVB. Dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD, P values shown above plots, bold shows significance (P < 0.05), scale bars, 10 μm.
Extended Data Figure 9 | NRX-1 long isoform functions in DVB to control DVB neurite outgrowth and NRX-1 expression in DVB controls neurite outgrowth of nlg-1 mutants. a, Genetic loci of nrx-1 showing long and short isoforms, PDZ binding motif, and locations of point mutation gk246237 and deletions ok1649 and wy778. b, c, Quantification of total neurite length (b) and number of neurite junctions (c) in controls and long-isoform-specific mutants nrx-1(ok1649) and nrx-1(gk246237) at day 3. d, e, Quantification of total neurite outgrowth (d) and number of neurite junctions (e) at day 3 in control, Ex[lim-6int4::birA::nrx-1LONG], nrx-1(wy778);Ex[lim-6int4::birA::nrx-1LONG], nrx-1(wy778);Ex[lim-6int4::birA::nrx-1LONG], nrx-1(wy778);Ex[lim-6int4::birA::nrx-1LONG], nrx-1(wy778); and nrx-1(wy778); Ex[lim-6int4::birA::nrx-1LONG] worms. f, Time to spicule protraction at day 3 in control, nrx-1(wy778), nrx-1(wy778); Ex[lim-6int4::birA::nrx-1LONG], and Ex[lim-6int4::birA::nrx-1LONG] worms. g–i, Confocal images of lim-6::wCherry expression (g) and quantification of total neurite length (h) and number of neurite junctions (i) of day 3 nlg-1(ok259), nlg-1(ok259);Ex[lim-6int4::birA::nrx-1LONG], nrx-1(wy778), nrx-1(wy778);Ex[lim-6int4::birA::nrx-1LONG], nlg-1(ok259); and nlg-1(ok259); nlg-1(ok259); Ex[lim-6int4::birA::nrx-1LONG] males. j, Confocal images of lim-6::wCherry and Ex[lim-6int4::gfp::nrx-1LONG] in control, nrx-1(wy778), and nlg-1(ok259) males at day 1 and 3. Dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD. P values shown above plots, bold shows significance (P < 0.05) scale bars, 10 µm.
**Extended Data Figure 10 | DVB in hermaphrodites does not show neurite branching upon gar-3b::ChR2::yfp activation or NRX-1 or NLG-1 manipulation.**

**a**, Confocal images of lim-6(ex4)wCherry and Ex[gar-3b::ChR2::yfp] expression in day 1 hermaphrodites showing DVB axon projection after activation with retinal (488-nm light for 3 x 15 s every 45 min for 4.5 h). **b**, Confocal images of lim-6(ex4)::wCherry or lim-6(ex4)::gfp in control, nrx-1(wy778), nlg-1(ok259), and Ex[lim-6(ex4)::gfp::nrx-1↑] hermaphrodites at day 3. **c**, Quantification of the percentage of hermaphrodites with DVB axon abnormalities or neurites (in almost all cases, a single neurite off the axon just posterior to the pre-anal ganglion) in day 1 control and Ex[gar-3b::ChR2::yfp] with activation, day 3 control, nrx-1(wy778), nlg-1(ok259), and Ex[lim-6(ex4)::gfp::nrx-1↑] worms. n shows number of worms, data points represent average percentage for each replicate of multiple hermaphrodites. Dot represents one worm; magenta bar, median; boxes, quartiles; one-way ANOVA and post-hoc Tukey HSD. P values shown above plots, bold shows significance (P < 0.05), scale bars, 10 μm.
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- Experimental design

1. Sample size
   Describe how sample size was determined.
   No sample size calculations were performed. Sample size was uniformly determined by analyzing 5-10 animals for each condition for each replicate, with most experiments having 2-3 independent replicates. In some instances, like experiments with more inherent variability (behavioral assays) more animals were analyzed per replicate or more replicates were performed.

2. Data exclusions
   Describe any data exclusions.
   No data was excluded

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   All experiments were independently replicated at least twice, almost all experiments were replicated three independent times.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   There was no method of randomization, experiments were performed based on condition and genotype.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Investigators were blinded to genotype, condition, and age of animals used in experiments.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   | n/a | Confirmed |
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   | ✗   | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.) |
   | ✗   | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
   | ✗   | A statement indicating how many times each experiment was replicated |
   | ✗   | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section) |
   | ✗   | A description of any assumptions or corrections, such as an adjustment for multiple comparisons |
   | ✗   | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted |
   | ✗   | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range) |
   | ✗   | Clearly defined error bars |

See the web collection on statistics for biologists for further resources and guidance.
Software

7. Software

Describe the software used to analyze the data in this study.

R and R studio statistical software for statistical tests. Zeiss Zen Black software for confocal image acquisition and processing. FIJI and Simple Neurite Tracer for quantification of neurites.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

No restrictions

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

No antibodies were used in this study

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

No eukaryotic cell lines

b. Describe the method of cell line authentication used.

No eukaryotic cell lines

c. Report whether the cell lines were tested for mycoplasma contamination.

No eukaryotic cell lines

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No eukaryotic cell lines

Animals and human research participants

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

No vertebrate or higher invertebrate animals

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

No human research subjects