



Figures and figure supplements

EM connectomics reveals axonal target variation in a sequence-generating network

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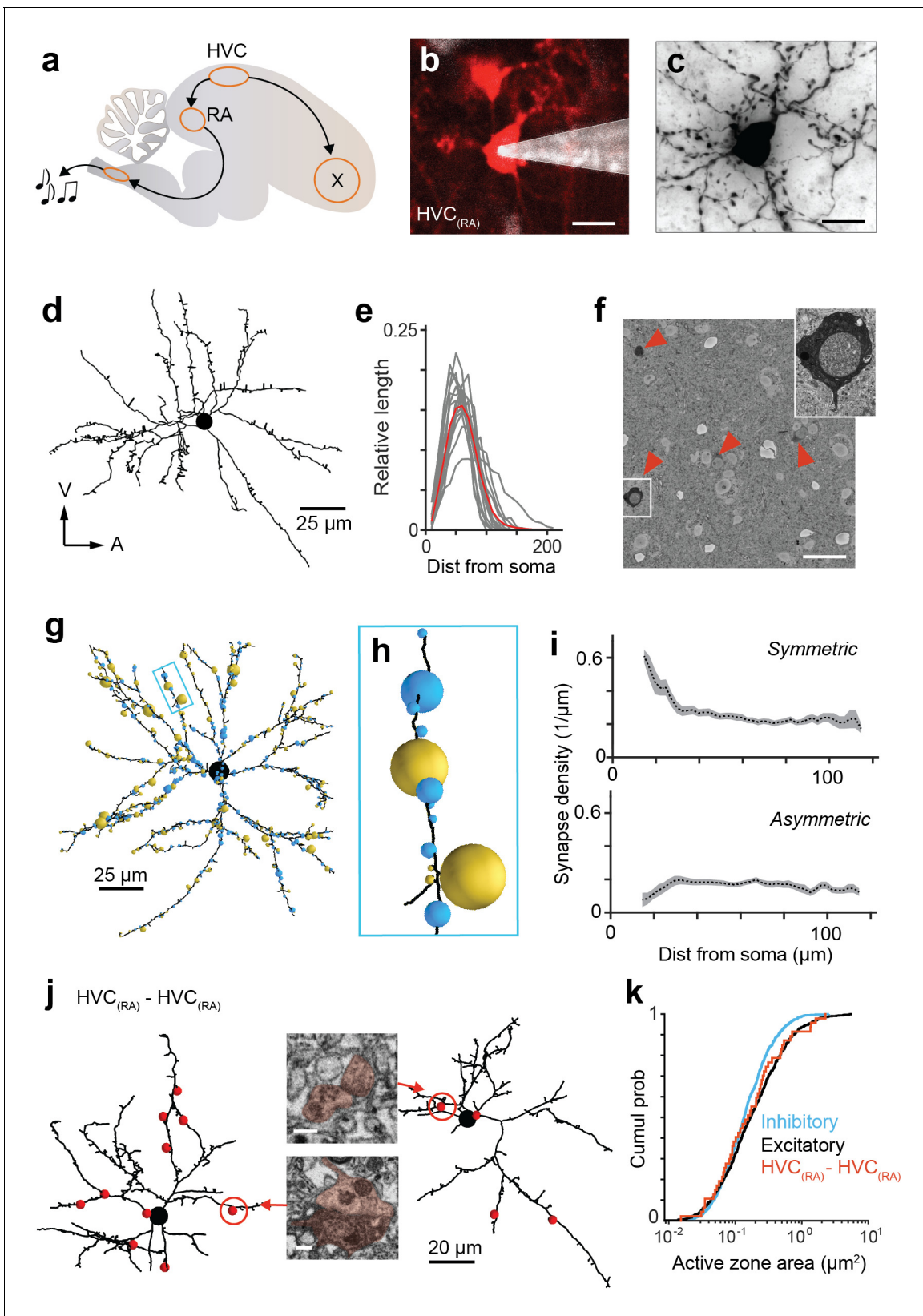


Figure 1. Analysis of synaptic inputs onto $HVC_{(RA)}$ dendrites. (a) A schematic of the songbird brain showing HVC and its two main downstream targets, RA and Area X. (b) A backlabeled $HVC_{(RA)}$ neuron (red) during juxtacellular filling (pipette shown in white) guided by 2-photon imaging of fluoro-Ruby. Figure 1 continued on next page

Figure 1 continued

(c,d) A Neurobiotin-filled cell from (b) in brightfield LM after histochemical processing (c) and dendritic reconstruction (d). (e) Normalized count of dendritic path length vs. soma distance for 15 HVC_(RA) neurons; individual cells (gray) and average (red). Bin size: 10 μm . (f) Cross-section through a SBEM stack showing BDA-labeled HVC_(RA) somata. (g) Inhibitory (blue spheres) and excitatory (gold spheres) synapses onto an HVC_(RA) dendrite in the SBEM volume. Sphere cross-sectional areas are proportional to the active zone area. (h) Higher magnification of a dendritic branch from (g). (i) Density of asymmetric and symmetric synapses vs. the distance to the soma. (j) Two HVC_(RA) dendrites; red spheres indicate double-labeled synapses, with cross sections through two synapses (insets). Inset, cross sections through the synapses circled in red. (k) Active zone size distributions of inhibitory (blue), excitatory (black), and double-labeled (red) synapses. Scale bars are 10 μm in b and c, 25 μm in f, and 0.25 μm in j.

DOI: [10.7554/eLife.24364.003](https://doi.org/10.7554/eLife.24364.003)

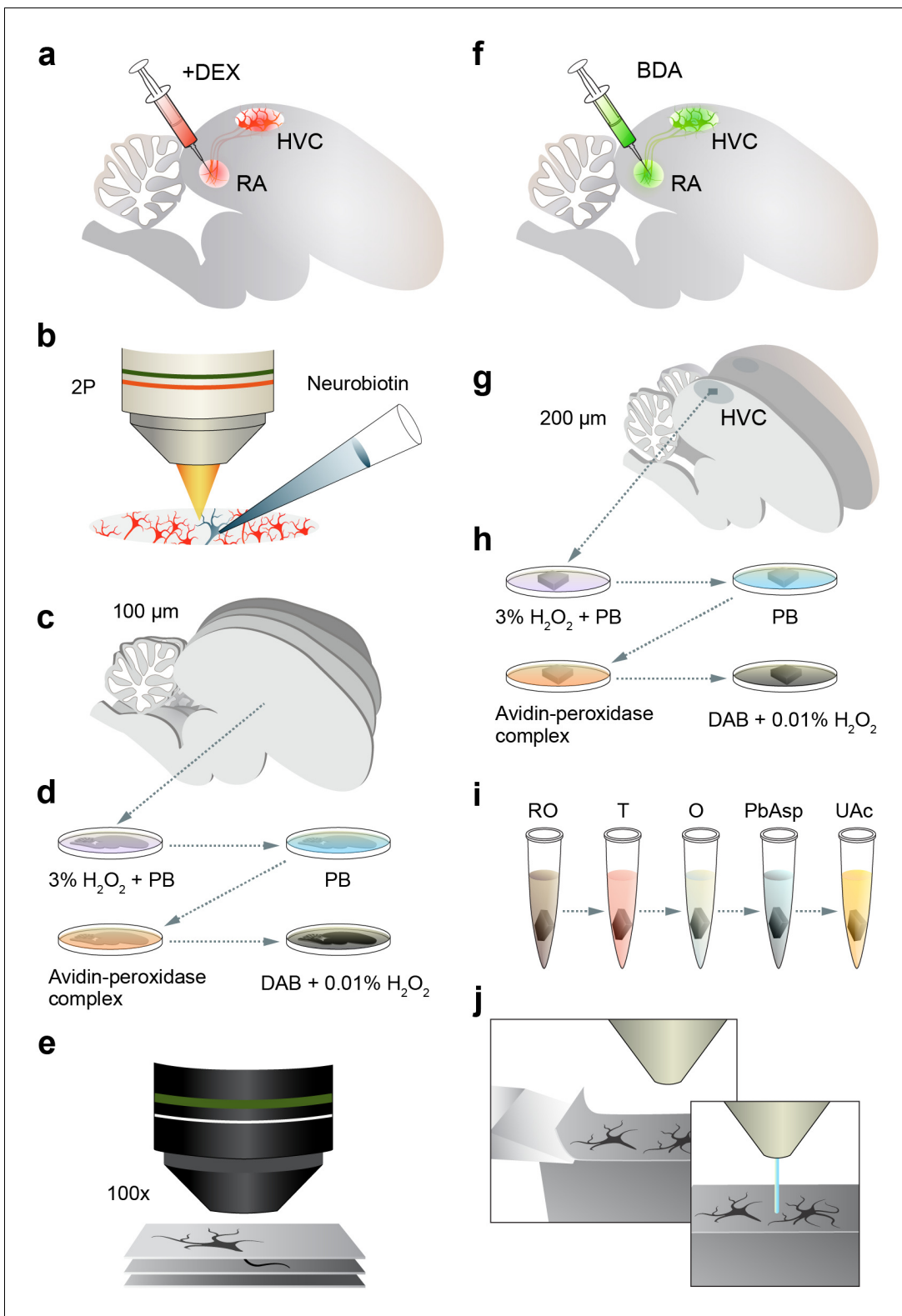


Figure 1—figure supplement 1. Sample preparation for LM and EM. (a,b) For LM, $\text{HVC}_{(\text{RA})}$ neurons are retrogradely labeled by injecting a fluorescent dextran (fluoro-Ruby) into RA (a), and a single labeled neuron is targeted and filled with Neurobiotin under the guidance of 2-photon microscopy (b). (c) The tissue is then fixed and 100 μm parasagittal sections are sliced through the entirety of HVC. (d,e) Sections are further processed to stain Neurobiotin-labeled neurites (d) and then imaged in brightfield with a 100x objective (e). (f) For EM, $\text{HVC}_{(\text{RA})}$ neurons are retrogradely labeled with biotinylated dextran (BDA). (g,h) A single 200 μm parasagittal section is taken from the center of HVC (g) and further processed to stain BDA-labeled Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

neurites (h). (i,j) A cube of tissue from within the center of HVC is extracted and stained by ROTO (reduced osmium OTO) (i) before being imaged via SBEM (j).

DOI: [10.7554/eLife.24364.004](https://doi.org/10.7554/eLife.24364.004)

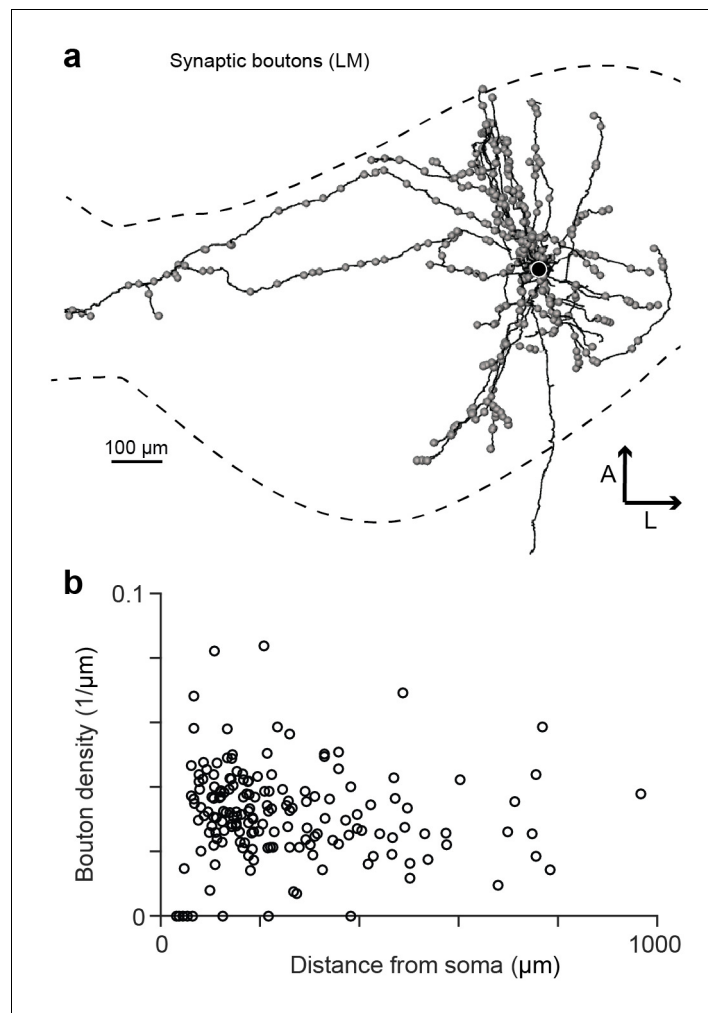


Figure 1—figure supplement 2. Synaptic boutons on HVC_(RA) axon collaterals. (a) An LM reconstruction of HVC_(RA) axon collaterals of one neuron with the HVC border indicated by dashed lines. Locations of all synaptic boutons are marked by grey spheres. (b) Bouton density of collateral branches as a function of their midpoint distances from the soma.

DOI: [10.7554/eLife.24364.005](https://doi.org/10.7554/eLife.24364.005)

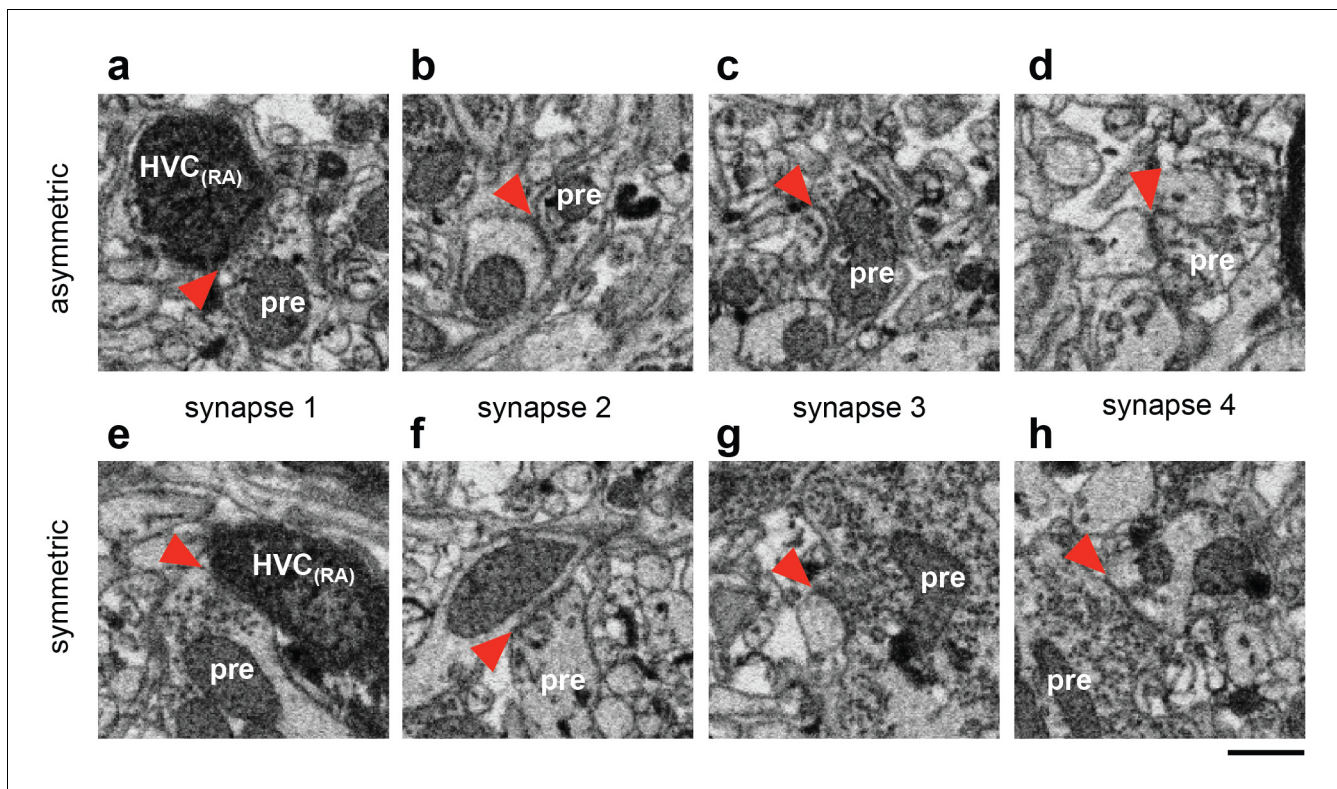


Figure 1—figure supplement 3. Ultrastructural classification of synapses. (a) An asymmetric synapse onto a BDA-labeled $HVC_{(RA)}$ dendrite whose morphology is partially obscured by the label. Red arrows indicate the synaptic cleft. (b to d) Other synapses made by the same axon onto unlabeled dendrites. The pronounced postsynaptic density (PSD), especially for synapse 4, confirms the classification of this connection as excitatory. (e) A symmetric synapse onto a BDA-labeled $HVC_{(RA)}$ dendrite. (f to h) Synapses from the same neuron onto other unlabeled dendrites display a lack of a PSD and a different appearance of synaptic vesicles compared with the asymmetric synapses. Scale bar: 0.5 μ m.

DOI: [10.7554/eLife.24364.006](https://doi.org/10.7554/eLife.24364.006)

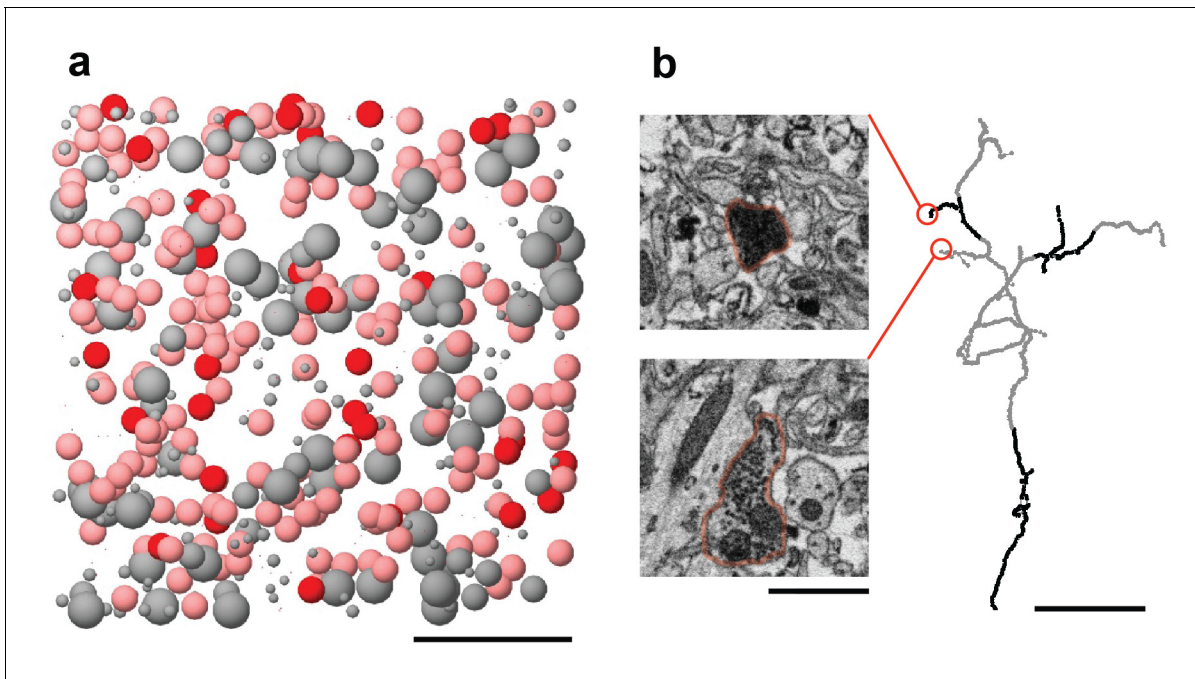


Figure 1—figure supplement 4. The BDA label is inefficient and incomplete. (a) All cells within our SBEM dataset are represented by spheres at the location of the cell body. Known HVC_(RA) neurons, which were labeled with BDA, were colored red. Also shown are putative HVC_(RA) neurons, classified by morphological features of the soma and dendrite (pink), other neuron types (large gray spheres), and glia (small gray spheres). Scale bar: 50 μm . (b) Incomplete labeling of axonal collaterals of HVC_(RA) neurons inside HVC is demonstrated with a skeleton reconstruction (labeled axon in black and unlabeled axon in gray). The inset electron micrographs correspond to the portions of the axonal field indicated. Scale bars: left: 1 μm , right: 5 μm . DOI: [10.7554/eLife.24364.007](https://doi.org/10.7554/eLife.24364.007)

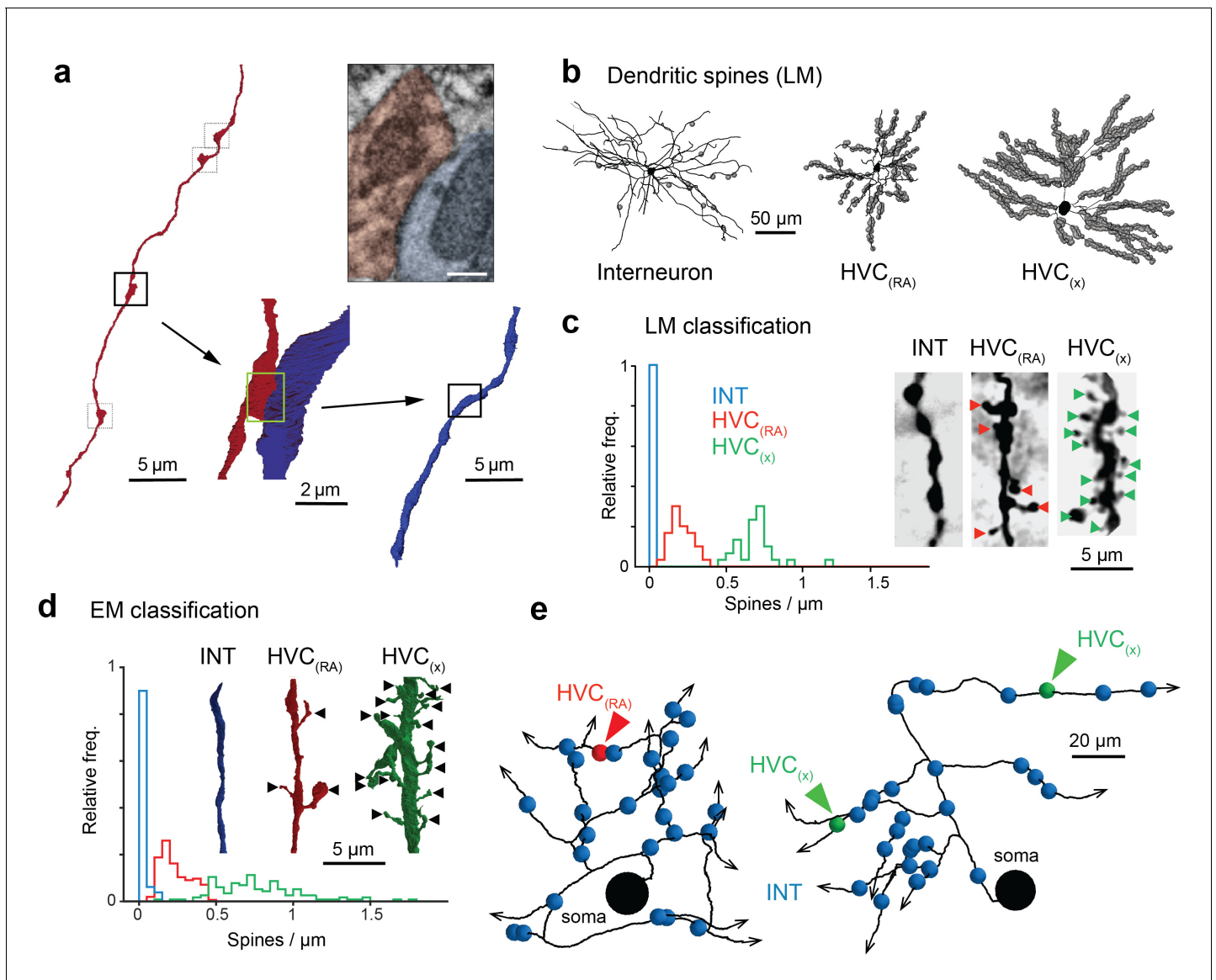


Figure 2. Classification of postsynaptic targets. (a) A BDA-labeled axon with four synaptic boutons (boxes). One bouton and its postsynaptic structure labeled in red and blue, respectively: In cross section (top right) and as a surface reconstruction (bottom center). (b) Dendrites from an inhibitory interneuron, an $\text{HVC}_{(\text{RA})}$ neuron, and an $\text{HVC}_{(\text{x})}$ neuron (left to right) in LM. Spine locations are indicated by grey spheres. (c,d) Spine densities for each of these neuron classes from LM (c) and EM (d) reconstructions. Insets show examples with spines indicated by arrowheads. (e) SBEM-based reconstructions of two $\text{HVC}_{(\text{RA})}$ somata with their proximal axons. Blue, green, and red spheres mark the location of synapses with inhibitory interneurons, $\text{HVC}_{(\text{RA})}$ neurons, and $\text{HVC}_{(\text{x})}$ neurons, respectively. Scale bar is 0.25 μm in a.

DOI: [10.7554/eLife.24364.011](https://doi.org/10.7554/eLife.24364.011)

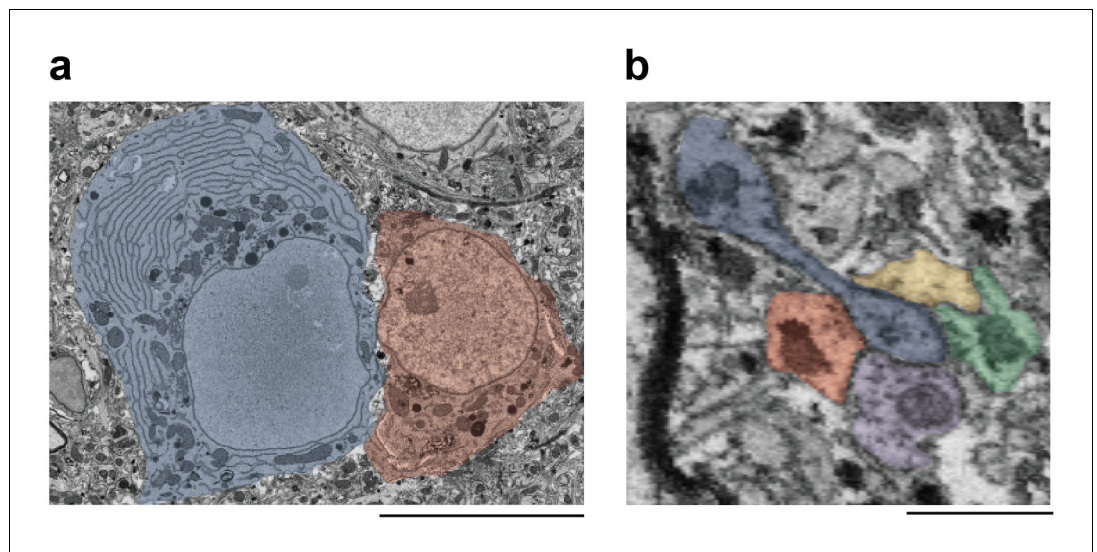


Figure 2—figure supplement 1. Morphological markers of interneurons. (a) Ultrastructural and morphological differences of the somata of an HVC interneuron (left, blue shade) and an HVC_(RA) neuron (right, red shade). Compared with HVC_(RA) neurons, interneurons had large amounts of endoplasmic reticulum (ER), many mitochondria, and a large cell body. Scale bar: 7.5 μm . (b) An electron micrograph (cut plane rotated to show spine attached to dendrite) showing a polysynaptic protrusion (blue label) that receives four synapses at its tip, that was previously misclassified as a dendritic spine of an excitatory cell. The four presynaptic axons are colored in yellow, green, purple and red. Scale bar: 1 μm .

DOI: [10.7554/eLife.24364.012](https://doi.org/10.7554/eLife.24364.012)

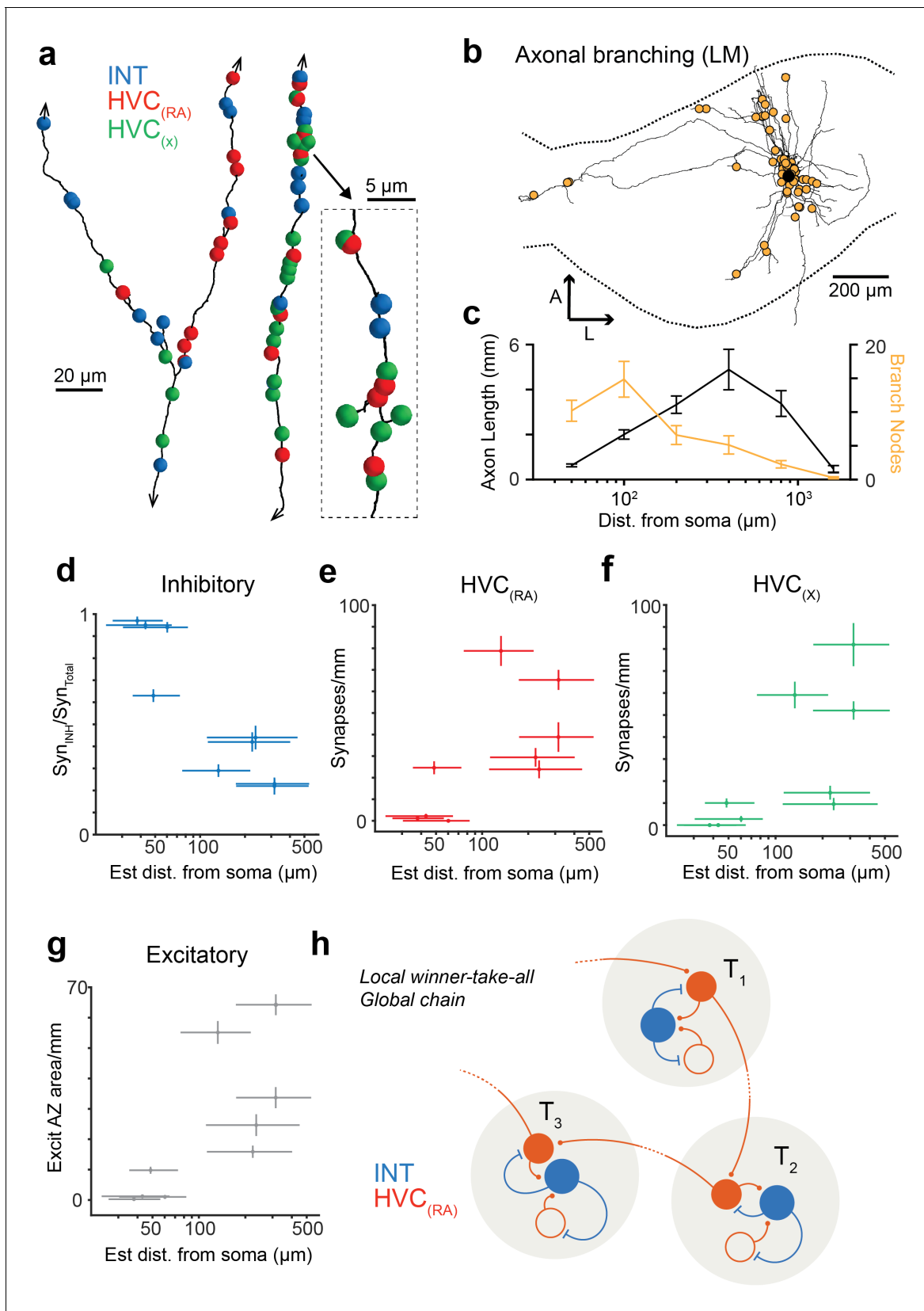


Figure 3. Spatial variation of postsynaptic cell type. (a) SBEM-based reconstructions and synaptic targets for two orphaned axon segments. Colored spheres mark the locations and types of synapses. (b) Axon collaterals (LM-based reconstruction) of an HVC_(RA) neuron with branch nodes (gold circles), Figure 3 continued on next page

Figure 3 continued

the soma (black circle), and the HVC border (dashed lines). (c) Mean axon length (black) and branch node densities (gold) vs. soma distance ($n = 15$ cells). (d) The ratio of synapses onto inhibitory interneurons vs. estimated distance from the soma ($p < 0.005$, Pearson's correlation). (e,f) The density of synapses onto $HVC_{(RA)}$ (e) and $HVC_{(X)}$ (f) vs. estimated distance from soma ($p < 0.002$, Pearson's correlation, combining $HVC_{(RA)}$ and $HVC_{(X)}$ values). (g) Total synaptic size (summed active zone area, $\mu\text{m}^2/\text{mm}$) onto excitatory neurons vs. estimated distance of the presynaptic axon from the soma ($p < 0.05$, Pearson's correlation). Vertical error bars: SEM of the Poisson-distribution means estimated from the number of synapses on each axon segment (e–g) or the SEM of an assumed underlying binomial count distribution (d). Horizontal error bars from quantiles 0.16 to 0.84 of the distance distribution based on the nearest neighbor sampling approach (see Materials and methods). (h) Proposed circuit architecture. $HVC_{(RA)}$ neurons (red) target inhibitory interneurons (blue) proximally and other $HVC_{(RA)}$ neurons distally.

DOI: [10.7554/eLife.24364.015](https://doi.org/10.7554/eLife.24364.015)

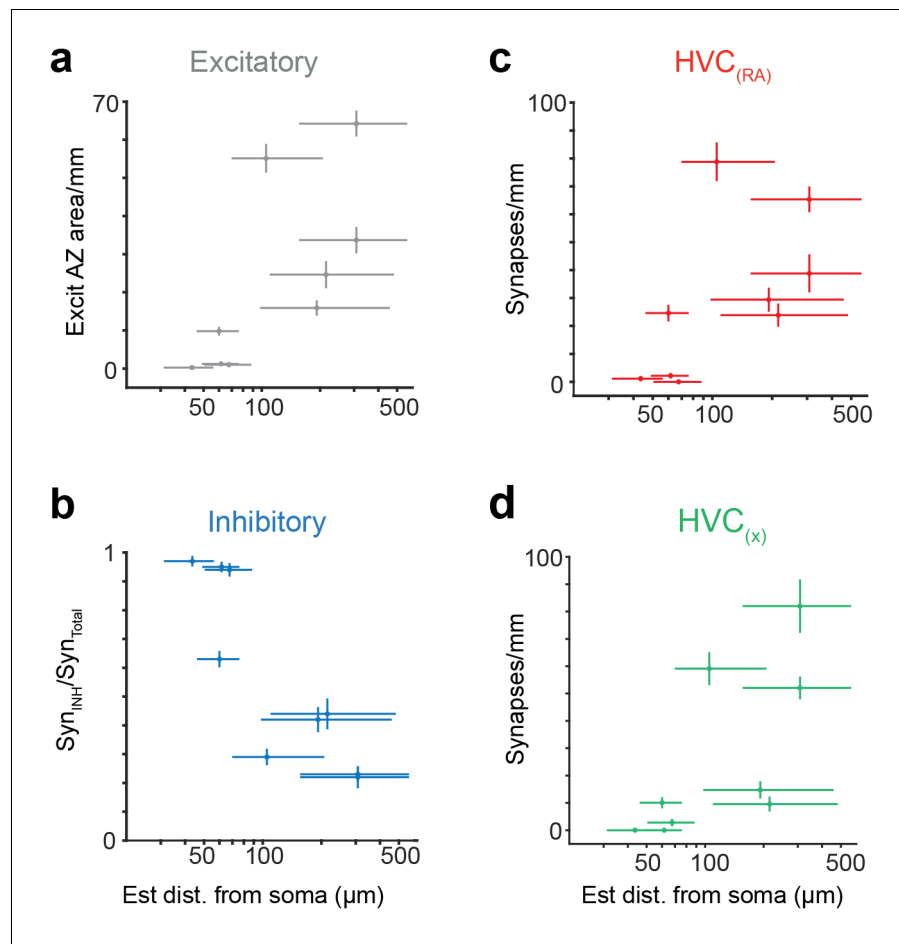


Figure 3—figure supplement 1. Synaptic properties of $HVC_{(RA)}$ axons, using a Bayesian approach to estimate distance from soma. (a) Total synaptic strength (summed active zone area/pathlength) onto excitatory neurons vs. estimated distance of the presynaptic axon from the soma ($p < 0.05$, Pearson's correlation). (b) The ratio of synapses onto inhibitory interneurons vs. estimated distance from soma ($p < 0.01$, Pearson's correlation). (c,d) The density of synapses onto $HVC_{(RA)}$ (c) and $HVC_{(X)}$ (d) vs. estimated distance from soma ($p < 0.002$, Pearson's correlation, combining $HVC_{(RA)}$ and $HVC_{(X)}$ values). Vertical bars represent the SEM of an assumed underlying Poisson distributed synapse counting process (a,c,d) or (b) the SEM of an assumed underlying Binomial count distribution. (a–d) Horizontal error bars correspond to a 0.16 and 0.84 quantile of the Bayesian posterior distribution, see Materials and methods.

DOI: [10.7554/eLife.24364.016](https://doi.org/10.7554/eLife.24364.016)

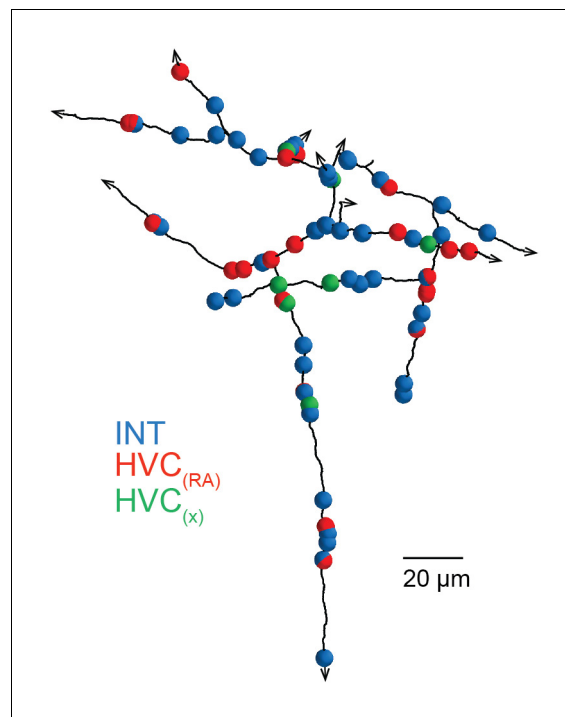


Figure 3—figure supplement 2. A SBEM-based reconstruction and synaptic targets for an orphaned axon with high branch density. Small spheres mark the location of synapses, with the color indicating the target type. Note the higher frequency of inhibitory targets (blue) along the length of the reconstruction compared with other orphaned axons with low branch density (see **Figure 3a**).

DOI: [10.7554/eLife.24364.017](https://doi.org/10.7554/eLife.24364.017)

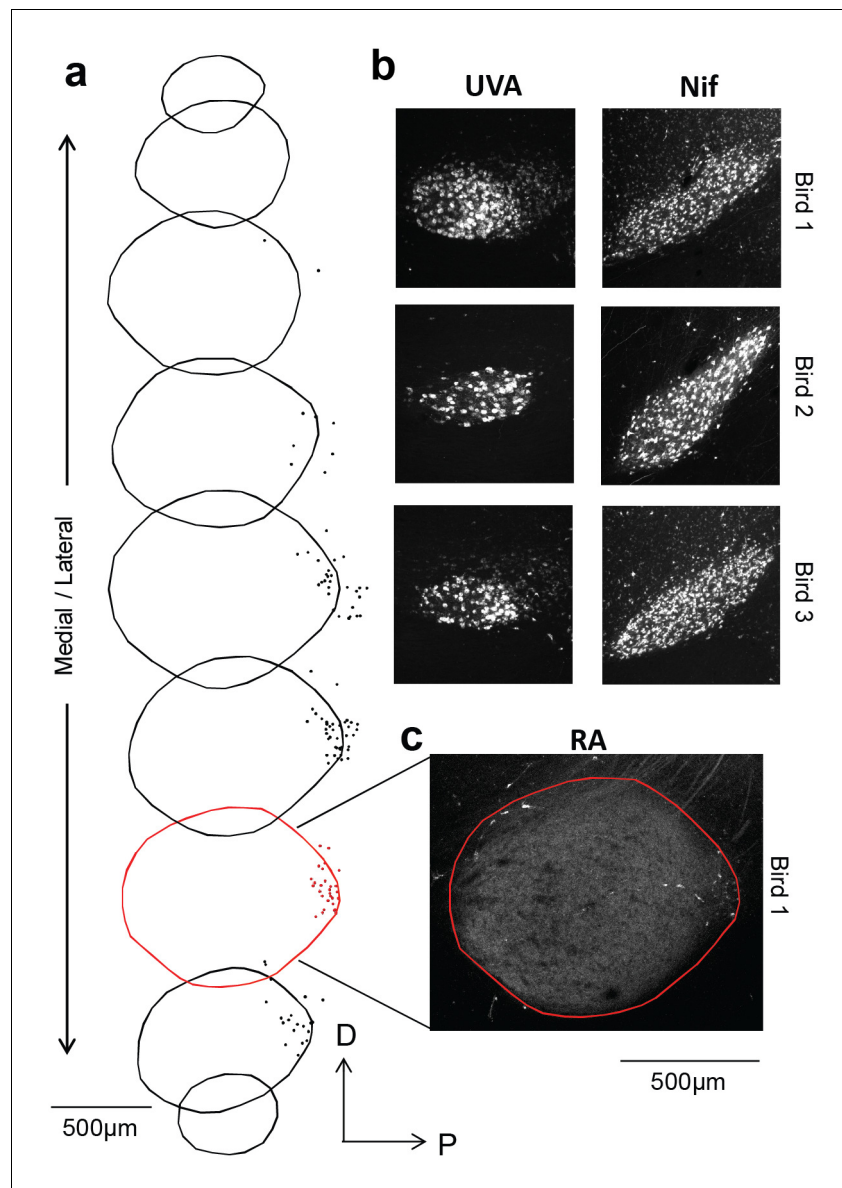


Figure 3—figure supplement 3. A small population of RA neurons project to HVC. (a) The borders of RA were traced across nine sequential 100 μm thick sagittal sections, and the location of each retrogradely labeled HVC-projecting RA neuron is marked with a dot. (b) Dil injection in HVC resulted in robust retrograde labeling of upstream motor nuclei Uva and Nif, while only a small percentage (<1%) of cells in RA were labeled (a,c). An example confocal image of RA is shown in (c), revealing a sparse population of retrogradely labeled neurons in the posterior region of the nucleus.

DOI: [10.7554/eLife.24364.018](https://doi.org/10.7554/eLife.24364.018)