Structural and functional analysis of single neurons to correlate synaptic connectivity with grooming behavior

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We describe a protocol to image the complex axonal branching structure of identified mechanosensory neurons in *Drosophila*, combined with a behavioral assay to evaluate the functional output of the neuron. The stimulation of identified mechanosensory neurons in live animals produces a stereotyped grooming reflex. The mechanosensory axonal arbor within the CNS is subsequently labeled with a lipophilic fluorescent dye and imaged by fluorescence microscopy. The behavioral output can therefore be correlated to the axonal morphology of the stimulated neuron in the same animal. Combining this protocol with genetic analysis provides a powerful tool for identifying the roles of molecules involved in different aspects of hard-wired neural circuit formation underlying an innate behavior. From behavioral analysis to axonal imaging, the protocol takes 4 d.

INTRODUCTION

The investigation of how neurons form precise connections with their partners during circuit development is crucial for our understanding of brain function and behavior. For hard-wired neural circuits underlying innate behaviors, it is generally thought that neurons distinguish between correct versus incorrect synaptic targets by using instructions encoded in the genome. To better understand how molecules can encode prespecified synaptic targeting patterns, the hard-wired and stereotyped neural circuitry of invertebrates has been used in conjunction with genetic analyses to identify these molecules[1,2].

The mechanosensory system of *Drosophila melanogaster* in particular offers several advantages for investigating the mechanisms of synaptic targeting. First, specific mechanosensory neurons are uniquely identifiable from one animal to another on the basis of the location of its corresponding bristle[3]. Each mechanosensory neuron is named after the bristle that it innervates, because a single neuron innervates a single bristle, and each of the large bristles (macrochaetae) on the back of the fly is identifiable on the basis of its position (**Fig. 1a**). For example, on the posterior scutellum, there are only two bristles, the left and right posterior scutellars (pSc), each innervated by a pSc neuron (**Fig. 1a**,**b**). Second, the axonal branching pattern of each mechanosensory neuron is complex yet highly stereotyped between animals (**Fig. 1c**,**d**)[3]. This indicates a tight genetic regulation of the synaptic connectivity for each neuron, and the axonal branching pattern can thus be used as a readout for differences in connectivity. Third, mechanosensory axonal branch lengths and positions can be quantitatively analyzed to measure the differences in axonal branch targeting between individuals (**Fig. 1c**,**d**)[2,4,5]. For example, we have characterized the wild-type branching variability of two mechanosensory neurons, the pSc and pDc, and we have identified a set of 'core' branches that are present in almost all flies (**Fig. 1c**,**d**). Thus, within a complex axonal arbor, we can now identify with certainty even the same axonal branch among different individuals. Fourth, different mechanosensory neurons are easily labeled using lipophilic dyes to allow for multicolor imaging of their axons.

Other techniques exist to label neurons in flies. Most commonly, transgenic lines that express Gal4 in restricted populations of cells are used to drive *UAS*-based expression of fluorescent proteins. This strategy is limited, however, because no *Gal4* line has been found to be specific for a single neuron, and this results in multiple axons being labeled. Highly specific *Gal4* promoters that can identify a small number of neurons typically do not express Gal4 for a long enough time for fluorescent proteins to label the entire axon until adulthood, such as the two lines used in this protocol (*455-Gal4*, *DC-Gal4*). Complex genetic methods can be used to circumvent these limitations, for example, by using combinations of spectrally distinct genetically encoded fluorophores to distinguish single axons[6–8]. However, our protocol, using spectrally distinct dyes, provides the simplest method to resolve single axons. This protocol also has broad applicability to other sensory axonal projections within *Drosophila*, such as chemosensory neurons[9], as well as other insect model systems that do not have *Gal4* lines[10]. Dye-labeling of axonal projections has also been used in vertebrate systems, where Cre-based expression shares similar limitations of the *Gal4* system in invertebrates[11].

Taking advantage of the above characteristics of the *Drosophila* mechanosensory system, we developed a technique to assess the functional output of the mechanosensory neuron by stimulating its corresponding bristle to elicit a grooming behavior from the animal[5] (**Fig. 2**). In the fly, touching mechanosensitive bristles can activate a cleaning reflex from the legs to repeatedly brush the stimulated bristles[5,12–16]. For example, stimulation of the notopleural bristle located on the lateral notum, and hence activation of the notopleural neuron, always results in the fly sweeping its front leg over the stimulated area, whereas stimulation of the pSc bristles evokes a stereotyped response from the third (rear) pair of legs. This specificity of stimulating single mechanosensory neurons to activate specific legs was first used in 1980 to demonstrate an independence between synaptic targeting of an axon versus its axon guidance into the CNS, where axons can be misrouted into different nerve roots but still activate the correct cleaning reflex leg[13].

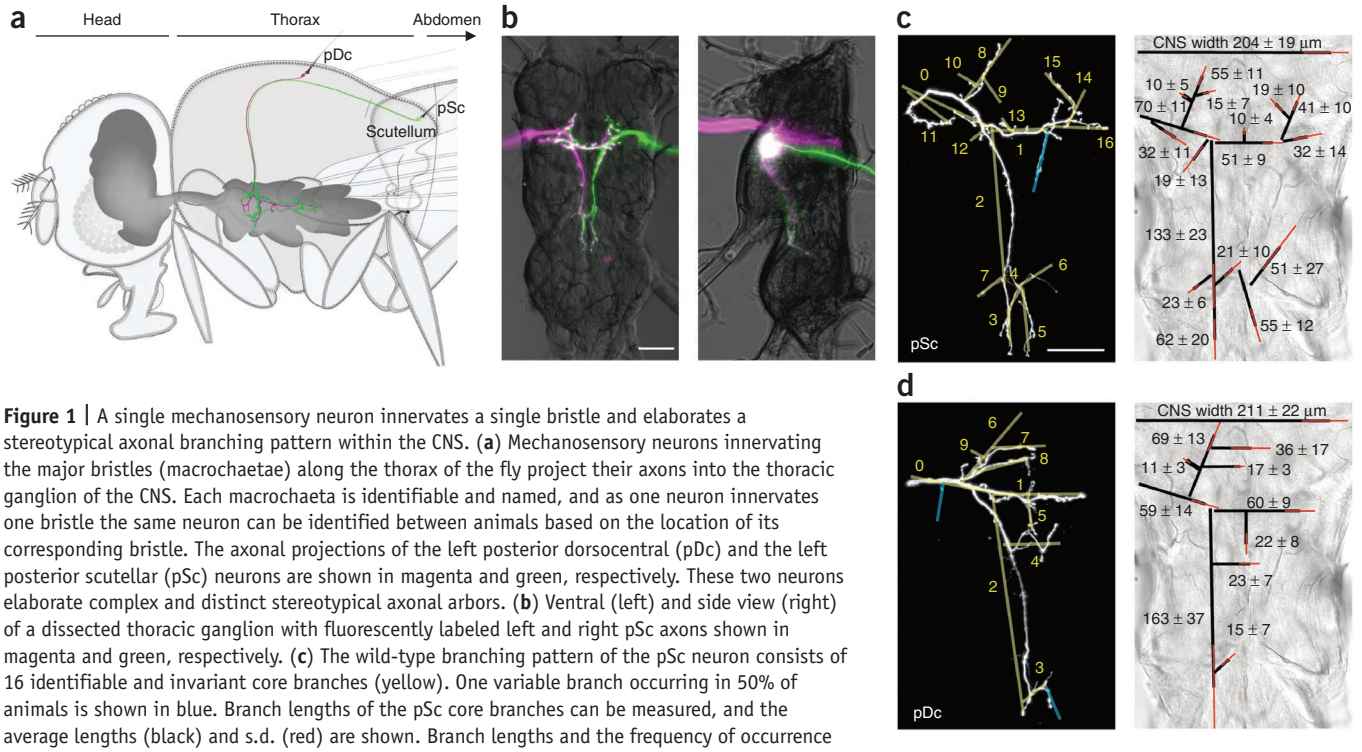


Figure 1 | A single mechanosensory neuron innervates a single bristle and elaborates a stereotypical axonal branching pattern within the CNS. (**a**) Mechanosensory neurons innervating the major bristles (macrochaetae) along the thorax of the fly project their axons into the thoracic ganglion of the CNS. Each macrochaeta is identifiable and named, and as one neuron innervates one bristle the same neuron can be identified between animals based on the location of its corresponding bristle. The axonal projections of the left posterior dorsocentral (pDc) and the left posterior scutellar (pSc) neurons are shown in magenta and green, respectively. These two neurons elaborate complex and distinct stereotypical axonal arbors. (**b**) Ventral (left) and side view (right) of a dissected thoracic ganglion with fluorescently labeled left and right pSc axons shown in magenta and green, respectively. (**c**) The wild-type branching pattern of the pSc neuron consists of 16 identifiable and invariant core branches (yellow). One variable branch occurring in 50% of animals is shown in blue. Branch lengths of the pSc core branches can be measured, and the average lengths (black) and s.d. (red) are shown. Branch lengths and the frequency of occurrence of these invariant branches can be used as a readout of synaptic connectivity of the pSc neuron. (**d**) The wild-type branching pattern of the pDc neuron consists of ten identifiable core branches (yellow), and, on average, three variable branches, the most variable (blue) occurring at 50%. Scale bars, 50 µm.

The behavioral assay that we developed, which we describe in this protocol, allows for functional analysis of an identified mechanosensory neuron by using the cleaning reflex, along with morphological analysis of its axonal branching pattern, all within the same fly. Combining this with genetic analysis for molecular manipulations in single neurons can identify cell-autonomous roles of molecules in axonal targeting and circuit function[5]. Our protocol is inexpensive, reliable, can be performed in 4 d (**Fig. 3**) and uses an established and well-characterized system for the study of synaptic connectivity and circuit function.

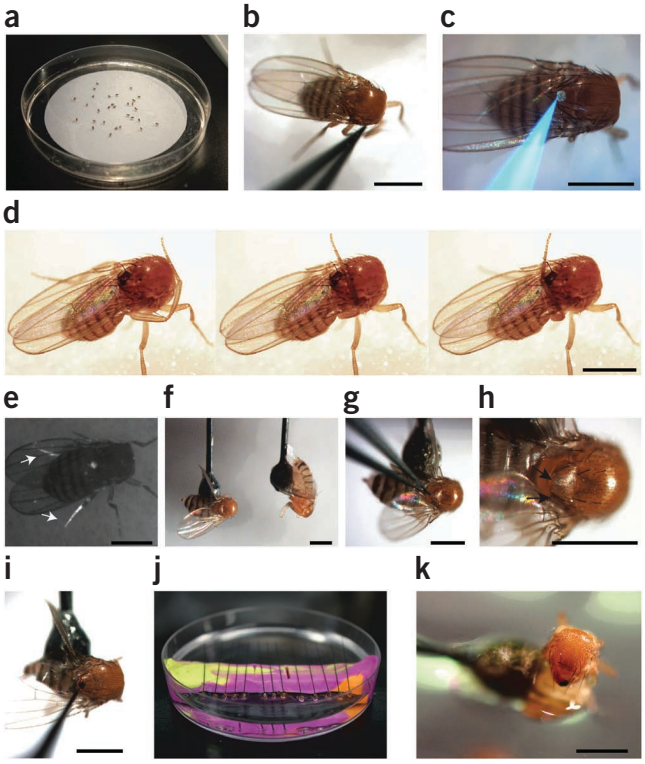
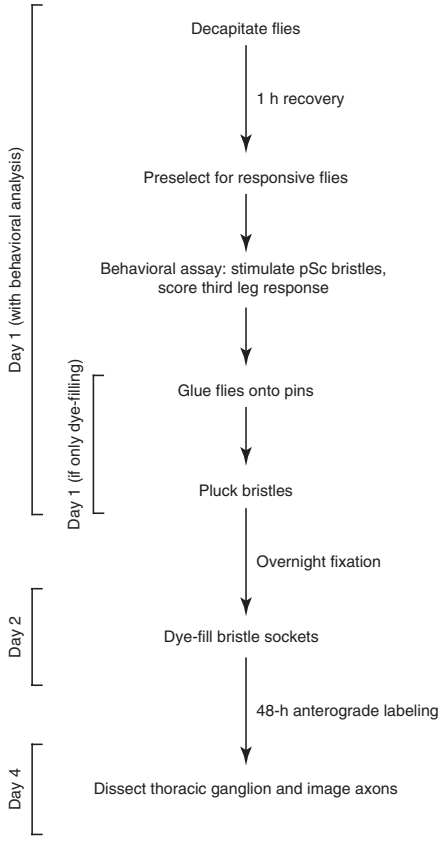


Figure 2 | The behavioral output and structure of a single mechanosensory neuron can be measured in single flies. (**a**) After decapitation, flies are left to recover for 1 h in a humidified chamber made from a 15-cm Petri dish with PBS-soaked filter paper. The decapitated flies should be standing on the filter paper after recovery from CO_2 anesthesia. (**b**) Behaviorally responsive flies are preselected by stimulating the anterior notopleural bristle by using fine forceps. The fly will react using its first leg to brush the stimulated area. (**c**) Stimulation of the pSc bristle is performed by applying DiO fluorescent dye only at the tip of the scutellum, and the dye should only deflect the two pSc bristles. (**d**) Upon pSc stimulation, the third leg of the fly will brush the entire notum, starting at the anterior dorsocentral region and progressing to the posterior tip of the scutellum. Blue DiD dye is shown for clarity. (**e**) The fluorescent dye will be transferred to the rear legs upon a positive cleaning response (arrows). (**f**) Flies are glued onto insect pin heads for bristle plucking. The lateral side of the flies can be either glued perpendicular to the pin head for access to the dorsally located bristles, or along the abdomen, parallel to the pin head for access to the laterally located bristles. (**g**) Bristles are plucked by using fine forceps by pulling against the direction of growth and close to the base. Plucking of a pSc bristle is shown. (**h**) Bristle sockets should be completely exposed (arrows) to allow access of the dye to the neuron underneath. (**i**) Bristle sockets are dye-filled with DiI (red, left pSc) and DiD (blue, right pSc). (**j**) Dye-filled flies are placed on clay and left undisturbed and protected from light in a 10-cm Petri dish with 0.2 M carbonate-bicarbonate buffer. (**k**) A close-up view of a half-submerged fly is shown. The abdomen should be submerged in buffer, and the upper thorax with the dye-filled sockets should be kept above the surface of the buffer. Scale bars, 1 mm.

Figure 3 | Flowchart of the experimental procedure, starting from behavioral assay to imaging axonal morphology, performed in 4 d. The protocol can be separated to perform only the behavioral assay or the morphological analysis; if the mechanosensory neuron is not being dye-filled, then the experiment takes only 1 d.



Experimental design

Here we describe how to measure mechanosensory circuit function through a behavioral response with subsequent imaging of the axonal morphology of the primary mechanosensory neuron involved in the behavior. The behavioral assay involves the mechanical stimulation of only a left and right pair of bristles by applying a controlled amount of fluorescent dye onto the bristles to elicit a cleaning reflex. To visualize the axonal branching patterns of single mechanosensory neurons, the axons are labeled with lipophilic carbocyanine fluorescent dyes. Anterograde labeling with carbocyanine dyes has been especially useful in examining the morphology and fine branching of single axons within insect nervous systems[2–5,17]. High-quantum-yield carbocyanines, such as DiI, DiD and DiO, can diffuse in lipids such as plasma membranes, are not toxic and can persist in both living and fixed tissue for extended periods of time. When these fluorescent dyes are applied peripherally, they diffuse anterogradely, progressing along the axonal branches to label all of the axon's fine projections within the CNS. Standard fluorescence microscopy can be used to image the axonal arbors within the CNS, and the axonal branching pattern can be quantitatively analyzed by measuring the lengths and positions of each identifiable branch. We routinely dye-fill pairs of axons with red and far-red dyes for multicolor imaging (**Fig. 4**), and in theory up to four different dyes can be used in the same animal.

Simple controls can be performed at different steps to verify the fidelity of the technique. To ensure that the neural circuitry underlying the grooming response is intact, *Drosophila* are preselected

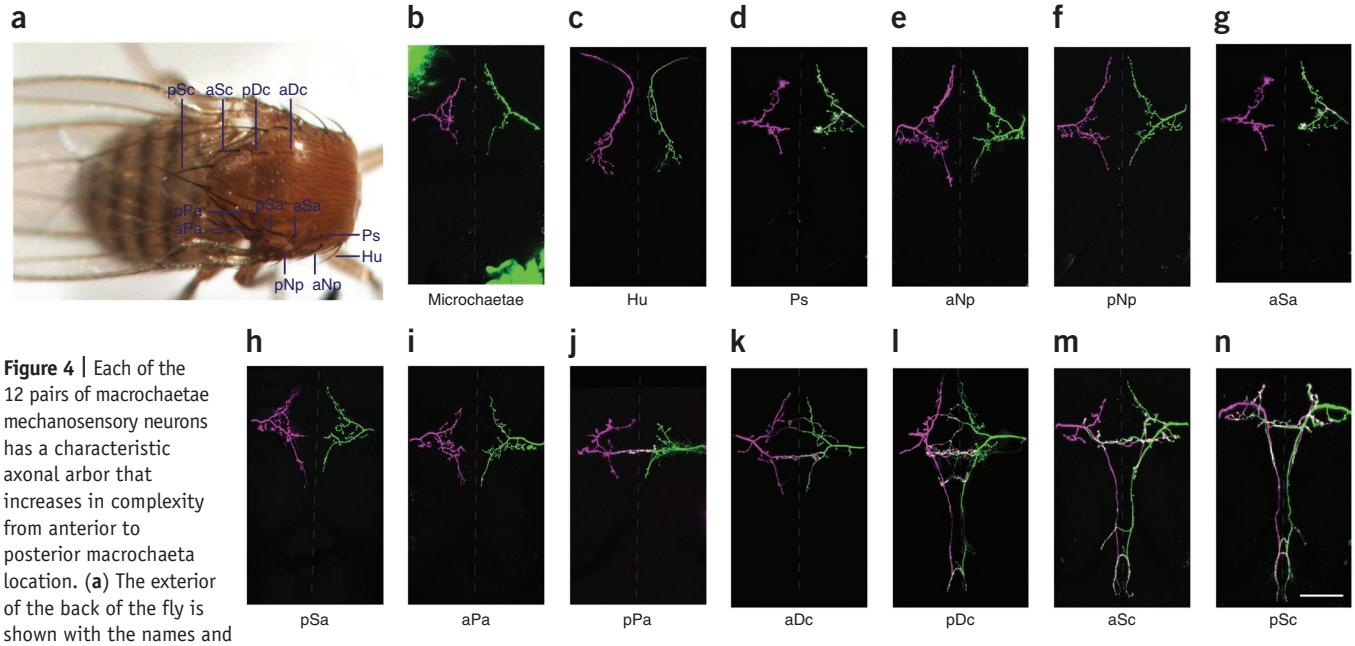


Figure 4 | Each of the 12 pairs of macrochaetae mechanosensory neurons has a characteristic axonal arbor that increases in complexity from anterior to posterior macrochaeta location. (**a**) The exterior of the back of the fly is shown with the names and positions of the macrochaetae labeled. The humoral bristles (Hu) are the most anterior, and they are located on the postpronotum. The presutural bristles (Ps) are located dorsally on the presutural area. The anterior and posterior notopleural bristles (aNp and pNp) are located laterally on the notopleuron. The anterior and posterior supraalar (aSa and pSa) and anterior and posterior postalar (aPa and pPa) bristles are located laterally on the prealar and postalar calluses, respectively. The anterior and posterior dorsocentral bristles (aDc and pDc) are located dorsally on the scutum. The anterior and posterior scutellar bristles (aSc and pSc) are the most posterior, and they are located on the scutellum. Microchaetae line the dorsal thorax, except on the scutellum. (**b**) The simple axonal arbors of the left and right microchaetae from two different regions of the thorax are shown. Depending on the location, microchaetae mechanosensory neurons have slightly different arbors. (**c**–**n**) Each pair of macrochaeta was labeled with DiI on the left (magenta) and DiD on the right (green) in the same fly. The symmetrical axonal arbors are shown for each pair. Dotted lines mark the midline of the CNS. Scale bar, 50 μm.

using stimulation of the notopleural bristles, which produces a cleaning response at close to 100% reliability in healthy flies. To ensure that the fluorescent dye can label entire axonal arbors with strong fluorescent signal, multiple bristles along the notum can be plucked to label several mechanosensory neurons at once with a large volume of dye covering the thorax, and this ensures overlabeling of many axons within the CNS. To control for variability between experiments, flies of the same age and sex should be used, and experiments should be performed at the same time of day to control for circadian cycle effects, and within the same season to control for differences in food consistency that can occur with changes in relative humidity.

Depending on the nature of the experiment, the entire structural and functional analysis can be performed, or this protocol can be separated into only the behavioral assay or the morphological analysis.

MATERIALS

REAGENTS

- Flies (e.g., wild-type flies from the Bloomington *Drosophila* Stock Center)
- DiD (Life Technologies, cat. no. D7757)
- DiI (Life Technologies, cat. no. D282)
- DiO (Life Technologies, cat. no. D275)
- Sodium carbonate anhydrous (Fisher, cat. no. S263500)
- Sodium bicarbonate (Fisher, cat. no. BP328)
- PBS (generic supplier)
- Paraformaldehyde (Sigma-Aldrich, cat. no. 16005) ! CAUTION Paraformaldehyde is toxic. Wear gloves and eye protection and handle the compound in a fume hood.
- Cyanoacrylate glue (e.g. Krazy Glue advanced gel formula, generic supplier)
- Sylgard 184 silicone elastomer (generic supplier)
- Ethanol, 100% (generic supplier)
- ddH_2O
- Gaseous CO_2

EQUIPMENT

- Borosilicate capillary glass outer diameter (o.d.)/inner diameter (i.d.) = 1.00 mm/0.78 mm (Harvard Apparatus, cat. no. W4 64-0778)
- Glass slides, 75 × 25 mm (Fisher, cat. no. 12-550-A3)
- Glass coverslips, 18 × 18 mm (Fisher, cat. no. 12542A)
- Double-sided tape (generic supplier)
- Insect pins (Fine Science Tools, cat. no. 26000-25)
- Micromanipulator (Harvard Apparatus, cat. no. 60-0569)
- Micropipette holder for behavior experiments (Harvard Apparatus, cat. no. W4 64-1228)
- Micropipette holder for dye-filling (Harvard Apparatus, cat. no. W4 60-0604)
- Dumostar nos. 5 and 55 forceps (Fine Science Tools, cat. nos. 11295-00 and 11295-51)
- Vannas spring scissors (Fine Science Tools, cat. no. RS 5618)
- Eppendorf 2-ml tubes (Fisher, cat. no. 22363344)
- Gel loading tips (Fisher, cat. no. 213802M)
- Sylgard-coated 35-mm dish
- Petri dishes, 10 cm
- Petri dishes, 15 cm
- Beakers
- Magnetic stirrer with heater
- Thermometer
- Shaking heat block
- Kimtech wipes
- Parafilm
- Whatman no. 1 filter paper
- Syringe, 30 ml
- Tygon R-3603 tubing, 3.2 mm i.d. × 4.8 mm o.d. × 0.8 mm wall (generic supplier)
- Micropipette puller (P-97 Flaming/Brown micropipette puller, Sutter Instruments)
- Dissecting microscope with epifluorescence lamp (Zeiss Stereo Discovery V12)
- Imaging microscope (Zeiss Axio Scope A1)
- Charge-coupled device (CCD) camera
- Visible light source
- Fluorescent light source (laser or epifluorescence lamp)

REAGENT SETUP

▲ CRITICAL All buffers can be stored at 4 °C for up to 3 months.

Carbonate buffer A Prepare 500 ml of a 0.4 M solution of anhydrous sodium carbonate. Dissolve 21.2 g of anhydrous sodium carbonate in 500 ml of ddH_2O.

Bicarbonate buffer B Prepare 500 ml of a 0.4 M solution of sodium bicarbonate. Dissolve 16.8 g of sodium bicarbonate in 500 ml of ddH_2O.

Carbonate-bicarbonate buffer, 0.2 M, at pH 9.5 Mix 13 ml of buffer A with 37 ml of buffer B and 50 ml of ddH_2O to obtain a final volume of 100 ml.

Paraformaldehyde, 37% (wt/vol) Weigh out and dissolve 5.87 g of paraformaldehyde in 10 ml of ddH_2O on a heated magnetic stirrer in a fume hood. You must add a few drops of 10 N NaOH for the solid to dissolve. ! CAUTION Paraformaldehyde is toxic and NaOH is corrosive. Wear gloves and eye protection.

Paraformaldehyde, 3.7%, in 0.2 M carbonate-bicarbonate buffer at pH 9.5 Mix 13 ml of buffer A with 37 ml of buffer B, 40 ml of ddH_2O and 10 ml of 37% (wt/vol) paraformaldehyde to obtain a final volume of 100 ml. Divide 250 µl of the solution in 2-ml centrifuge tubes and store them at –20 °C for up to 3 months.

DiI dye, 20 µg µl^{-1} Dissolve 100 mg of DiI in 2 ml of 100% ethanol to make a stock solution of 50 µg µl^{-1}. Store it at 4 °C protected from light for up to 6 months. Dilute 20 µl of stock DiI (50 µg µl^{-1}) in 30 µl of 100% ethanol to make a working solution of 20 µg µl^{-1} DiI. Store it at 23–25 °C protected from light for up to 1 month.

DiD dye, 32 µg µl^{-1} Dissolve 10 mg of DiD in 250 µl of 100% ethanol to make a stock solution of 40 µg µl^{-1}. Store it at 4 °C protected from light for up to 6 months. Dilute 20 µl of DiD stock (40 µg µl^{-1}) in 5 µl of 100% ethanol to make a working solution of 32 µg µl^{-1} DiD. Store it at 23–25 °C protected from light for up to 1 month.

DiO dye, 10 µg µl^{-1} Dissolve 100 mg of DiO in 2 ml of 100% ethanol to make a stock solution of 50 µg µl^{-1}. Store it at 4 °C protected from light for up to 6 months. Dilute 10 µl of DiO stock (50 µg µl^{-1}) in 40 µl of 100% ethanol to make a working solution of 10 µg µl^{-1}. Store it at 23–25 °C protected from light for up to 1 month.

EQUIPMENT SETUP

Micropipette design guidelines We have found that for optimal dye application in the behavioral assay, a taper length of 2–3 mm and a tip diameter of 20 µm allow the use of only slight pressure to eject a small volume (~20 nl) onto the fly. As a guide, our settings for a Sutter P-97 Flaming/Brown micropipette puller using standard borosilicate glass of o.d./i.d. 1.00 mm/0.78 mm with filament are as follows: heat = 515, pull = 30, velocity = 40, time = 165.

For optimal mechanosensory neuron dye labeling, micropipettes with a medium taper length of 3–4 mm and tip diameters that are half as wide as the bristle sockets (10 µm) perform best. The tip diameter needs to be wide enough for the dye to flow out upon contact with the bristle socket without the need to pressure-eject the dye. Our settings for a Sutter P-97 Flaming/Brown micropipette puller using standard borosilicate glass of o.d./i.d. 1.00 mm/0.78 mm with filament are as follows: heat = 550, pull = 30, velocity = 40, time = 165.

Micromanipulator Set up the micromanipulator on a flat, sturdy surface near the stereomicroscope stage (to the right of the microscope if you are right-handed) and adjust the micromanipulator so that its three axes are in the center of their dynamic range. Insert a micropipette holder with a pressure port (for example, Harvard Apparatus) connected to a 30-ml syringe via a 15-cm tubing (for example, Tygon R3603 tubing, 3.2 mm i.d. × 4.8 mm o.d. × 0.8 mm wall). For optimal visualization of DiO, we recommend using a fluorescence stereomicroscope with a green filter.

Dye filling Set up the microscope and micromanipulator. Use a dual-electrode holder. White light can be used for illumination of the concentrated DiI and DiD dyes; however, if preferred, epifluorescence illumination and a red filter can be used to visualize the dye flow.

Sylgard-coated dishes Mix 10 parts Sylgard 184 silicone elastomer base with 1 part elastomer curing agent (10:1). Coat the bottom of several

35-mm dishes and cure them at 37 °C for 8 h. The dishes can be stored at 23–25 °C.

Imaging For fly manipulation, bristle plucking, behavioral assays and dissection, we use a fluorescence stereomicroscope with an ×1 objective and a green filter for the visualization of DiO. For imaging axonal arbors, standard epifluorescence or laser-scanning confocal imaging microscopes can be used, with an ×40 objective, numerical aperture 1.0 and red and far-red filters for DiI and DiD, respectively.

PROCEDURE

Day 1, behavioral assay and bristle plucking ● TIMING ~2 h

▲ CRITICAL If the mechanosensory neuron is not being labeled, the experiment just comprises Steps 1–8. If the behavioral assay is not being performed, then the protocol can begin at Step 9.

1| Anesthetize the adult flies with CO_2.

▲ CRITICAL STEP To reduce behavioral variability between animals, we recommend using animals of the same sex (females), age (2 d old), circadian cycle (afternoon) and season (owing to relative humidity effects during food preparation).

2| Decapitate the flies by using spring scissors.

▲ CRITICAL STEP Do not damage the front legs or anterior thorax.

3| Place the flies in a 150-mm dish with wet Whatman filter paper as a humidity chamber. After a 1-h recovery period, the flies should be standing on all six legs and generally responsive (**Fig. 2a**).

▲ CRITICAL STEP If at any point during the experiment the filter paper begins to dry out, rehydrate it with drops of PBS.

? TROUBLESHOOTING

4| To verify the integrity of the cleaning reflex neural circuitry within the thoracic ganglion, use forceps or a pin to gently deflect the anterior notopleural bristle (**Fig. 2b**) and preselect flies that respond with movement of the ipsilateral first (anteriormost) leg. Proceed to pSc stimulation on preselected flies.

? TROUBLESHOOTING

5| Backfill a micropipette with 1 µl of DiO dye solution.

6| Attach the micropipette to a micromanipulator. Adjust the micromanipulator so that all the axes are in the center of their dynamic range. Attach a 30-ml syringe to the electrode holder's pressure port (see Equipment Setup).

7| By using the micromanipulator, bring the micropipette to the posterior scutellum and gently eject the dye by applying pressure to the syringe. Ideally, the dye should cover the two pSc bristles (**Fig. 2c**).

▲ CRITICAL STEP Always approach the scutellum posteriorly, parallel to the scutellar bristles. This prevents you from accidentally touching the dorsocentral bristles. Avoid spilling dye on the side of the scutellum, the thorax or the wings, as this may result in a false positive behavioral response.

8| Assay the fly's cleaning response. A positive cleaning response from stimulation of the pSc bristle is defined as the third (rear) pair of legs reaching dorsally and brushing the thorax (**Fig. 2d**), and it can be verified by the transfer of the fluorescent dye onto the legs (**Fig. 2e**). Individual animals that respond or do not respond are identified and separated.

9| Select and anesthetize the flies from the responder or nonresponder groups.

10| Place a drop of cyanoacrylate glue into a disposable receptacle (e.g., a 35-mm Petri dish), and dip the round head of an insect pin into the glue to lightly coat the head of the pin; dab to remove any excess glue.

? TROUBLESHOOTING

11| By using a stereomicroscope for visualization, glue the insect pin head to the fly at a region between the posterior thorax and anterior abdomen, slightly ventrally. Pull the wing over and onto the pin head to help stabilize the fly. This orientation is best for accessing the dorsal bristles. For accessing laterally located bristles, glue the ventral abdomen of the fly parallel to the pin head (**Fig. 2f**).

▲ CRITICAL STEP Glue the left side of the fly if you are right-handed, and the right side if you are left-handed.

? TROUBLESHOOTING

12| Insert the pin into a block of clay and repeat this for all flies, lining the pins in parallel. The pins are generally stable enough for dye-filling if they extend ~3 cm out of the clay.
▲ **CRITICAL STEP** Allow ~5 min for the glue to dry. All the flies should still be alive and active.
? TROUBLESHOOTING

13| Zoom in (×5) on the bristle of choice, and by using a pair of fine forceps (e.g., Dumont no. 55) grab the bristle close to, but not at, the base and quickly pull from posterior to anterior, against the direction of growth of the bristle (**Fig. 2g**,**h**).
▲ **CRITICAL STEP** Avoid breaking the bristle near or at the base, as this will prevent the dye from entering the bristle socket.
? TROUBLESHOOTING

14| Remove ~50% of the abdomen (**Fig. 2i**) (and the head if the behavioral assay, Steps 1–8, was not previously performed) by using spring scissors, and immediately immerse the fly on the pin in a 2-ml tube containing a 250-µl solution of 3.7% (wt/vol) paraformaldehyde in 0.2 M carbonate-bicarbonate buffer, pH 9.4.
! CAUTION Paraformaldehyde is toxic. Wear gloves and eye protection.

15| Gently agitate the tube to evacuate any air bubbles. Seal the tube with the cap open with Parafilm to avoid damaging the flies.

16| Place the tube at 23–25 °C for 2 h or at 4 °C overnight.
■ **PAUSE POINT** Flies can be stored in fixative at 4 °C for up to 14 d without a noticeable decrease in dye-filling efficiency. Best results are always obtained after overnight fixation at 4 °C.

Day 2, dye filling ● TIMING ~1 h for 15–20 flies
17| Reconstitute the fluorescent carbocyanine tracer DiI or DiD solution by shaking it at 1,200 r.p.m. in a 40 °C heat block, for 5 min. If the dye particles do not dissolve, add 2–3 µl of 100% ethanol and resume shaking.
▲ **CRITICAL STEP** If a shaking heat block is not available, incubate it at 40 °C with periodic vortexing.

18| Attach the micropipette to a micromanipulator. Adjust the micromanipulator so that the axes are in the center of their dynamic range.

19| Use coarse forceps to remove one of the pins from the fixative and immerse the fly in 0.2 M carbonate-bicarbonate buffer for a few seconds, and then in ddH_2O for a few seconds. Gently blot the fly dry on a Kimwipe and allow 1–2 min for it to dry. Flies need to be completely dry for dye-filling to prevent spilling of dye across the back of the fly.
! CAUTION Paraformaldehyde is toxic. Wear gloves and eye protection.

20| Backfill a micropipette with 1–2 µl of dye solution using gel-loading tips.

21| Place the flies under a stereomicroscope and zoom to ×5 to inspect the empty socket.

22| Gently touch the tip of the micropipette on the socket; the dye should immediately flow into and slightly around the socket (**Fig. 2i**).
▲ **CRITICAL STEP** Do not insert the micropipette tip inside the socket, as this can damage the tissue; simply make contact with the socket surface.
▲ **CRITICAL STEP** It is normal for the tips to break slightly during contact with the sockets; however, it is best to change micropipettes after dye-filling 4–6 flies.
? TROUBLESHOOTING

23| Coat the entire socket with dye and allow a small protective bubble of dye to solidify to prevent washout in subsequent steps. Be careful not to spread dye to neighboring bristles, especially if neighboring bristles are also plucked.
? TROUBLESHOOTING

24| Retract the micropipette and allow the dye to dry for 1 min.

25| Carefully take the pinned fly and lay the pin onto clay placed in a 10-cm Petri dish filled with 0.2 M carbonate-bicarbonate buffer (**Fig 2j**), such that the fly's remaining abdomen is immersed but the dye-filled bristle socket is above the buffer (**Fig 2k**). Repeat this for all flies.

26| Store the finished flies in a dark area at 23–25 °C where they can remain undisturbed for 2 d.
▲ **CRITICAL STEP** The dye requires 2 d to completely label the axonal arbor, and incubating the flies for longer may result in the dye diffusing beyond the axonal membrane.

Day 4, dissection and imaging ● **TIMING ~2.5 h for 15–20 flies**
27| Turn on and prepare all imaging hardware and software.

28| Take one fly and use the shank of a closed pair of dissecting forceps to gently nudge it off the head of the pin into a Sylgard-coated 35-mm dish filled with 1–2 ml of 0.2 M carbonate-bicarbonate buffer.
? TROUBLESHOOTING

29| Gently dissect out the fly's thoracic ganglion. The ganglion is located on the ventral side of the thorax, and it extends from anterior to posterior throughout the ventral thorax (**Fig. 1a**). It is translucent but not as clear as the muscle tissue you will encounter (**Fig. 1b**). It is ~500 × 300 µm in size, with the thinnest part being 40 µm wide.
▲ **CRITICAL STEP** The thinnest part of the thoracic ganglion is easily damaged or detached. Carefully dissect around the thoracic cuticular extension that grows around the thinnest part of the ganglion. This 'clamp' is located ventrally, and it is nearly transparent.
? TROUBLESHOOTING

30| Gently move the ganglion away from the dissection debris and carefully clear out any remaining tracheal tubes or connective tissue.

31| Take two small pieces of double-sided tape and place them ~1 cm apart on a glass slide; put a drop of 0.2 M carbonate-bicarbonate buffer in between.

32| Place the ganglion in the drop of buffer on the slide. Zoom in on the ganglion to manipulate it so that the ventral side of the ganglion is facing up (**Fig. 1b–d**).

33| Place a cover slip on the slide, gently pressing against the tape. Use a pipette to fill any remaining air space with buffer.

34| Image the slide immediately by using standard fluorescence microscopy with a ×40 objective, so that the entire axonal arbor (~135 µm × 245 µm for the pSc) is within one field of view.
▲ **CRITICAL STEP** Once the thoracic ganglion has been dissected out of the thorax, all of its afferent axons are severed and the dye will slowly begin to leak out of the mechanosensory axon. Therefore, Steps 28–34 should be done within 5–10 min.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
3	Flies do not recover 1 h after decapitation	Damage to the thorax during decapitation	Use spring scissors, not a razor blade or forceps, for decapitation
4	Many flies do not respond to notopleural stimulation	Flies could be dying or unable to move their front legs. Notopleural responses should be close to 100%	Ensure proper hydration of filter paper If the filter paper is too wet, flies can get stuck to it Always use filter paper. Fly legs can get stuck on the fibers of wet Kimwipes
10–12	Glue is covering the thorax	Too much glue on the pin head Cyanoacrylate glue will spread from the pin head to the fly thorax if placed in fixative before it is dry	Allow enough time for glue to dry before starting bristle plucking

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
13	Base of the bristle remains in the socket	Pulling the bristle in the wrong direction	Pull with a sharp motion from posterior to anterior, parallel to the cuticle surface. Orient the fly appropriately
22, 23	Dye spills onto the thorax and other bristles	Fly is not completely dry	Allow at least 2 min for fly to dry after washing steps
		Dye-filling tip diameter is too large	Change dye-filling tips if broken or irregular
		Salt is left over from buffer wash	Wash longer in ddH_2O
28, 29	Fly tissue appears to be dehydrated	Flies were not partially submerged between dye-filling and dissection	Ensure that the pins are stabilized in the clay
			Examine the Petri dish at eye level to ensure that the abdomen is touching the buffer
34	Multiple axons are labeled	Spilling of dye into other bristles/sockets	Pluck only the bristle of choice and avoid covering a large area with dye
		Dye is too concentrated	Persistent overlabeling may require slight dye dilution
	Labeling is not continuous	Physical damage to CNS during dissection	Carefully dissect and mount ganglion with minimal stress on the tissue. Do not tug, squeeze, warp or torque the ganglion
		Optical obstruction in front of the axon	Remove any air bubbles, tracheal tubes and other physical obstructions from the ventral surface of the thoracic ganglion
	Faint or no labeling	Dye did not enter the socket or got washed out	Avoid occlusions of the socket (bristle remains, glue and salt) and ensure the formation of a solidified, protective dye bubble over the socket during dye-filling
		All afferent axons are ripped as the thoracic ganglion is dissected out of the thorax, so the dye labeling the mechanosensory axon will slowly begin to leak out	Imaging should be completed within 5–10 min of dissection
		Prolonged exposure of the sample to UV light reduces the signal intensity	Do not expose the dye to unnecessary UV light

● TIMING

Steps 1–16, day 1, behavioral assay and bristle plucking: ~2 h
Steps 17–26, day 2, dye filling: ~1 h for 15–20 flies
Steps 27–34, day 4, dissection and imaging: ~2.5 h for 15–20 flies

ANTICIPATED RESULTS

We used fluorescent dye-filling to reveal the fine morphology of all 13 major thoracic mechanosensory bristles in wild-type *Drosophila* (**Fig. 4**). As previously shown, the complexity of the mechanosensory axonal arbor increases from anterior to posterior positioned mechanosensory neurons[3]. The stereotyped branching pattern of these axons has allowed us to construct a model of the invariant branches of the pSc and pDc axonal arbors (**Fig. 1c**,**d**). Several parameters of the axonal branching pattern can be quantitatively analyzed, such as their frequency of occurrence, positions and lengths, and this can be used to examine the mechanisms underlying different aspects of synaptic targeting of these neurons[2,5]. High-resolution fluorescence imaging also allows for individual axonal varicosities along the axon to be quantified, and these varicosities are thought to be sites of synaptic contact. For example, we have previously used the stereotyped targeting of the pSc neuron to identify roles for the PlexinA receptor in suppressing axonal branch growth and axonal varicosity formation, and for PlexinB receptor in promoting axonal branch growth[4].

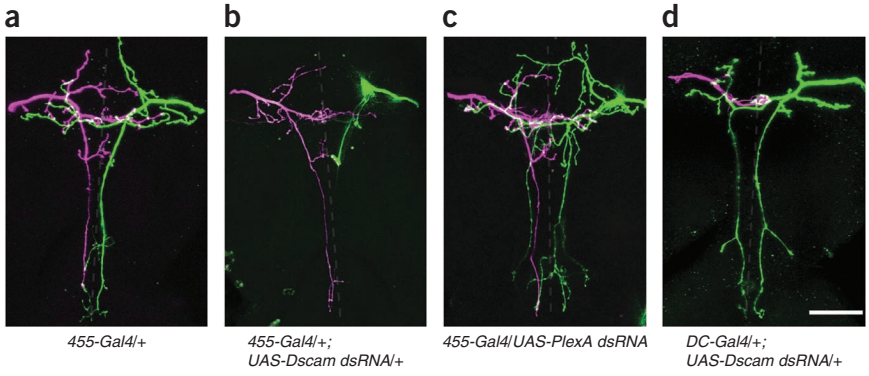


Figure 5 | Different *Gal4* drivers can be used to express dsRNA solely within specific mechanosensory neurons. (**a**) The *455-Gal4* driver is scutellar specific and it expresses only in the four scutellar mechanosensory neurons, the left and right aSc and pSc neurons. A representative example of the pDc (magenta) and pSc (green) neurons' axonal arbors in a control heterozygous *455-Gal4/+* animal is shown. (**b**) RNAi-mediated knockdown of *Dscam* only in the scutellar mechanosensory neurons in a *455-Gal4/+; UAS-Dscam dsRNA/+* animal results in a characteristic *Dscam*-null phenotype in the pSc axon (green) with clumped axonal branches, whereas the pDc axon (magenta) remains unaffected. (**c**) *Gal4* driving UAS expression of double-stranded RNA against *PlexA* in a *455-Gal4/UAS-PlexA dsRNA* fly results in a characteristic PlexinA knockdown phenotype in the pSc neuron (green) with excessive, supernumerary branches, whereas the pDc (magenta) remains unaffected. (**d**) The *DC-Gal4* driver expresses *UAS* constructs only in the four dorsocentral neurons. The specificity of the driver is shown in a *DC-Gal4/+ ; UAS-Dscam dsRNA/+* fly, where the pSc neuron is unaffected (green) and the pDc neuron (magenta) has a Dscam-null phenotype. Dotted lines mark the midline of the CNS. Scale bar, 50 μm.

To examine how different molecules affect the mechanosensory neuron's targeting decisions, *Gal4* lines can be used to drive expression solely within mechanosensory neurons, also allowing for mosaic analysis of a molecule's cell-autonomous effects. By using *Gal4* to drive different *UAS* lines, different molecules can be either overexpressed using the *UAS* promoter or knocked down using *UAS-dsRNA* constructs to target molecules for RNA interference. As an example, we used the *455-Gal4* driver to express double-stranded RNA against *Dscam* and *PlexA* solely within scutellar neurons[18], and the *DC-Gal4* driver to knock down *Dscam* solely within dorsocentral neurons[19] (**Fig. 5**). Dscam has been previously shown to have an essential function within mechanosensory neurons for axonal branch targeting. Loss of Dscam causes a severe collapsed axonal arbor phenotype[2], whereas the loss of PlexinA causes supernumerary axonal branches to form throughout the arbor[4].

The specificity of each *Gal4* line allows for manipulation of a few identifiable neurons within the context of a mosaic animal that is otherwise completely wild type. By using multicolor dye-labeling of the pSc and posterior dorsocentral neurons, we confirmed that the *455-Gal4* driver solely affected the scutellar neurons and produced wild-type phenotypes in dorsocentral neurons, and vice versa for the *DC-Gal4* driver (**Fig. 5**). Thus, multicolor imaging can serve as a within-animal control to verify cell-autonomous phenotypes (**Fig. 5**).

By combining the behavioral assay with fluorescence imaging of single mechanosensory axons, we can correlate the synaptic connectivity of the primary sensory neuron with the grooming behavioral output (**Fig. 6**). The use of specific *Gal4* drivers to manipulate only mechanosensory neurons ensures that neurons and cells within the cleaning reflex circuitry that are downstream of the mechanosensory neuron remain unaffected, and any changes in behavioral response are due to changes within the mechanosensory neuron itself. By using this structural and functional analysis, we have previously shown that excessive protein levels of the Dscam receptor impairs the pSc axonal targeting, and animals were less able to perceive mechanical stimulation of their affected bristles[5].

We performed our combined structural/functional protocol using the *455-Gal4* line to express *Dscam* dsRNA within scutellar neurons, and confirmed that the loss of Dscam structural phenotype was always obvious qualitatively and quantitatively. For example, *Dscam* RNAi pSc 'yes response' neurons (n = 9) had, on average, 5.2 ± 2.4 s.d. total axonal branches with a total arbor size of 260.6 ± 146.9 μm, and the *Dscam* RNAi pSc 'no response' neurons (n = 20) had an average of 4.7 ± 1.8 total branches and an arbor size of 279.1 ± 112.3 μm. In comparison, control *455-Gal4* 'yes response' neurons (n = 14) had

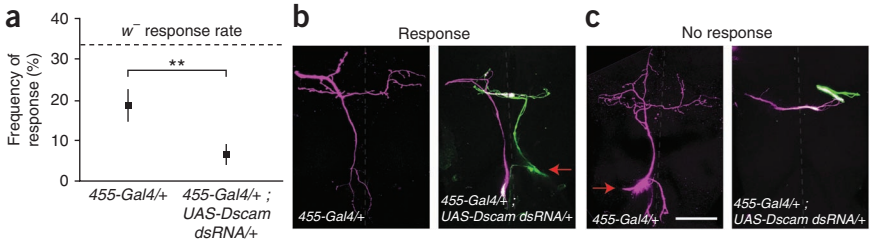


Figure 6 | Specific *Gal4* drivers for the presynaptic mechanosensory neuron can be combined with a behavioral assay to examine the functional output of the neuron after experimental manipulations. (**a**) Knockdown of Dscam specifically in the scutellar neurons severely impairs behavioral output and synaptic targeting. Stimulation of the pSc neurons in *455-Gal4/; UAS-Dscam dsRNA/+* flies resulted in severely impaired behavioral output, compared with *455-Gal4/+* controls (t test for proportions, $^{**}P < 0.01$). Only 7% of RNAi flies (n = 114) responded to pSc stimulation, whereas 19% of *455-Gal4/+* controls (n = 118) elicited a cleaning reflex. For comparison, the response rate of wild-type w^- flies are shown as a reference (dotted line). (**b**,**c**) Representative images of dye-filled pSc axons in *455-Gal4/+* controls and *455-Gal4/+; UAS-Dscam dsRNA* animals that succeeded or failed to respond to pSc stimulation. *455-Gal4/+; UAS-Dscam dsRNA* flies have severely reduced axonal arbors. Correlating axonal morphology with behavioral output can provide a link between specific synaptic connectivity and neural function, such as the extending branches in *455-Gal4/+; UAS-Dscam dsRNA*-positive responding neurons, shown in the representative image. Red arrows indicate axon guidance errors. Dotted lines mark the midline of the CNS. Scale bar, 50 μm.

an average of 25.2 ± 3.2 axonal branches and an arbor size of 926.7 ± 92.9 μm, and the *455-Gal4* 'no response' neurons (n = 13) had 22.2 ± 5.2 average total branches and an arbor size of 870.5 ± 150.5 μm (**Fig. 6**). Over 90% of the *Dscam* RNAi pSc neurons could not detect bristle stimulation, as the pSc is the primary sensory neuron in the reflex pathway (**Fig. 6a**), and those *Dscam* RNAi pSc neurons that could respond were likely due to the variability in dsRNA expression (**Fig. 6b**,**c**). Dscam loss of function clearly highlights the relationship between structural connectivity and neural function. Further negative controls can be used to verify *Gal4* drivers to produce a complete loss of response, such as knockdown of molecules involved in synaptic transmission.

It is important to note that in this protocol we define a cleaning response simply as the rear pair of legs brushing the notum and the consequent transfer of the fluorescent dye onto the legs (**Fig. 2d**,**e**). Thus, the data are grouped into a binary set of 'yes response' and 'no response.' By analyzing only total branch numbers and lengths in *455-Gal4* control and *Dscam* RNAi genotypes, we found no significant difference between the 'yes response' and 'no response' neurons (P > 0.05) (**Fig. 6b**,**c**). Our representative grooming behavior results illustrate that very large data sets are required to correlate specific synaptic connectivity patterns with behavioral outputs even when comparing only within control animals. In addition, the cleaning response rate is dependent on the genetic background of the animal and the type of stimulus presented. For example, the response rate for pSc neurons in w^{-1} wild-type flies at 34% (n = 68) is significantly higher than for *455-Gal4/+* controls at 19% (n = 118, P < 0.01) (**Fig. 6a**)[5]. Previous studies examining the response rates from stimulation of single macrochaeta have reported widely varying rates from 10% to 80% for the pSc neuron, by using mechanical stimuli such as an eyelash or hair and different stimulus protocols[12–14,16].

We have previously reported that response rates increase with viscosity of the solvent used to dissolve the fluorescent dye, so that when DiO was dissolved in dimethylformamide, a more viscous solvent than ethanol, pSc neurons produced greater response rates proportionally across all genotypes being examined[5]. Thus, our behavioral assay can be further enhanced by optimizing the viscosity of the solvent used for the fluorescent dye, which may allow for increased differentiation between 'yes response' and 'no response' synaptic connectivity patterns. For the 'yes response' group, our assay could also be further developed to potentially quantify the level of cleaning efficiency, for example, in the amount of fluorescent dye removed[20], to further correlate specific synaptic connectivity with a quantitative behavioral output. Finally, future experiments combining functional imaging (i.e., optical physiology)[21] with single neuron sequencing[22,23] of physically isolated mechanosensory neurons will further bridge the relationships between molecules, neuronal structure, synaptic targeting and function and animal behavior.

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