BAcTrace, a tool for retrograde tracing of neuronal circuits in Drosophila

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Animal behavior is encoded in neuronal circuits in the brain. To elucidate the function of these circuits, it is necessary to identify, record from and manipulate networks of connected neurons. Here we present BAcTrace (Botulinum-Activated Tracer), a genetically encoded, retrograde, transsynaptic labeling system. BAcTrace is based on Clostridium botulinum neurotoxin A, Botox, which we engineered to travel retrogradely between neurons to activate an otherwise silent transcription factor. We validated BAcTrace at three neuronal connections in the Drosophila olfactory system. We show that BAcTrace-mediated labeling allows electrophysiological recording of connected neurons. Finally, in a challenging circuit with highly divergent connections, BAcTrace correctly identified 12 of 16 connections that were previously observed by electron microscopy.

The development of genetic tools to elucidate connectivity and manipulate neurons and circuits has advanced our understanding of how the brain works. Increasingly, these tools are being used to study diseases of the nervous system and develop effective treatments1,2.

In the context of circuit research, the ability to identify and manipulate pre- or postsynaptic cells to neurons of interest is of crucial importance. Tools to label downstream neurons are termed anterograde, while retrograde tools reveal the input neurons to a given population.

Drosophila melanogaster is a key model organism for studying the genetic and circuit basis of animal behavior. The fly has a rich behavioral repertoire encoded in a relatively small nervous system. This simplicity is paired with extensive collections of genetic reagents, both for investigating gene function and for labeling and manipulating most neuronal classes1. While these reagents offer genetic access to neurons, until recently, tools for mapping synaptic connections in Drosophila have been lacking. This has recently changed with the development of electron microscopy methods for mapping connections in larval and adult brains4–6. Furthermore, contact-based, genetically encoded systems for anterograde tracing such as trans-Tango and TRACT have been developed7,8. Despite these additions to the experimental toolbox, a retrograde-labeling system for Drosophila is still missing. While rabies virus and its modifications constitute examples of retrograde transsynaptic tools for mice9, their applicability to flies is far from simple, both because of the experimental difficulties of delivering viruses via brain injections and because the virus neurotropism may not extend to flies.

Here, we introduce BAcTrace, a genetically encoded tracing tool designed for retrograde tracing in Drosophila. We first established the system in tissue culture and then implemented and refined BAcTrace in flies, showing that it can reveal the connectivity between olfactory projection neurons (PNs) and three different classes of presynaptic neurons, olfactory receptor neurons (ORNs), Kenyon cells (KC s) of the mushroom bodies (MBs) and lateral horn neurons (LHNs). BAcTrace provides a way to test connectivity and manipulate components in circuits with high sensitivity and specificity.

Results

System design. In contrast to contact-based systems, BAcTrace shares two key feature with rabies virus-based methods. First, labeling is triggered by protein transfer between connected neurons; and second, this transfer is followed by a signal amplification step. C. botulinum neurotoxin A1 (BoNT/A) is at the core of our system. BoNT/A is a modular protein (Fig. 1a and Supplementary Fig. 1) with a well-studied mechanism of action (Extended Data Fig. 1). BoNT/A is made as a single polypeptide that is cleaved by proteases, generating a light chain (LC) and a heavy chain (HC). In vertebrates, the receptor-binding domain (RBD), located in the C-terminal half of the HC, enables enrichment on neuronal membranes by interacting with a neuron-specific lipid (polysialoganglioside GT1b). Upon neurotransmitter–vesicle fusion in the axon, the RBD gains access to the vesicle lumen and binds with high affinity to a second partner, synaptic protein SV2 (ref. 9). When the vesicle is recycled and acidified, the translocation domain undergoes a conformational change, injecting the LC across the vesicle membrane. The LC is a protease that is highly specific for the human protein SNAP25 (hSNAP25), and once in the cytosol, it cleaves its target, preventing further release of neurotransmitters9,11.

In BAcTrace, BoNT/A is expressed in postsynaptic ‘donor’ neurons and, similarly to rabies virus, is transferred to connected presynaptic ‘receiver’ cells, where it triggers the expression of an effector gene. BAcTrace can be broken into three steps (Fig. 1b). In the first step, BoNT/A is made in donor neurons that are attached to the extracellular portion of a transmembrane protein (CD2). The tobacco etch virus protease (TEV)12 is made in receiver neurons that are attached to the extracellular portion of a second transmembrane protein. At synapses, both proteins interact, and TEV cleaves two recognition sites, releasing the toxin from the membrane and allowing the LC and HC to separate after translocation (Fig. 1c,d). While we initially considered TEV cleavage as essential, we later found...
that TEV was dispensable for toxin transfer (see below). Because flies lack the vertebrate BoNT/A receptor SV2, we exchanged the RBD for a single-chain anti-GFP nanobody (GFPnb) to create BoNT/A–GFPnb–CD2 (Fig. 1c and Supplementary Fig. 2). In the second step, modified BoNT/A is targeted to neurotransmitter vesicles of receiver neurons that express a synaptobrevin (Syb)–GFP fusion that is oriented with GFP inside the vesicle. Retargeting prevents binding of the toxin to vertebrate neurons and renders it safe for the researchers who handle the flies. In the third step, we made a toxin sensor by linking the QF2 transcription factor to Drosophila syntaxin (Syx) via amino acids 141–206 of hSNAP25 (ref. 15) (QF2–hSNAP25–Syx). Syx targets the transcriptionally inactive sensor to the synaptic membrane. However, after translocation, the cytosolic LC releases QF2, triggering expression of the QUAS (QF2 binding site) reporter. The BoNT/A LC is highly specific and does not cleave Drosophila SNAP2515; therefore, toxin expression should not be harmful to flies.

BAcTrace is active in Drosophila cells. We tested the feasibility of BAcTrace in Drosophila S2 cells. We synthesized the BoNT/A–GFPnb toxin in bacteria and added the purified toxin to S2 cells that were rendered sensitive by the expression of a human transferrin receptor (hTfR)–GFP chimera; this chimeric hTfR constitutively cycles between the plasma membrane and a cellular compartment with low pH (ref. 16 and Supplementary Note 1). We also transfected the cells with a FLAG-tagged hSNAP25 (Fig. 2a). We determined whether FLAG–hSNAP25 was cleaved by observing a small shift of 0.9 kDa (9 amino acids) by western blot. All tested toxin concentrations induced efficient cleavage (Fig. 2b, Extended Data Fig. 2a and Supplementary Note 2). Furthermore, the hTfR–GFP chimera was strictly required for cleavage (Fig. 2b).

We also tested and confirmed that the sensor used in flies, QF2–hSNAP25–Syx, was cleaved by BoNT/A–GFPnb (Fig. 2b). Furthermore, the released QF2 induced tandem dimer (td)Tomato expression, confirming that the transcription factor activity of QF2 was not inhibited by the 62 amino acids of hSNAP25 remaining on its C terminus following cleavage.

Both TEV and BoNT/A are originally cytosolic proteins. We therefore verified that they remained active when produced as extracellular membrane fusions in Drosophila cells. We found that TEV was inactive unless we removed a predicted glycosylation site (TEVT173V) (Extended Data Fig. 2b–f and Supplementary Note 3). Next, we tested the function of plasma membrane-targeted BoNT/A using a cell-mixing experiment. We transfected two cell populations, donor cells with either membrane-targeted BoNT/A–GFPnb–CD2 or cytosolic LC protease (negative control) and receiver cells with receptor and sensors (Fig. 2c). One day later, we mixed the cell populations and 2 d later, we could detect hSNAP25 cleavage, confirming that the BoNT/A–GFPnb–CD2 fusion protein was able to pass from the donor cell to the cytoplasmic compartment of the receiver cell and cleave the hSNAP25-based sensor proteins (Fig. 2d). Curiously, we found that TEV T173V–CD2 was not essential for this transfer, perhaps due to TEV-independent cleavage and release of BoNT/A–GFPnb from the donor cell membrane.
Fig. 2 | BAcTrace in tissue culture. a. Experiment for testing the activity of toxin purified from Escherichia coli. TM: transmembrane. b. Western blot analysis of S2 cell extracts. Empty and solid arrowheads indicate uncleaved and cleaved toxin sensors, respectively. c. Experiment for testing the activity of toxin produced in insect cells. d. Western blot analysis of S2 cell extracts from cell-mixing experiments. The lane marked with * contains the components shown in c. 

Critically, just as for the experiments with bacterial BoNT/A–GF Pnb (Fig. 2b), we observed a strict requirement for hTIR–GFP in receiver cells. Increasing the amount of receptor past a certain threshold did not increase its efficiency (Extended Data Fig. 2g). We expressed donor components in upstream activation sequence (UAS) vectors, from which the expression was driven by Gal4, and the expression of receiver components was driven by LexA on vectors containing LexA operator sequences (LexAop). For most experiments, we crossed flies containing all BAcTrace components with flies containing Gal4 and LexA drivers (Fig. 3a). One important limitation of BAcTrace is that LexA and Gal4 expression cannot overlap. If they do, activation will take place in the cells with overlapping expression, producing a false-positive result. Furthermore, the toxin is likely to be internalized and become unavailable for transsynaptic labeling. We chose the well-characterized fly olfactory system for initial testing of BAcTrace in vivo (Fig. 3b). Briefly, Drosophila has 50 types of peripheral ORNs. ORNs of each type express a general coreceptor, odorant receptor coreceptor (Orco) and one of 50 different olfactory receptor genes, conferring responses to a specific set of odors. ORNs expressing the same receptor relay information to one of 50 glomeruli in the brain’s antennal lobe (AL). In each glomerulus, the axons of 20–100 ORNs make strong connections (for example, ~1,215 synapses per PN in glomerulus DM6 (ref. 17)) onto the dendrites of 1–8 PNs. These in turn make a modest number of reciprocal synapses (for example, ~40 synapses per PN in glomerulus DM6 (ref. 17)) onto ORNs. PN axons project onto KCs in the calyx of the MB and to the lateral horn (LH)18. It is important to note that PNs connect to KCs almost randomly19–22; therefore, expressing toxin even in a small number of KCs should label most or all PNs. 

We expressed donor components in KCs using the MB247-Gal4 driver and receiver components in PNs using the broad LexA driver VT033006-LexA–P65 (Fig. 3c and Extended Data Fig. 3). We confirmed that BoNT/A–GF Pnb–CD2 could be expressed and trafficked throughout KCs, and we detected expression in soma, dendrites and axons (Fig. 3d). We first established the background signal of the system in vivo by bringing together all components except the Gal4 driver. We observed low-frequency, stochastic labeling of PNs (Fig. 3e, left), which was likely due to weak, Gal4-independent toxin expression in PNs (BAcTrace working in cis instead of in trans). To reduce this background, we added a transcriptional stop cassette to BoNT/A–GF Pnb–CD2, which can be removed by the B3 DNA recombinase23. This stop cassette reduced background in the absence of Gal4 (Fig. 3e, right). We mapped the source of this background signal to the V5 tag present in the sensor (Supplementary Fig. 3 and Supplementary Note 5), for simplicity we decided to omit the V5 tag from the remaining experiments. Critically, the Syb–GFP receptor was required for transfer of toxin in vivo (Fig. 3f, right), indicating that the system should select for synaptic rather than non-synaptic cell contacts. 

Next, we repeated the experiment including Gal4 and found that toxin expression in the MB induced strong labeling of most PNs in the VT033006 line, even in the absence of wild-type TEV (Fig. 3f). Although further experiments hinted at a small improvement in efficiency with mutant TEVTR173V (Extended Data Fig. 4 and Supplementary Note 5), for simplicity we decided to omit it from the remaining experiments. Critically, the Syb–GFP receptor was required for transfer of toxin in vivo (Fig. 3f, right), indicating that the system should select for synaptic rather than non-synaptic cell contacts.
We confirmed the results from experiments with MB247-Gal4 using a panel of split-Gal4 lines and found that toxin expression in KC subtypes also labeled presynaptic PNs (Extended Data Fig. 5 and Supplementary Note 6).

**BacTrace expression in ORNs labels connected PNs.** Next, we examined the labeling specificity of BacTrace using an experimental configuration in which only a subset of receiver cells were connected to toxin-expressing donor cells. We selected the

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*Fig. 3 | BacTrace in vivo. a, Genetic strategy for expressing BacTrace components. The female fly contributes all UAS, LexAop and QUAS components, while the male fly contributes a Gal4 driver defining the donor cells and a LexA driver defining the receiver cells. b, First three layers of the fly olfactory system, adapted from ref. 41. c, Expression patterns of MB247-Gal4 (blue) and VT033006-LexA–P65 (green). d, BoNT/A–GFPnb–CD2 expression in the MBs. e, BacTrace negative controls. Left, no Gal4 driver. Middle, a B3 DNA recombinase-dependent transcriptional stop cassette (B3RT.STOP) was added in front of the toxin transgene; UAS-B3 was present. Right, stop cassette and MB247-Gal4 were present, but UAS-B3 was absent. Brp, Bruchpilot. f, BacTrace experiments. Left, all components were present as described in Fig. 1d. Middle, TEV transgene was absent. Right, Syb-GFP receptor transgene was absent. e and f are full and c and d include partial maximum intensity projections of confocal stacks. Epitopes detected by antibodies are indicated in bold in c–f. All animals were 3–4 d old. Genotypes for the organisms in each panel are described in Supplementary Table 16. Results shown in c–f are representative of at least four brains. Scale bars, 30 µm.*
reciprocal synapses from PNs to ORNs (Fig. 3b) for two reasons. First, highly specific Gal4 driver lines are available for ORN subtypes. Second, while ORN axons make strong connections to PN dendrites, electron microscope connectomics identified a reciprocal connection of moderate strength between these two cell types; for example, DM6 glomerulus PNs make ~40 reciprocal synapses onto ORNs (Fig. 4a).

We expressed toxin in the majority of ORNs using Orco-Gal4 (Fig. 4b; for the reverse experiment, see Supplementary Note 7). In 2–4-d-old animals, we detected little labeling above...
background. However, at 9–10 d after eclosion, we found consistent labeling in most glomeruli covered by the VT033006-LexA–P65 line (Fig. 4b). Given this time dependence, BAcTrace labeling could be used to characterize the strength of a connection under different conditions (for example, mutant backgrounds, level of stimulation, etc.).

Toxin expression in single ORN types (Fig. 4c,d and Extended Data Fig. 6) resulted in strong labeling in connected PNs in animals as young as 2 d old, for example, Or83c, Or88a, Or92a, Or65a and Or98a, while in other cases, labeling took longer, for example, 10–13 d for Or47a. While these differences may be due to cell type-specific differences in the number of ORN–PN reciprocal synapses, they could also be the result of variability in the expression level of BAcTrace components in the different cell types (that is, detection system components in PNs or toxin in ORNs).

In some cases, BAcTrace-labeled PNs targeted glomeruli that were not known to be innervated by donor cells (Fig. 4c,d and Extended Data Fig. 6). This could be the result of artifactual background labeling, as characterized before (Fig. 3e). It might be triggered by non-synaptic contacts from the ORN axons as they traverse the AL, or it could be due to unexpected synaptic contacts. For instance, some ORNs and PNs have small processes in neighboring glomeruli.

For an initial assessment of neuronal health, we used BAcTrace to label DC3 PNs while expressing the light-gated cation channel CsChrimson in connected donor Or83c ORNs8 (Fig. 4e). We stimulated the ORNs using light while performing electrophysiological recordings on the tdTomato-labeled PNs in 14-d-old flies (we obtained similar results from 5-d-old animals, not shown).

Graded light stimulation induced increasing spiking responses in PNs, indicating that ORNs are able to release neurotransmitter and stimulate connected PNs and that BoNT/A–GFPnb–CD2 was not overtly toxic (Fig. 4f). These responses could be partially blocked by the nicotinic acetylcholine receptor blocker mecamylamine, indicating that the responses were due to synaptic transmission from ORNs. Furthermore, light responses were absent from tdTomato–control neurons.

Mapping connections in the LH. In the LH, connectivity is less well understood and projections are more divergent, with each olfactory PN type having many postsynaptic LHN partners. These partners share fewer synapses than those we assessed in our previous experiments (Fig. 3b)20,31. We expressed receiver components in PNs using the broad VT033006-LexA–P65 and toxin using a panel of nine Gal4 and split-Gal4 lines that express in eight donor LHN cell types (Fig. 5a,b and Extended Data Fig. 7a)11. All eight donors strongly labeled the ORN–PN copy dataset also identified as connected by BAcTrace (Fig. 5e). In the case of AV1a1, seven of nine connected PN types in the electron microscopy dataset were also identified as connected by BAcTrace.

Discussion

In this study we present BAcTrace, a genetically encoded retrograde-labeling system, as well as the application of C. botulinum neurotoxin as a circuit tracer.

We used the fly’s olfactory system7,17,18,20,30,32,33 to explore three important and related questions about BAcTrace. First, does labeling occur in the retrograde direction? Second, is labeling specific? Third, how many synapses are required? Support for the retrograde direction of labeling comes from the PN–LHN experiments; PD2a1/b1 and AV1a1 have no reciprocal synapse in the PN–LHN connection that could mediate anterograde transfer from LHNs to PNs. While BAcTrace labeling observed at these particular connections must be retrograde, and by design BAcTrace should have a strong retrograde bias, our results do not conclusively prove that BAcTrace works exclusively in the retrograde direction. BAcTrace is specific, because it most frequently labeled the correct PNs when toxin was expressed in ORNs. This was confirmed by the good match between our PN–LHN results and electron microscopy connectivity data. Regarding BAcTrace’s sensitivity, in our experiments, we could detect labeling in connections ranging from ten synapses (PN–LHN) to >200 synapses (PN to KC). Does this range encompass functional connections? Recent work has found that seemingly low connection strengths, constituting only a handful of synaptic contacts, can generate measurable effects on postsynaptic activity17,20,34–36. While these numbers are tentative and could be different for different neuronal classes, they indicate that BAcTrace sensitivity falls within the range of known functional connections.

Current BAcTrace limitations include the lack of pan-neuronal coverage, which is related to the impossibility of Gal4 and LexA drivers overlapping and the toxicity of the Syx sensor when expressed widely. Furthermore, the presence of false positives in some of our experiments suggests that, when BAcTrace is used as a discovery tool, other methods should be considered to confirm the identified connections; we expect this to become less of a requirement as community usage extends our understanding of the system’s performance.

Given rapid advances in the field of electron microscope connectomics35, which can reveal dense connectivity for all the neurons within a brain, one might wonder if it will supplant transsynaptic tracing in the future. However, transsynaptic labeling has numerous advantages, suggesting that these approaches should remain complementary rather than competitive for the foreseeable future. First, electron microscope connectomics is limited to postmortem specimens, while transsynaptic labeling methods can rapidly reveal connectivity in living animals, enabling neurons to be targeted for recording or manipulation or to be followed over time. Furthermore, connection-based labeling can enable more precise labeling of neurons than can be achieved with genetic drivers alone, for instance, in cases where neurons that are genetically similar, such as olfactory PNs, but make connections with different partners, such as specific
**Fig. 5** BAcTrace reveals connections between PNs and LHNs. **a**, Top, split-Gal4 lines LH989 and LH1983 drive GFP expression in LHN cell types PD2a1/b1 and AV1a1, respectively. Bottom, single slices of the LH shown in the top panel. nc82 staining from an average brain. Images are shown for a representative brain of five animals for each line. **b**, Schematic of the experiment shown in c. Single slices from a representative AL and the corresponding LH showing PNs labeled by toxin expression in PD2a1/b1 and AV1a1 cells (see d for sample sizes). Examples of glomeruli differentially labeled between the two lines (VA1d, VM3, DM3 and VM4) are indicated with dotted lines. **d**, Stacked bar plot showing labeling frequency per glomerulus for PD2a1/b1 (n = 10 ALs), AV1a1 (n = 13 ALs), PV5c1 (n = 18 ALs) and AV6a1 (n = 13 ALs). Glomeruli coloring is the same as in Fig. 4d. **e**, Three-dimensional renderings of ALs. BAcTrace (BT), glomeruli with BAcTrace signal >3; electron microscopy (EM), glomeruli with more than ten synapses; and BT ∩ EM, meeting both conditions. Glomeruli coloring in BT and EM is the same as in d, in BT ∩ EM, green, blue and red show regions satisfying BT and EM criteria, EM only and BT only, respectively. Epitopes detected by antibodies are indicated in bold in a and c. Genotypes for the organisms in each panel are described in Supplementary Table 16. Scale bars: a, 50 um; c, 10 um.
ORNs, are to be functionally manipulated. Second, electron microscope connectomics will continue to be prohibitively expensive and resource intensive for many laboratories and for studies requiring the comparison of multiple specimens. Third, densely reconstructed connectomics datasets are still missing important information, for example, the identification of excitatory versus inhibitory synapses, electrical synapses and extrasynaptic communication. In contrast, genetic tools can provide a readout for these properties, for example, by using BAC Trace with neurotransmitter or peptidergic-specific LexA lines or in combination with methods such as electrophysiology or effectors such as Chrimson and/or GCaMP. There are also cases in which the two approaches are synergistic. Transsynaptic tools are particularly well suited to unequivocally link neurons identified in electron microscopy volumes to those labeled by genetic drivers, because they simultaneously reveal neuronal morphology and connectivity. This process will be essential for the functional exploitation of the small number of reference connectomes that will become available over the next few years.

Online content
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Methods

Molecular cloning and transgenic flies. Backbones of plasmids used in experiments with S2 cells were based on the Drosophila Gateway vector collection (Drosophila Genomics Resource Center, cat. no. 1071–1138). Backbones of plasmids used for making transgenic flies were derived from pJFRC19 (ref. 41), pJFRC81 (ref. 41) and pJFRC161 (ref. 41). Synthesized DNA sequences were codon optimized for Drosophila expression and made by GeneArt (Thermo Fisher Scientific) or IDT (Integrated DNA Technologies).

Plasmids were made using Gibson assembly44. All fragments were PCR amplified with overlapping primers, yielding scarless products. All fusion were sequenced to control for mutations introduced during cloning. GenBank accession numbers for all constructs can be found in Supplementary Tables 9, 12, 13 and 14. Transgenic flies were made by BestGene.

Transfection of S2 cells. S2 cells were acquired from Thermo Fisher Scientific (R69007) and cultured according to the manufacturer’s recommendations. Once the culture was established, the cells were transferred from serum-containing Schneider’s medium into medium containing increasing proportions of serum-free Express Five medium (Thermo Fisher Scientific, 10486025).

Plasmid DNA for transfecting S2 cells was prepared using a Midiprep DNA purification kit according to the manufacturer’s instructions (QiaGen, 12143). Transfection of S2 cells was performed in six-well plates, with each well containing 2 ml of 1 × 10^5 cells per ml. A total of 2 μg of plasmid DNA was diluted with culture medium to a volume of 100 μl, and the mixture was vortexed. FuGENE HD transfection reagent (3 μl; Promega, E2511) was added to the diluted DNA, mixed gently, and incubated for 10 min at room temperature. This mixture was then added dropwise to the well with cells, and the plate was put back into the incubator for 24 h. Cells were then rinsed to remove the transfection mix.

Depending on the experiment, either toxin was added as required (as shown in Fig. 2a), or cells were mixed at a 1:1 ratio (as shown in Fig. 2b), followed by incubating further for 48 h before staining or western blot analysis. Plasmids used for the experiments shown in Fig. 2 are listed in Supplementary Table 3, those for Fig. 2d in Supplementary Table 4, those for Extended Data Fig. 2a in Supplementary Table 5, those for Extended Data Fig. 2g in Supplementary Table 6 and those for Extended Data Fig. 2f in Supplementary Table 7.

Western blot analysis. Cells for western blot analysis were resuspended in culture medium, pelleted, rinsed with PBS and pelleted again. Pellets were resuspended in 1× sample buffer (Thermo Fisher Scientific, NP0007) with a reducing agent (Thermo Fisher Scientific, NP0004) and heat denatured. Samples were then loaded on 12% Bis-Tris gels (Thermo Fisher Scientific, NP0342BOX) and run using MOPS buffer (Thermo Fisher Scientific, NP0001). Samples were transferred from gels to PVDF membranes (Millipore, IPVH0001), which were developed using MOPS buffer (Thermo Fisher Scientific, NP0007) with a reducing agent. All fusions were sequenced to control for mutations introduced during cloning. GenBank accession numbers for all constructs can be found in Supplementary Tables 9, 12, 13 and 14. Transgenic flies were made by BestGene.

Image acquisition and processing. Confocal stacks of fly brains were imaged at 768 × 768 pixels every 1 μm (vocal size 0.46 × 0.46 × 1 μm, zoom factor of 0.6) using an EC Plan-Neofluar x40, 1.30 numerical aperture, oil DIC M27 objective with a zoom factor of 0.6). All images were acquired using ZEN software on Zeiss LSM 710 and Zeiss LSM 880 confocal microscopes. For batch processing of images (for example, brain registration), a combination of Fiji and CMTK (https://www.itrc.org) was used.

Fluorescence quantification. To quantify the expression levels of Syb-GFP and Q2E–V5–hsNAP25–Sx driven by the VD33006-LexA–P65 driver (Extended Data Fig. 3), brains were co-stained for the neuropil marker Bruchpilot and GFP or Bruchpilot and V5, respectively. We then acquired high-magnification confocal stacks of the stained AEs. Next, the Zeiss LSM files were split into NRRD files using Fiji, followed by segmentation of each glomerulus by drawing a region of interest in the center of the glomerulus (the plane that captured most of the area). Both glomerulus identification and segmentation were done using the ncb2 channel, blind in relation to the GFP and V5 fluorescence intensities. Segmentations were saved as ROI files. GFP and V5 mean intensities were obtained using Fiji and were normalized to the mean intensity of the ncb2 channel for each glomerulus. This normalization was intended to counter the drop in intensity due to light scatter when imaging deeper into the tissue.

Drosophila stocks. Fly stocks were maintained at 25°C on iberonian food. The driver lines used in this study are summarized in Supplementary Table 15, LexA responsive transgenes are summarized in Supplementary Table 14, those for Gal4 in Supplementary Table 13 and those for QAS in Supplementary Table 12. All brain images were from female flies.

Quantification of LHN BaTrace labeling. We took high-magnification confocal stacks of AEs and used the ncb2 channel to identify and annotate the 15 glomeruli with the highest expression level of receiver components (Extended Data Fig. 3c) using regions of interest in Fiji. Values of 0 (absent), 3 (present but weak) or 10 (strong) were assigned to annotated glomeruli by examining the intensity of the QUAS-Halo reporter channel. Once all AIs were blindly annotated in relation to LHN type, the toxin-labeling (CD2) channel was used to assign each specimen to the correct LHN cell type. Stacks are available online12 for readers to examine.

Light microscopy PN–LHN overlap score. To quantify the overlap between neuronal skeletons for PNs and LHNs, derived from both light-level and electron microscopy data, we employed the ‘overlap score’ from ref. 41:

\[ f(i, j) = \sum_{k=1}^{n} e^{-d_{ij}^2/2\sigma^2} \]

Skeltons were resampled so that we considered ‘points’ in the neuron at 1 μm intervals and an ‘overlap score’ was calculated as the sum of \( f(i, j) \) over all points \( i, j \). The sum of the scores between each point \( i \) and each point \( j \) was taken.

Overlap scores were calculated between light-level reconstructions from stochastic labeling experiments44,45 that were previously registered from hundreds of brains to a common template, categorized and identified14,15,16. We also made use of a complete set of uniglomerular PNs, reconstructed from a single electron microscopy dataset comprising a whole fly brain18.

Electrophysiology. Electrophysiological recordings were carried out as described in ref. 19 with minor modifications. Briefly, 1 d after eclosion, flies were anesthetized with CO2; females of the correct genotype were selected, transferred to all-trans retinal fly food and kept in the dark. Five days later, flies were cold anesthetized, placed in the recording chamber and dissected under dim light for recording as described in ref. 19. Data acquisition was performed as previously described, with the only difference being that a pco.edge 4.2 CMOS camera was used. For CxChrimson excitation of ORNs, a short (0.5–s) pulse of light (550 nm) was applied via a Cairn OptoLED controller.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data necessary for confirming the conclusions presented in the article are represented fully within the article. In addition, all raw and processed brain images and blots are freely available from the authors. All fly strains used are listed in Supplementary Tables 12–15 and are available either from the Bloomington Stock Center (Supplementary Table 17) or from the authors. All plasmids are available upon request from the authors and their sequences are deposited in GenBank as described in Supplementary Tables 9 and 12–14. Brain confocal stacks used for making ORN and LHN plots are available from the Zenodo repository. Source data are provided with this paper.

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Author contributions
S.C. and G.S.X.E.J. conceived the project, obtained funding and supervised the research. S.C., L.B., A.M. and A.S. designed, cloned, purified proteins, conducted tissue culture experiments and analyzed the results. S.C., M.G. and B.S. designed and conducted BACTrace experiments in flies, stained, imaged and analyzed the results. S.F. designed, conducted and analyzed the results of electrophysiology experiments. Y.A. made VT033006-LexA–P65 and obtained MFCO data. A.S.B. summarized electron microscopy connectivity data for LHNs. S.C. and G.S.X.E.J wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

Additional information
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Correspondence and requests for materials should be addressed to S.C. or G.S.X.E.J.
Peer review information Nina Vogt was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.
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Extended Data Fig. 1 | BoNT/A1 mechanism of action in vertebrates. Adapted from 52.
Extended Data Fig. 2 | Testing BAcT race in tissue culture. **a**, Western blot analysis of S2 cell extracts transfected and incubated with decreasing amounts of toxin. **b**, Constructs used in (C) and (F). **c**, Immunochemistry of S2 cells transfected with sensors and TEV variants. **d**, Prediction of N-glycosylation sites on TEV (http://www.cbs.dtu.dk/services/NetNGlyc/). **e**, TEV structure (PDB id 1lvb) with potential glycosylation sites from (D) highlighted in blue. Bound pseudo-substrate shown in yellow. **f**, Western blot analysis of wild type and TEVIT73V ability to cleave their target sequence. * non-specific band. **g**, Western blot analysis of cleavage efficiency under varying concentrations of hTfR::GFP receptor. Epitopes detected by antibodies are indicated in bold in (A), (C), (F) and (G). Experiments in (A), (F) and (G) were done once. Empty arrowheads in (A), (C), (F) and (G) indicate uncleaved while solid arrowheads indicate cleaved products.
Extended Data Fig. 3 | Expression strength of VT033006-LexA::P65. a, V5 tag immunostaining of QF2::V5::hSNAP25::Syx in VK000018. b, GFP immunostaining of Syb::GFP in VK000037. Images in (A) and (B) are representative of stainings from 3 animals. c, Boxplot showing the fluorescence quantification per glomerulus for (A) and (B); each glomerulus was measured in 6 ALs from 3 brains. Leftmost panel in (A) and (B) are maximum intensity projections while all other panels are single slices from confocal microscopy stacks. Epitopes detected by antibodies are indicated in bold in (A) and (B). Scale bars 30 µm. Boxplot shows the median of the measurements. Lower and upper hinges correspond to the first and third quartiles; the upper whisker extends from the hinge to the largest value no further than 1.5 * inter-quartile range from the hinge. The lower whisker extends from the hinge to the smallest value at most 1.5 * inter-quartile range of the hinge.
Extended Data Fig. 4 | TEVT173V modestly increases BAcTrace efficiency. a) BAcTrace labelling of PNs from sparse KC Donors driven by MB005C using TEV variants. The panels for each condition are representative of stainings from 3 animals. (b) Constructs used in (C). (c) Targeting TEV to the synapse using the toxin receptor. The panels for each condition are representative of stainings from 4 animals. Maximum intensity projections of registered confocal stacks from age matched animals; images taken using the same microscope settings. Epitopes detected by antibodies are indicated in bold in (A) and (C). Genotypes for each panel are described in Supplementary Table 16. Scale bars 30 µm.
Extended Data Fig. 5 | BAcTrace expression in subsets of KCs induces labelling in PNs. a, Negative control missing a split Gal4 hemidriver. Donor KCs: b, all, c, α'/β', d, γ and e, α/β. f, Higher magnification view of the MB calyx and LH of a brain with Donor MB607B neurons (same as in D). The brain was mounted dorsal side up to provide better image quality of the MB calyx and LH. Within each lobe subtypes are: γ lobe: main (m) and dorsal (d), α'/β' lobe: anterior-posterior (ap) and middle (m) and α/β: posterior (p), core (c) and surface (s). Images in A–F are representative of stainings from at least 3 animals. Maximum intensity projections of registered confocal stacks from age matched animals; (A), (B), (C) and (D) taken using the same microscope settings. Epitopes detected by antibodies are indicated in bold in each panel. Number of KCs per subtype from 24. Genotypes for each panel are described in Supplementary Table 16. Scale bars 30 µm.
Extended Data Fig. 6 | BAcTrace expression in ORNs labels connected PNs. (a) BAcTrace expression in specific ORNs induces labelling in connected PNs (dotted lines). Top panels show maximum intensity projections of affine registered confocal stacks. Bottom 5 panels show single slices from the same specimen. Epitopes detected by antibodies are indicated in bold in each panel. (b) BAcTrace labelling quantification in older animals (10-12 days old) for the same crosses shown in Fig. 4c. Sample size: Or83c n = 10ALs, Or88a n = 10ALs and Or92a n = 12ALs. Genotypes for each panel are described in Supplementary Table 16. Scale bars 30 µm.
Extended Data Fig. 7 | BAcTrace expression in LHNs induces labelling in PNs. a, Expression of LHN driver lines tested. Anti-GFP immunostaining against UAS-csChrimson::mVenus in attP18. The name of each line is indicated on the top left and the cell type in each line on the top right. Images adapted from31. b, For each line animals of two ages were dissected. Several LHs were imaged (sample size: n number on top left corner of each image), registered to a template and averaged to produce the different panels. Bottom two panels on the right are a negative control made from line LH196 with one hemidriver missing. Epitopes detected by antibodies are indicated in bold in each panel. Genotypes for each panel are described in Supplementary Table 16. Scale bars (A) 30 µm and (B) 10 µm.
Extended Data Fig. 8 | BAcTrace variability for PD2a1/b1 and AV1a1 LHNs. BAcTrace labelled PNs by toxin expression in: a, PD2a1/b1 neurons (LH989) and b, AV1a1 neurons (LH1983). Images in (A) and (B) are representative of stainings from 5 and 7 animals respectively. Images are maximum intensity projections of confocal stacks, affine registered to a template for the AL region. The surface outside the neuropil region was masked out to avoid obscuring the glomeruli. Epitopes detected by antibodies are indicated in bold. Genotypes for each panel are described in Supplementary Table 16. Scale bars 10 µm.
Extended Data Fig. 9 | BAcT race reveals connections between PNs and LHNs. a, Split Gal4 lines LH173 and LH1139 drive GFP expression in cell types PV5c1 and AV6a1, respectively. Images for a representative brain of 5 animals for each line. AL: Antennal Lobe. b, Single slices from a representative AL and corresponding LH showing PNs labelled by expression of toxin in PV5c1 and AV6a1. Images are representative of PV5c1 (9 animals) and AV6a1 (7 animals). c, 3D renderings summarizing BAcT race results for PV5c1 and AV6a1. d, Forward and reciprocal synapses between PNs for the 15 glomeruli analyzed in Fig. 5 and PD2a1/b1 (top) and AV1a1 (bottom) as assessed by EM. The number next to the glomerular identity of the PN is its EM identification number. Epitopes detected by antibodies are indicated in bold in (A) and (B). Genotypes for each panel are described in Supplementary Table 16. Scale bars (A) 50 μm and (B) 10 μm.
Extended Data Fig. 10 | LHN–PN connectivity. Summary of published data for the 4 LHN cell types analyzed. R/S: average expression level for Receptor and Sensor (see Extended Data Fig. 3). BT: BAcTrace results. EM: Electron Microscopy connectivity data. EPhys: Electrophysiological recordings from LHNs during opto-stimulation of PNs \(^5\). Cell types PD2a/b1: ML9+ML8 and AV1a1: V3 from ref. \(^5\). LM: Light Microscopy overlap between single cell PNs labelled with a membrane marker and imaged by confocal microscopy and EM LHNs \(^4\). The number in column 1 (Glom.) indicates how many individual PNs contribute to the total number of synapses counted by EM.

|        | PD2a/b1 |        |        |        | AV1a1 |        |        |        | AV6a1 |
|--------|---------|--------|--------|--------|--------|--------|--------|--------|--------|
| Glom.  | R/S     | BT     | EM     | EPhys  | LM     | BT     | EM     | EPhys  | LM     |
| VMc (x3) | 1.3   | 1.3    | NA     | 3.0    | 5.1    | 23.2   | NA     | 2.2    | 8.7    |
| ULc (x3) | 1.9   | 1.9    | 0.4    | 2.7    | 8.9    | 1.0    | 0.8    | 0.8    | 6.6    |
| VMc (x3) | 0.3   | 0.3    | 0.0    | 0.7    | 7.8    | 138.0  | 0.0    | 5.0    | 54.1   |
| VCc (x6) | 1.9   | 3.2    | 17.9   | NA     | 3.3    | 6.3    | 100.0  | NA     | 3.9    |
| OMCc (x3) | 1.3   | 16.0   | 17.6   | 5.2    | 2.5    | 7.0    | 0.1    | 1.4    | 9.4    |
| VA2c (x3) | 1.3   | 0.3    | 3.0    | 2.7    | 4.8    | 20.0   | 0.5    | 3.0    | 6.6    |
| DCc (x3) | 1.5   | 0.3    | 1.4    | 3.2    | 4.2    | 53.0   | 3.1    | 1.6    | 3.3    |
| VMc (x2) | 1.5   | 6.0    | 77.0   | 0.0    | 21.2   | 0.0    | 0.0    | 0.0    | 1.6    |
| VA2c (x3) | 1.3   | 1.2    | 43.0   | 0.5    | 18.7   | 0.2    | 0.0    | 0.0    | 1.6    |
| DLc (x1) | 1.3   | 0.0    | 0.0    | 0.0    | 6.1    | 1.2    | 52.0   | 0.0    | 19.1   |
| VA2c (x3) | 1.3   | 0.0    | 150.0  | 0.0    | 65.1   | 0.0    | 0.0    | 0.0    | 19.1   |
| DLc (x1) | 1.3   | 1.9    | 0.0    | 0.0    | 0.2    | 7.4    | 100.0  | 1.3    | 14.4   |
| DAa (x8) | 0.9   | 1.0    | 0.0    | 0.0    | 2.0    | 3.0    | 12.0   | 2.6    | 0.9    |
| OMCc (x1) | 0.9   | 7.1    | 198.0  | 2.1    | 54.5   | 1.3    | 1.0    | 0.4    | 0.0    |
| OMCc (x1) | 0.8   | 5.6    | 11.0   | 0.8    | 27.9   | 0.0    | 4.0    | 0.3    | 4.1    |

|        |        | BT     | EM     | EPhys  | LM     | BT     | EM     | EPhys  | LM     |
|        |        |        |        |        |        |        |        |        |        |
|        |        | 8.7    | 2.6    | 7.7    | 5.8    | 6.6    | 1.5    | 4.8    | 0.1    |
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

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Data collection: All images were collected on Zeiss LSM 710 and 880 microscopes using the Zen software (2.3 SP1 FP3).

Data analysis: All image analysis was done using the open source Fiji image analysis suite(2.0.0), as described in the manuscript. Plots were done in R (3.6.2) using freely available packages as described in the manuscript.

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- Sample size: Sample sizes are indicated in the legends of each figure.
- Data exclusions: We have only excluded datapoints where methodological problems made it not possible to evaluate the data. For example antennal lobes which were extensively damaged/lost during brain dissection.
- Replication: Tissue culture experiments were preparatory for work in flies and were done once for paper presentation purposes but repeated 2-3 times in different experimental configurations (e.g. not assaying all concentrations shown in the figures or testing all conditions shown at once). Experiments in flies, which corroborated tissue culture results, were done in at least 3 animals and in general in 5-10 (exact n numbers indicated in each figure). In the case of flies, simultaneous experiments shown in figures were done once, but most panels were repeated independently at different times with equivalent results.
- Randomization: Based on our experience, we did not consider that the research presented in our work required randomisation.
- Blinding: We carried out blind assessment as described in Methods for fluorescence and LHN tracing quantifications.

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| [ ] Clinical data | |

Antibodies

Antibodies used for western blot experiments

| Species | Target | Concentration | Supplier | Cat no |
|---------|--------|---------------|----------|--------|
| Rabbit  | FLAG   | 1:1000        | Cell Signalling | 2368   |
| Mouse   | CD2    | 1:2000        | GeneTex  | GTX75123 |
| Chicken | GFP    | 1:5000        | Abcam    | ab13970 |
| Rat     | HA     | 1:4000        | Roche    | 11 867 423 001 |

Antibodies used for brain immunostainings

| Species | Target | Concentration | Supplier | Cat no |
|---------|--------|---------------|----------|--------|
| Mouse   | Bruchpilot | 1:40        | DSHB     | AB_2314866 |
| Mouse   | CD2    | 1:200        | GeneTex  | GTX75123 |
| Rabbit  | Tomato | 1:1000       | antibodies-online | ABIN179578 |
| Chicken | V5     | 1:800        | Bethyl   | A190-118A |
| Chicken | GFP    | 1:1000       | Abcam    | ab13970 |

Validation

All antibodies used are of commercial origin and have been extensively used elsewhere for the same applications as in our manuscript. Furthermore signals in our blots are identified based on size and are internally controlled by un-transfected lanes. In the case of immunolabelling of brains, antibody signals can be controlled using neurons in the same brain which do not express the detected transgenes (e.g. GFP negative neurons when labeling for GFP).
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| Category                         | Description                                                                                                                                 |
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| Laboratory animals               | We used laboratory strains of *Drosophila melanogaster* with a number of genetic modifications described in the manuscript.                     |
| Wild animals                     | None                                                                                                                                          |
| Field-collected samples          | None                                                                                                                                          |
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