

Synaptic Connectivity of Sensorimotor Circuits for Vocal Imitation in the Songbird

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The songbird vocal motor nucleus HVC contains cells that project to the basal ganglia, the auditory system, or to downstream vocal motor structures. In this **fundamental** study, the authors conduct optogenetic circuit mapping to clarify how four distinct inputs to HVC act on these distinct HVC cell types. They provide **compelling** evidence that all long range projections engage inhibitory circuits in HVC and can also exhibit cell-type specific preferences in monosynaptic input strength. Understanding HVC at this microcircuit level is critical for constraining models of song learning and production.

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Abstract

Sensorimotor computations for learning and behavior rely on precise patterns of synaptic connectivity. Yet, we typically lack the synaptic wiring diagrams for long-range connections between sensory and motor circuits in the brain. Here we provide the synaptic wiring diagram for sensorimotor circuits involved in learning and production of male zebra finch song, a natural and ethologically relevant behavior. We examined the functional synaptic connectivity from the 4 main sensory afferent pathways onto the 3 known classes of projection neurons of the song premotor cortical region HVC. Recordings from hundreds of identified projection neurons reveal rules for monosynaptic connectivity and the existence of polysynaptic ensembles of excitatory and inhibitory neuronal populations in HVC. Circuit tracing further identifies novel connections between HVC's presynaptic partners. Our results indicate a modular organization of ensemble-like networks for integrating long-range input with local circuits, providing important context for information flow and computations for learned vocal behavior.

Introduction

Patterns of synaptic connectivity anchor how activity flows within sensory and motor networks in the brain. Consequently, these synaptic connections delimit and help define how sensory and premotor circuits interact. Mapping synaptic pathways between these circuits is therefore fundamental to understanding the neural computations involved in learning and producing behaviors. Great strides have been made in developing technologies for large-scale recording of neuronal activity across brain regions^{1,2,3} and in building functional connectomes of local (< 1 mm) synaptic circuits⁴. Less progress has been made at intermediate levels, which aim to build wiring diagrams for long-range synaptic connections in the brain⁵. Moreover, while most evidence for long range synaptic connectivity has been provided piecemeal, we now have the opportunity to systematically apply circuit dissection methods to other animal models in which we still know relatively little about patterns of long-range synaptic connectivity.

We were inspired to understand the wiring diagram for sensorimotor circuits critical for birdsong, a complex and volitionally produced skilled behavior that, like speech and language, is dependent on forebrain circuits for its fluent production^{6,7}. The dedicated neural circuits associated with song provide a powerful model in which to study how synaptically linked sensory and motor networks of neurons control a complex behavior. The courtship song of male zebra finches is one of the better studied naturally learned behaviors^{8,9,20}. Zebra finch song is controlled by a relatively discrete set of interconnected forebrain regions located in the dorsal ventricular ridge (DVR)^{21,22-30}. The DVR is the avian analogue of the mammalian neocortex, and like the neocortex it contains parcellated regions that (i) receive sensory information from the thalamus, (ii) regions that process information connected by mostly ipsilaterally projecting intratelencephalic projection neurons (PNs), and (iii) output motor circuits projecting back to the thalamus and to motor regions throughout the brainstem^{31,32}. The vocal premotor nucleus HVC (proper name) is a central hub in the DVR song network. HVC is necessary for juvenile song learning and adult song production. It also serves as the primary synaptic interface between sensory pathways and the premotor circuits involved in learning and controlling singing behavior^{21,27,28,33-36}.

Although anatomical evidence delineated the main input and output pathways of HVC decades ago^{21,27,37-40}, a cell-type specific synaptic wiring diagram of this core circuitry has remained out of reach. HVC receives intratelencephalic input from at least 4 sensory and sensorimotor regions in the DVR (Nif, Av, mMAN and RA) and one thalamic brain region, Uva (**Fig. 1A, B**; see **Fig. 1** legend for anatomical descriptions). Several lines of evidence support the idea that song learning, and vocal motor control involves some of these pathways projecting into HVC^{27,34,36,40,46}. In addition, HVC has 3 distinct classes of intratelencephalic projecting neurons that function as the output pathways important for song learning and song motor control²⁷. HVC's projection onto RA through HVC_{RA} neurons forms the descending song motor pathway necessary for song production. HVC's projection onto Area X through HVC_X neurons forms a DVR-striatal circuit that is important for song plasticity. HVC's projection onto Avalanche through HVC_{Av} neurons forms the song circuit's projection back to the auditory DVR, a pathway that is important for evaluating motor performance during song learning²⁷.

Mapping the synaptic connectivity between HVC's input and output pathways has been hindered by the lack of tools for robustly manipulating these circuits. We have overcome this issue by employing better performing optogenetic channels packaged in an adeno associated virus (AAV) that we optimized for expression throughout various portions of the song circuitry. Specifically, we made use of the axonal expression of the opsin eGtACR1^{47,48}. GtACRs are blue light-driven Cl⁻ channels that, while hyperpolarizing at the soma and dendrites, are depolarizing at axon terminals due to the shifted internal Cl⁻ concentration in axonal compartments^{49,51}. Here we

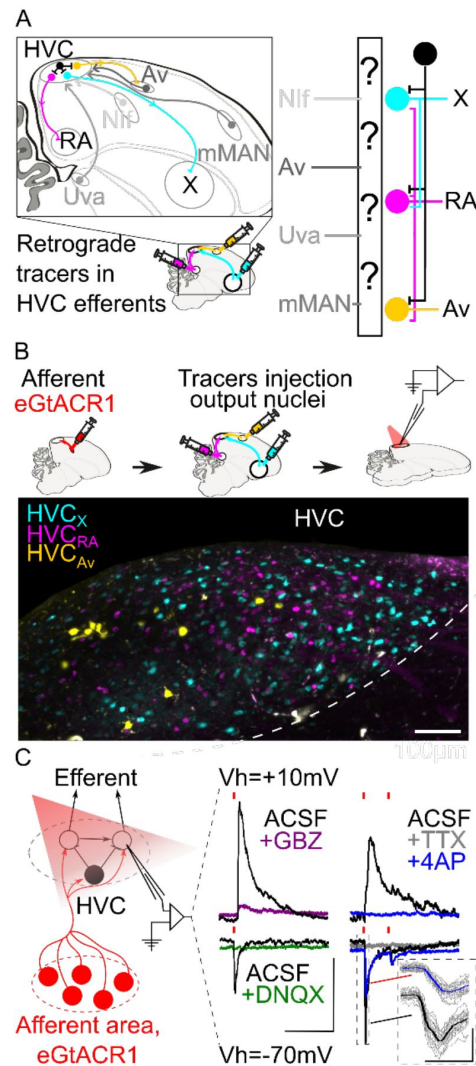


Fig. 1

Strategy for opsin assisted HVC circuit mapping.

A) (left) Parasagittal schematic of the zebra finch brain illustrating HVC afferents (grey) from nucleus interface of the nidopallium (Nif), nucleus avalanche (Av), medial magnocellular nucleus of the anterior nidopallium (mMAN) and nucleus Uvaeformis (Uva), and the 3 classes of HVC-PNs: HVC_X (projecting to Area X, cyan), HVC_{RA} (projecting to RA, magenta) and HVC_{Av} (projecting to Av, yellow). (right) Schematic illustrating the known synaptic connectivity of HVC's input and output pathways (interneurons schematized in black). **B)** (top) Schematic of the workflow including opsin expression in afferent areas, retrograde tracer injection in afferent areas and whole cell patch-clamp recording of light-evoked currents in acute brain slices. (bottom) Sample image of retrogradely labeled HVC-PN classes in a brain slice used for patch-clamp recordings (scalebar 100µm). **C)** Schematic of whole-cell recording of light evoked synaptic currents in HVC-PNs receiving monosynaptic inputs from one of the afferent areas expressing eGtACR1 (red), as well as polysynaptic inputs from local HVC-PNs (white circle) and interneurons (black circle). Recordings are performed at holding potential (Vh)= +10mV and -70mV. Sample traces report the effect of bath application of DNQX (green) and gabazine (GBZ, purple), which suppress oEPSC and oIPSC, respectively. Glutamatergic monosynaptic currents are pharmacologically isolated by bath application of TTX+4AP: sample traces (average of 20 sweeps) portray a typical case of a cell displaying oEPSC with a monosynaptic component (inset shows 20 oEPSCs sweeps in grey, averaged in the thick traces in black and blue), and polysynaptic oIPSC, as 4AP (blue) application results in a partial restoration of oEPSC, but not of oIPSC (red lines represent light stimuli, 1ms; scalebar: 100ms, 100pA).

use viral expression of eGtACR1 to achieve independent control of synaptic release from Nif, Uva, mMAN, and Av. We paired this with whole-cell patch-clamp recordings from visually targeted HVC projection neurons (HVC-PNs; HVC_X , HVC_{RA} , HVC_{Av}), identified using retrograde tracer injections into each efferent region (**Fig. 1C**). This permitted the first large-scale cell-type specific synaptic connectivity mapping, using gold-standard electrophysiological methods, in songbirds. In ex-vivo brain slices from adult male zebra finches, we mapped the properties of synaptic transmission at the 12 afferent-HVC-PN combinations. Via recordings from hundreds of HVC PNs, we provide a comprehensive polysynaptic and monosynaptic mapping of the connectivity between these afferent sensory pathways and the output pathways of HVC. Through this research, we provide the first long-range cellular resolution synaptic connectivity map in the avian DVR, a fundamental step in the effort to identify common themes and differences in synaptic connectivity between the DVR and mammalian neocortical circuits.

Results

To conduct opsin-assisted circuit mapping in zebra finches, we expressed eGtACR1 in Nif, Uva, mMAN, or Av using AAVs (**Fig. 1C**; a cocktail of AAV2.9-Cbh-FLP and AAV2.9-Cbh-fDIO-eGtACR1 injected in HVC afferent areas). 4-6 weeks later we injected retrograde tracers in HVC projection targets (RA, Area X, and Av) to label HVC-PN classes in different fluorescent channels. After 2-7 days we obtained acute brain slices and performed whole-cell patch-clamp recordings from visually identified HVC-PNs to examine optically evoked post-synaptic currents (oPSCs). We confirmed that oPSCs measured while holding the membrane voltage at -70mV were excitatory (oEPSCs) and were mediated by AMPA receptors (current suppressed by bath application of DNQX; **Fig. 1C**). Holding the membrane potential to +10mV allowed us to measure inhibitory currents (oIPSCs) mediated by GABA_A receptors (current suppressed by bath application of gabazine; **Fig. 1C**).

For each pathway we report the likelihood of observing oEPSCs and oIPSCs, the amplitude of oEPSCs and oIPSCs, and the excitatory-inhibitory ratio (oEPSC/oIPSC). Although we calculated the paired-pulse ratio, the slow kinetics of eGtACR1 make interpretation of these results difficult, therefore it is not being reported. We also report whether observed synaptic currents have a monosynaptic component, by applying the voltage-gated Na⁺ channel blocker tetrodotoxin (TTX) followed by the K⁺ channel blocker 4-aminopyridine (4AP)^{52,53} (**Fig. 1C**). TTX blocks action potentials and the addition of 4AP blocks the rapid K⁺ dependent repolarization of the axon. This allows local opsin-driven depolarizations to reach threshold for calcium-dependent vesicle docking and release, thus revealing optically evoked monosynaptic currents⁵². We consistently observed that, while oEPSCs registered in ACSF displayed multiple peaks consistent with a polysynaptic source, currents suppressed by TTX that returned following bath application of 4AP had a monotonic rising slope and a single peak, consistent with a monosynaptic origin of the current (**Fig. 1C**).

Uva monosynaptically innervates HVC_{RA} neurons

Nucleus Uvaeformis (Uva) is a small polysensory nucleus in the caudal thalamus that is reported to be necessary for song production. Uva is a recipient of somatosensory information from the trigeminal system, visual input from the optic tectum, auditory information from the lateral lemniscus, cholinergic input from the medial habenula, and information about respiratory timing from the ventral respiratory column⁵³⁻⁵⁸. Its inputs to HVC are thought to be pivotal in song motor control^{29,42,43}. However, how Uva synaptically influences HVC activity is still poorly understood. We found that Uva neurons are robustly transduced by our AAV expressing eGtACR1 (**Fig. 2A**). Thalamic regions around Uva do not project to HVC, allowing us to selectively map Uva's input to the 3 classes of HVC-PNs.

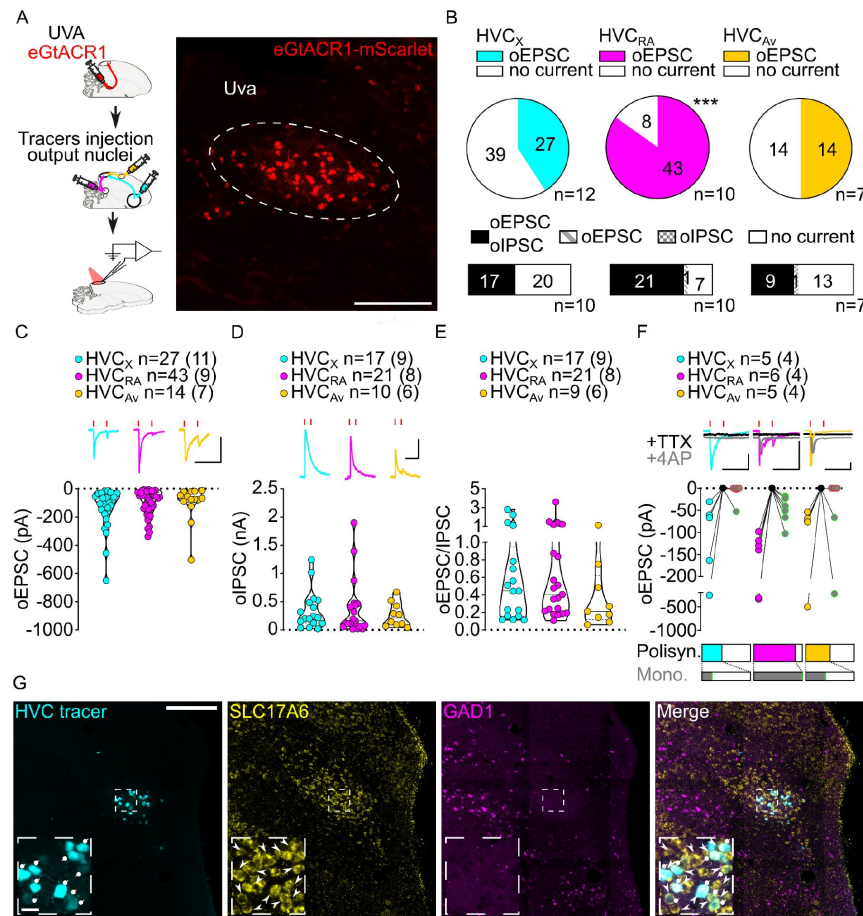


Fig. 2

Uva afferents reliably elicit polysynaptic oIPSCs and oEPSCs in all three HVC-PN classes and have monosynaptic inputs onto HVC_{RA} neurons.

A (left) Schematic of the experimental timeline, illustrating injection of AAV-eGtACR1 in Uva, followed by retrograde tracer injections in HVC efferent areas and whole-cell patch-clamp recording in acute brain slices; (right) sample image of eGtACR1-mScarlet expression in Uva (scalebar 200µm). **B** (top) pie charts representing the likelihood of observing oEPSCs in HVC_X (cyan), HVC_{RA} (magenta) or HVC_{Av} (yellow) (Fisher's exact test, $P < 0.001$). Numbers in the pie fragments represent the number of cells in which current (colored) or no current (white) was found. Numbers next to the pie charts represent the number of animals from which the data is obtained. (bottom) bar chart representing the number of cells where both oEPSCs and oIPSCs could be elicited (black), only oEPSCs but no oIPSCs (grey lines), only oIPSCs but no oEPSCs (grey checkers), or neither (white), for subsets of cells from the HVC-PN classes' pie charts aligned above (Fisher's exact test, $P = 0.0244$). **C** violin and scatter plot and sample traces reporting average measured oEPSC amplitude of each cell, by cell class (Kruskal-Wallis test $H(2) = 2.241$, $P = 0.3262$; n = cells (animals); red lines represent light stimuli, 1ms; scalebars, 100ms, 100pA). **D** violin and scatter plot and sample traces of average measured oIPSC amplitude of each cell, by cell class ($H(2) = 0.5946$, $P = 0.7428$). **E** violin and scatter plot of the ratio of oEPSC and oIPSC peak amplitude of each cell where both are measured and $\neq 0$, per cell class ($H(2) = 0.2716$, $P = 0.2572$). **F** (top) sample traces and plot representing the amplitude of post-synaptic currents evoked by lightly-driven release of neurotransmitter from Uva axonal terminals in HVC; oEPSCs amplitudes are reported before (HVC_X cyan, HVC_{RA} magenta, HVC_{Av} yellow) and after bath application of TTX (black) and 4AP (grey, green outline indicates polysynaptic oEPSC, see methods), (n = cells (animals); blue lines represent light stimuli, 1ms; scalebars, 100ms, 100pA) (bottom) bar charts representing the likelihood of observing polysynaptic oEPSCs in HVC_X (cyan), HVC_{RA} (magenta) or HVC_{Av} (yellow) (data from panel B), and (grey) likelihood of a subset of the corresponding oEPSCs to be monosynaptic. **G** Sample images reporting retrogradely labeled HVC-projecting neurons in UVA (cyan, white circles) together with in-situ labeling of glutamatergic (SLC17A6, yellow, white arrowheads) and GABAergic (GAD1, magenta, gray arrowheads) markers transcripts (scalebar 200µm, inset 20µm).

In acute brain slices we observed glutamatergic oEPSCs in all 3 classes of retrogradely identified HVC-PNs (27/66 HVC_X, 43/51 HVC_{RA}, 14/28 HVC_{AV} neurons; **Fig. 2B**, **Suppl. Fig. 1**). Optical stimulation of Uva axons resulted in significantly lower probability of observing oEPSCs in HVC_X and HVC_{AV} than in HVC_{RA} neurons (**Fig. 2B**, **Suppl. Fig. 1**). When we held the membrane voltage to +10mV we also observed relatively delayed GABAergic currents (53.93% probability, **Suppl. Fig. 1**), consistent with disynaptic inhibition. Uva axon stimulation elicited oIPSCs mainly in cells where we observed oEPSCs (oIPSC contingent with oEPSC: HVC_X, 100.0%, HVC_{RA} 96.6%, HVC_{AV}, 95.8%; **Fig. 2B**).

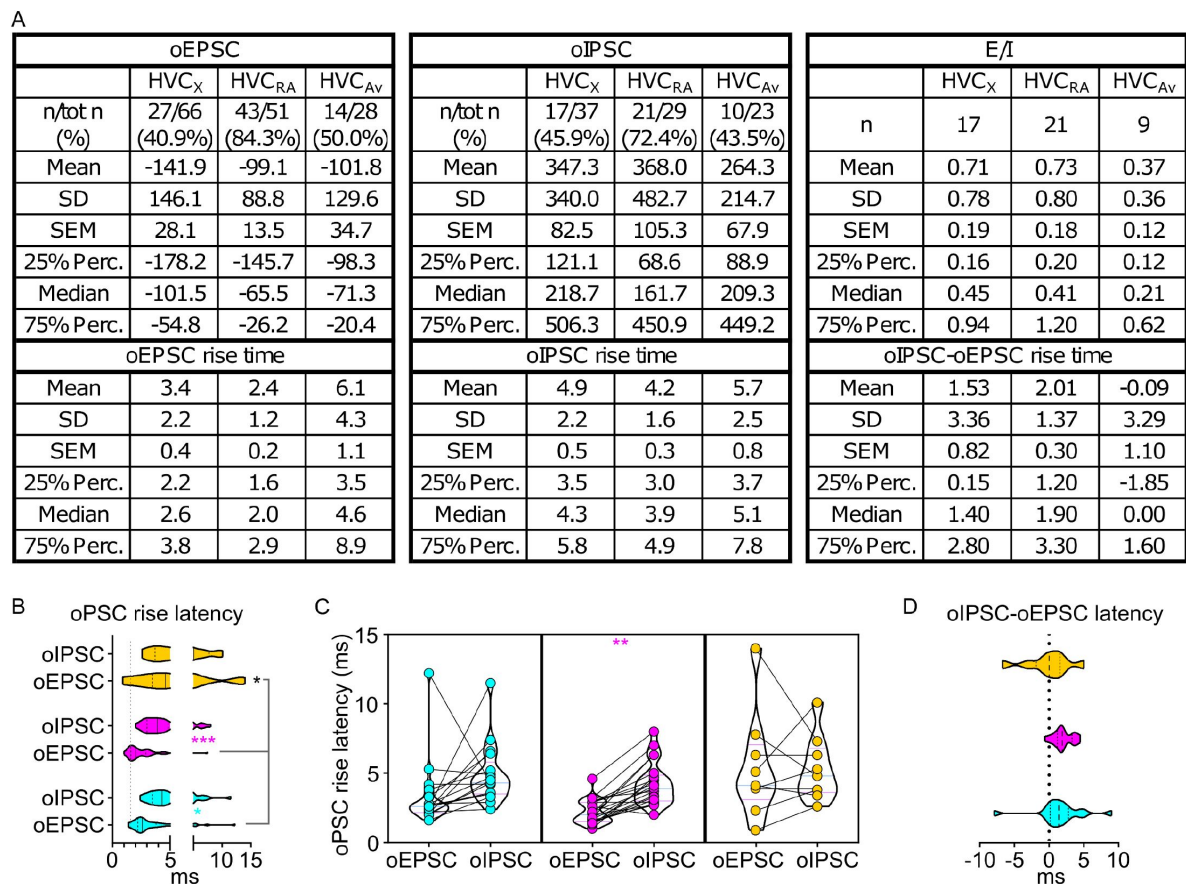
Despite the markedly higher probability of eliciting currents in HVC_{RA} neurons, oEPSC and oIPSC amplitudes were comparable across all the HVC-PN classes (**Fig. 2C, D**, **Suppl. Fig. 1**). The excitation/inhibition ratio revealed that Uva terminal stimulation generally led to higher amplitude oIPSCs than oEPSCs (**Fig. 2E**, **Suppl. Fig. 1**).

We next examined if HVC-PN classes receive monosynaptic input from Uva. Recent studies have suggested different views for how Uva may influence the propagation of activity in HVC. Uva has been proposed to provide high frequency (30 – 60Hz) synchronous synaptic inputs that facilitate activity propagation across HVC_{RA} premotor neurons⁴⁵. In another proposal, Uva selectively drives activity in only a fraction, ~16%, of HVC_{RA} neurons which are active during syllable transitions²⁹. Consistent with the higher likelihood of polysynaptic excitation in HVC_{RA} neurons, we found that oEPSC returned after application of TTX+4AP in all HVC_{RA} neurons tested (HVC_{RA}: 6/6 neurons) while HVC_X and HVC_{AV} were infrequently found to receive monosynaptic input from Uva (HVC_X: 1/5 cells, HVC_{AV}: 2/5 cells, **Fig. 2F**). Our synaptic circuit mapping provides the first conclusive evidence that Uva makes monosynaptic connections with HVC_{RA} neurons. Given that we observed oEPSCs in 84.3% of recorded HVC_{RA} and found monosynaptic connections from Uva onto 100% of the HVC_{RA} neurons tested, our data further suggests that Uva is unlikely to make monosynaptic connections with only a small percentage of HVC_{RA} neurons.

Previous studies have been equivocal to the excitatory, inhibitory, or neuromodulatory nature of the Uva to HVC circuitry^{29,53}. We did not observe monosynaptic oIPSCs in any cell class suggesting that Uva provides only a glutamatergic input to HVC (HVC_X: 0/3, HVC_{RA}: 0/5, HVC_{AV}: 0/4, data not shown). We combined retrograde labeling from HVC with in-situ hybridization labeling on brain slices. This revealed that Uva neurons projecting to HVC (Uva_{HVC}) selectively expressed the glutamatergic marker SLC17A6, but not the GABAergic marker GAD1³² (**Fig. 2G**). Interestingly, our labeling did not identify any GABAergic neurons within Uva, indicating that Uva_{HVC} may function as an excitatory relay of diverse polysensory input pathways. Although we cannot exclude the possibility that Uva neurons may also co-release neuropeptides, our results define the excitatory synaptic connectivity between Uva and HVC PNs.

NIf provides excitatory monosynaptic input to all HVC-PNs

We next examined the synaptic transmission between the nucleus NIf and the 3 classes of HVC-PNs. NIf is a higher order polysensory DVR nucleus located adjacent to the primary auditory DVR (Field L2 - region that receives thalamic input from the avian homologue of the medial geniculate nucleus). NIf is recipient of afferents from Uva and multiple areas of the auditory DVR and has an essential role in relaying auditory signals to HVC⁵⁹. The synaptic connection between NIf and HVC is obligatory in a young male zebra finch's ability to form a memory of their father's song^{34,46}. This memory is used as birds evaluate their song performances, permitting vocal imitation of song³³. Optogenetic manipulations of NIf inputs to HVC are also sufficient to implant song memories that guide song syllable imitation³⁴. Although NIf is selectively active during singing, it is not necessary for producing song in adulthood or the song imitation process once the memory of a father's song is acquired in juvenile birds^{34,46,60,61}.



Suppl. Fig 1 (related to Fig. 2).

A) Table reporting descriptive statistics relative to oEPSCs and oIPSCs as reported in **Fig. 2**. **B)** violin and scatter plots representing oPSC rise latencies reported in **fig. 2** (gray line marks the minimal latency (1_{st} quartile) to oPSC rise across the cell types, for ease of comparison (Mixed-effects analysis, oEPSC vs. oIPSC $F(1,44)=7.143$, $P=0.0105$ HVCX $P=0.0164$, HVCRA $P<0.001$; HVCAN $F(2,82)=10.24$, $P<0.001$, oEPSCs:HVCX vs HVCAN $P<0.001$, HVCRA vs HVCAN $P<0.001$); **C)** oPSCs rise latency for cells where both oEPSC and oIPSC were measured (2W ANOVA, oEPSC vs. oIPSC $F(1,44)=7.923$, HVCRA $P=0.0032$); **D)** relative delay of oIPSC compared to oEPSC across the cells reported in C) (Kruskal-Wallis test, $H=4.587$, $P=0.1009$)

To map synaptic connections from Nif to HVC, we expressed eGtACR1 in Nif. While the surrounding area, Field L, has been described to project to the ventral boarder of HVC^{38,62,63}, we recorded from visually identified, retrogradely labeled HVC projection neurons. Area Avalanche is the only other nearby brain region that also sends projections to HVC and we carefully checked for a lack of expression in this region in all brain hemispheres used in this study (Fig. 3A). This provides confidence that the currents we measured are evoked by stimulating neurotransmission from Nif terminals. Light stimulation (1 ms) reliably elicited glutamatergic oEPSCs in all three classes of HVC projection neurons (78.6% probability, Fig. 3B, Suppl. Fig. 2). We examined oIPSCs in a subset of neurons and found strong GABAergic currents evoked by optical stimulation in all three classes of projection neurons (65.9% probability, Fig. 3B, Suppl. Fig. 2). oIPSCs were predominantly found in cells in which we also observed oEPSCs (oIPSC contingent with oEPSC: HVC_X 90.0%, HVC_{RA} 76.5%, HVC_{AV} 94.1%; Fig. 3B).

We found oEPSC and oIPSC amplitudes to be comparable between HVC_X and HVC_{AV} PN (Fig. 3C, 3D, Suppl. Fig. 2). However, HVC_{RA} neurons had lower amplitude currents than the other HVC-PN classes (Fig. 3C, 3D, Suppl. Fig. 2). When we computed the amplitude of oEPSCs and oIPSCs for each cell to calculate the excitation/inhibition, we observed that optogenetic stimulation of Nif axonal terminals evoked relatively higher amplitude oIPSCs than oEPSCs in all HVC-PN classes (Fig. 3E, Suppl. Fig. 2).

We next examined monosynaptic connectivity from Nif to each HVC-PN. We found that application of TTX followed by 4AP reliably returned oEPSCs in all three HVC projection subtypes (monosynaptic oEPSCs: HVC_X: 5/5, HVC_{RA}: 4/5, HVC_{AV}: 5/5, Fig. 3F). This widespread monosynaptic connectivity is in stark contrast to thalamic excitatory inputs from Uva that preferentially target HVC_{RA} neurons. Similar to Uva projections however, we did not observe monosynaptic oIPSCs (HVC_X: 0/5, HVC_{RA}: 0/2, HVC_{AV}: 0/3, data not shown), suggestive of a glutamatergic projection from Nif to HVC. We combined retrograde labeling from HVC with in-situ hybridization labeling on brain slices. Nif neurons projecting to HVC (Nif_{HVC}) selectively expressed the glutamatergic marker SLC17A6, but not the GABAergic marker GAD1³² (Fig. 3G). This finding is consistent with previous studies indicating that Nif projections result in disinaptic inhibition onto HVC projection neurons⁶⁴.

Together, these results indicate that intratelencephalic DVR circuits are excitatory and that Nif provides excitatory glutamatergic monosynaptic projections onto all 3 known classes of HVC-PNs. Given that monosynaptic connectivity across regions of the DVR have not previously been examined, this suggests the possibility that, in contrast to the thalamo-DVR pathways, projections between regions of the DVR might not have cell type selectivity.

Novel connection from mMAN to Av and selective monosynaptic connections with HVC

To better define the specificity of regional DVR projections we next mapped synaptic connectivity of two other HVC afferents, mMAN and Av. The DVR is comprised of three large pallial fields separated by distinct lamina, the nidopallium, mesopallium, and the arcopallium. HVC, Nif, and mMAN reside in the nidopallium and Av is in the mesopallium. HVC and Nif are in caudal regions of the DVR's nidopallium while mMAN is located at anterior portions of the DVR, some 4-5mm from HVC. Av, on the other hand is only ~1.5 mm from HVC but is in a distinct pallial field.

Cell type specific synaptic connectivity between nidopallial regions and between mesopallial and nidopallial regions are poorly understood; however, the connectivity of mMAN and Av are better described than other portions of these pallial fields. mMAN receives synaptic input from the thalamic nucleus DMP and is part of a vocal motor DVR-thalamo-DVR loop (mMAN > HVC > RA > DMP > mMAN), a circuit architecture similar to premotor and motor control-related cortico-

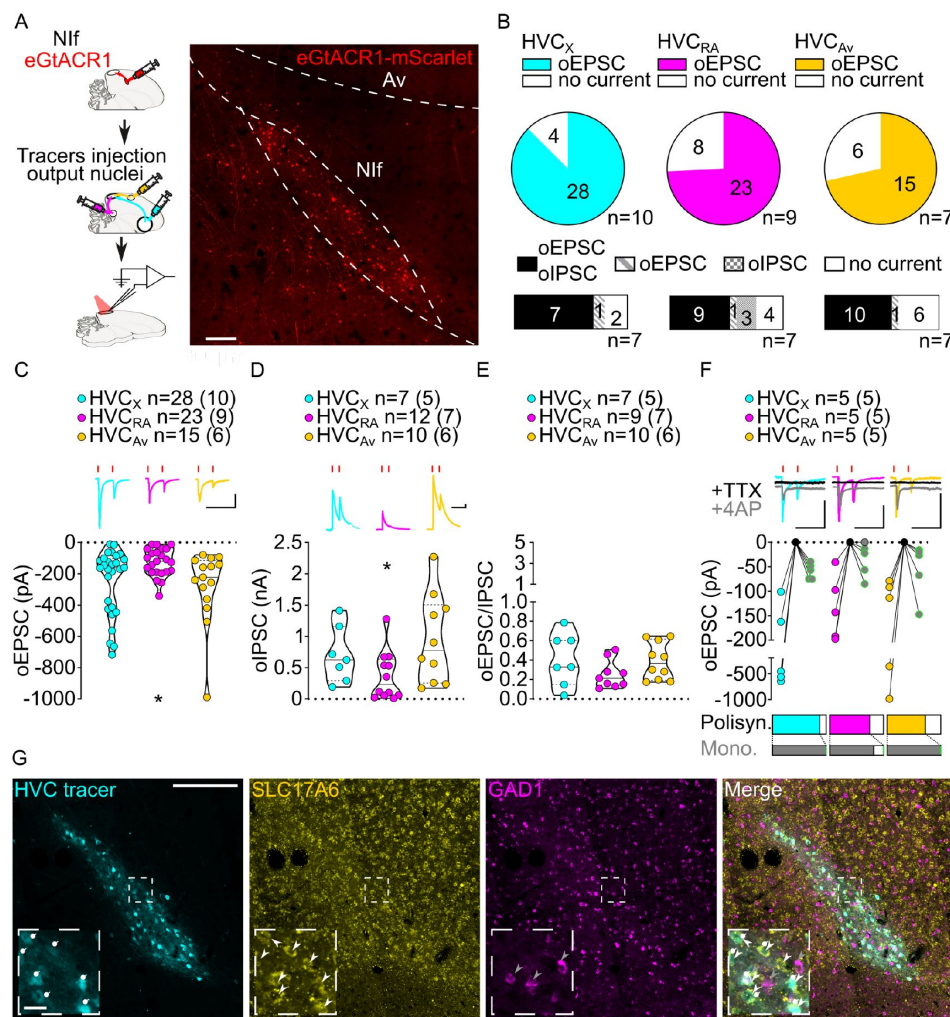
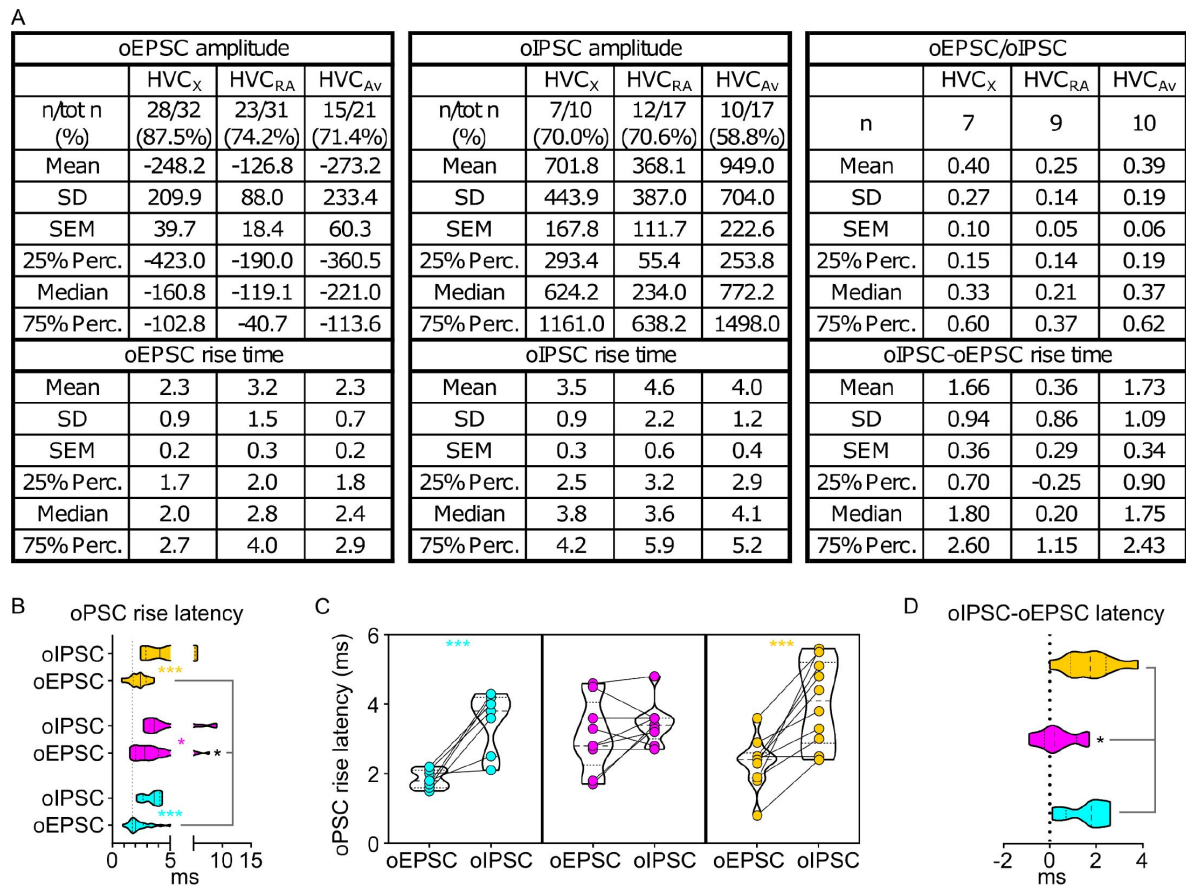


Fig. 3

Stimulation of Nif inputs to HVC reliably elicits polysynaptic oIPSCs and oEPSCs, and monosynaptic oEPSCs, in all three HVC-PN classes.

A (left) Schematic of the experimental timeline, illustrating injection of AAV-eGtACR1 in Nif, followed by retrograde tracer injections in HVC efferent areas and whole-cell patch-clamp recording in acute brain slices; (right) sample image of eGtACR1-mScarlet expression in Nif (scalebar 200µm). **B** (top) pie charts representing the likelihood of observing oEPSCs in HVC_X (cyan), HVC_{RA} (magenta) or HVC_{Av} (yellow) (Fisher's exact test, $P=0.2783$). Numbers in the pie fragments represent the number of cells in which current (colored) or no current (white) was found. Numbers next to the pie charts represent the number of animals from which the data is obtained. (bottom) bar chart representing the number of cells where both oEPSCs and oIPSCs could be elicited (black), only oEPSCs but no oIPSCs (grey lines), only oIPSCs but no oEPSCs (grey checkers), or neither (white), for subsets of cells from the HVC-PN classes' pie charts aligned above (Fisher's exact test, $P=0.5841$). **C** violin and scatter plot and sample traces reporting average measured oEPSC amplitude of each cell, by cell class (Kruskal-Wallis test, $H(2)=6.135$, $P=0.0465$; n = cells (animals)); red lines represent light stimuli, 1ms; scalebars, 100ms, 100pA). **D** violin and scatter plot and sample traces of average measured oIPSC amplitude of each cell, by cell class ($H(2)=6.182$, $P=0.0455$). **E** violin and scatter plot of the ratio of oEPSC and oIPSC peak amplitude of each cell where both are measured and $\neq 0$, per cell class ($H(2)=3.305$, $P=0.1916$). **F** (top) sample traces and plot representing the amplitude of post-synaptic currents evoked by lightly-driven release of neurotransmitter from Nif axonal terminals in HVC; oEPSCs amplitudes are reported before (HVC_X cyan, HVC_{RA} magenta, HVC_{Av} yellow) and after bath application of TTX (black) and 4AP (grey, green outline indicates polysynaptic oEPSC, see methods), (n = cells (animals)); blue lines represent light stimuli, 1ms; scalebars, 100ms, 100pA) (bottom) bar charts representing the likelihood of observing polysynaptic oEPSCs in HVC_X (cyan), HVC_{RA} (magenta) or HVC_{Av} (yellow) (data from panel B), and (grey) likelihood of a subset of the corresponding oEPSCs to be monosynaptic. **G** Sample images reporting retrogradely labeled HVC-projecting neurons in Nif (cyan, white circles) together with in-situ labeling of glutamatergic (SLC17A6, yellow, white arrowheads) and GABAergic (GAD1, magenta, gray arrowheads) markers transcripts (scalebar 200µm, inset 20µm).



Suppl. Fig 2 (related to Fig. 3).

A) Table reporting descriptive statistics relative to oEPSCs and oIPSCs as reported in **Fig. 3**. **B**) violin and scatter plots representing oPSC rise latencies reported in **fig. 3** (gray line marks the minimal latency (1_{st} quartile) to oPSC rise across the cell types, for ease of comparison (Mixed-effects analysis, oEPSC vs. oIPSC $F(1,23)=46.55$, $P<0.001$ HVC_X $P<0.001$, HVC_{RA} $P=0.0167$, HVC_{AV} $P<0.001$; HVC_{PN} $F(2,66)=3.358$, $P=0.0408$, oEPSCs:HVC_{RA} vs HVC_X $P=0.0029$, HVC_{RA} vs HVC_{AV} $P=0.0157$); **C**) oPSCs rise latency for cells where both oEPSC and oIPSC were measured (2W ANOVA, oEPSC vs. oIPSC $F(1,23)=41.60$, $P<0.001$, HVC_X $P<0.001$, HVC_{AV} $P<0.001$); **D**) relative delay of oIPSC compared to oEPSC across the cells reported in **C**) (Kruskal-Wallis test, $H=8.809$, $P=0.0122$).

thalamo-cortical loops in mammals. Although the synaptic connectivity between mMAN and HVC has not been well studied, lesions of mMAN in juvenile zebra finches result in song imitation deficits and lesions in adult Bengalese finches increase song syntax variability, suggesting a role in selection and planning of vocal motor sequencing^{41,65}. Av is one of the more recently described song related regions and unlike Uva, Nif, and mMAN, it is reciprocally connected to HVC. The role of Av is still poorly explored, but it is understood to be embedded in a higher order auditory portion of the DVR and thought to be an important node in the circuit comparing efferent copies of motor commands from HVC to auditory feedback^{35,39}.

While evaluating connectivity between HVC, mMAN, and Av we discovered that mMAN also provides a strong projection to Av (**Fig. 4**). Approximately half of the identified projection neurons in mMAN project to Av and our tracing experiments reveal that there are only partially overlapping classes of projection neurons in mMAN: mMAN_{HVC} PNs, mMAN_{Av} PNs, and mMAN_{HVC/Av} PNs. This newly identified pathway appears positioned to provide the auditory system with information about song motor commands via a DVR-thalamo-DVR loop (RA > DMP > mMAN > Av). It also opens the potential for synaptic loops relaying through HVC between these regions.

Brain regions nearby mMAN do not send projections to HVC and we were able to achieve robust expression of eGtACR1 in mMAN (**Fig. 5A**), allowing us to selectively examine the synaptic connectivity between mMAN and HVC-PNs. Optical stimulation of mMAN axon terminals in brain slices revealed glutamatergic oEPSCs in all retrogradely identified projection neuron classes, albeit with significantly lower success rate compared to what we observed when stimulating Nif terminals (48.15% probability; mMAN vs. Nif oEPSC probability: Fisher's exact test $P < 0.001$). In addition, we found that oEPSCs were more likely evoked in recordings from HVC_X and HVC_{Av} than from HVC_{RA} neurons (**Fig. 5B**, **Suppl. Fig. 3**).

Optogenetic stimulation of mMAN axon terminals also revealed GABAergic currents in all three HVC-PN classes (48.24% probability). Similar to Nif and Uva inputs, oIPSCs elicited by mMAN axons were most often found in cells where oEPSCs were also observed (oIPSC contingent with oEPSC: HVC_X: 93.5%, HVC_{RA}: 96.4%, HVC_{Av}: 90.9%; **Fig. 5B**).

While oEPSCs amplitudes across HVC-PN classes were statistically indistinguishable (**Fig. 5C**), we found that oIPSCs in HVC_{Av} had significantly higher amplitude than those evoked in HVC_{RA} neurons (**Fig. 5D**, **Suppl. Fig. 3**). Despite this difference, when computing the E/I ratio we didn't see any significant difference across HVC-PNs, and like the other inputs examined, GABAergic currents had higher amplitude than glutamatergic currents (**Fig. 5E**, **Suppl. Fig. 3**).

We next asked whether mMAN monosynaptically contacted any class of HVC-PNs. We found evidence that mMAN monosynaptically contacts all three classes of HVC PNs. The most reliable monosynaptic transmission is between mMAN and HVC_{Av} neurons and HVC_X neurons (HVC_{RA}: 2/5, HVC_X: 5/7, HVC_{Av}: 6/6, **Fig. 5F**). We did not observe monosynaptic oIPSCs (HVC_X: 0/5, HVC_{RA}: 0/5, HVC_{Av}: 0/6, data not shown), and confirmed that mMAN neurons projecting to HVC are glutamatergic using in-situ hybridization combined with retrograde tracing from injection of tracer in HVC (**Suppl. Fig. 3**). Together, this data indicates that mMAN predominantly provides robust monosynaptic excitatory input to HVC_{Av} and HVC_X neurons. HVC_{Av} neurons only comprise ~3% of the neurons in HVC²⁷. Therefore, finding that 100% of the HVC_{Av} neurons tested receive monosynaptic input from mMAN suggests that this is likely to be a highly selective synaptic target of mMAN inputs to HVC. Given that we have now also identified a projection from mMAN directly to Av, this supports a model in which mMAN is providing Av song related signals directly and indirectly via projections onto HVC_{Av} neurons.

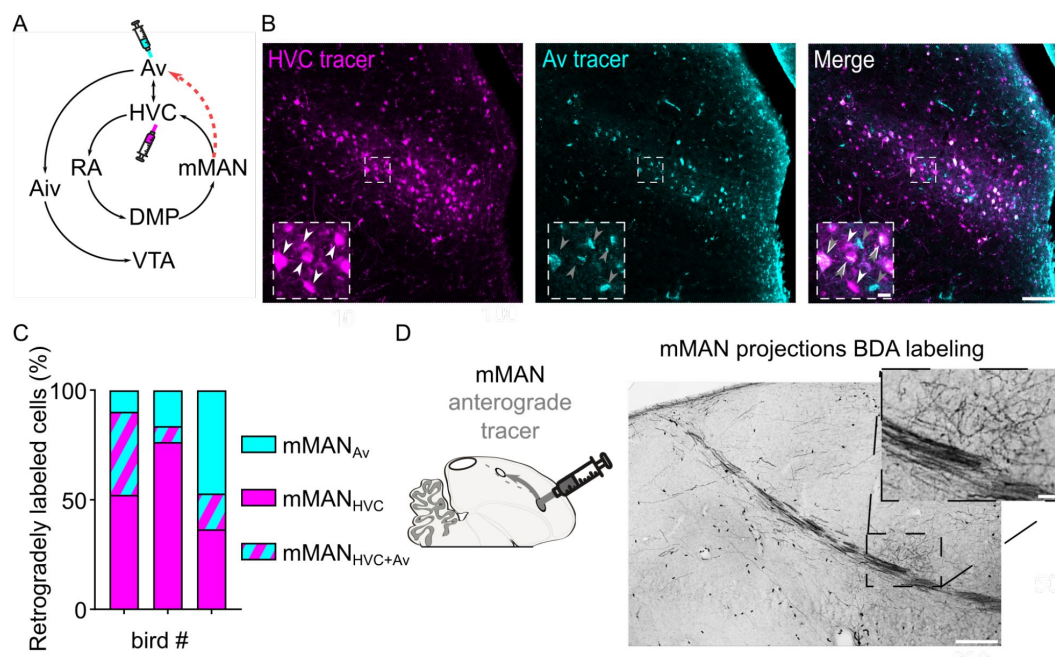


Fig. 4

Identification of mMAN neuronal subpopulations projecting to Av and to HVC.

A) Schematic of the known mMAN afferent and efferent circuitry, together with the proposed new connection towards Av (red dashed arrow), and schematized injection of retrograde tracers in HVC and Av. **B**) sample images of retrogradely labeled mMAN cells projecting to HVC (magenta) or to Av (cyan), and merged image. Insets report magnified selection (dashed square box in the images) illustrating the potential 3 subpopulations identified: mMAN_{HVC} (white arrowheads), mMAN_{Av} (grey arrowheads) and mMAN_{HVC+Av} (overimposed white and grey arrowheads) (scalebars: 100μm, inset: 10μm). **C**) Bar chart displaying the number of retrogradely mMAN_{HVC}, mMAN_{Av}, and mMAN_{HVC+Av} labeled cells, in 3 birds (averaged across hemispheres, 2-6 slices/bird). **D**) BDA labeling of anterograde projection to Av from mMAN, inset magnifies the terminal field in Av (scalebars: 200μm, inset: 50μm).

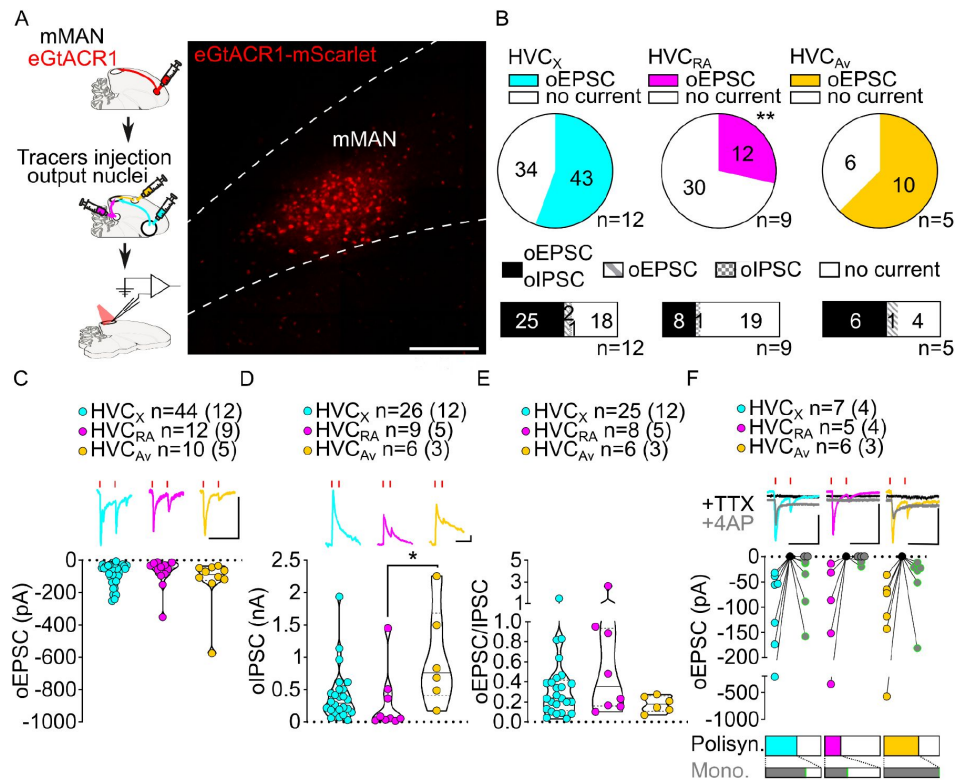
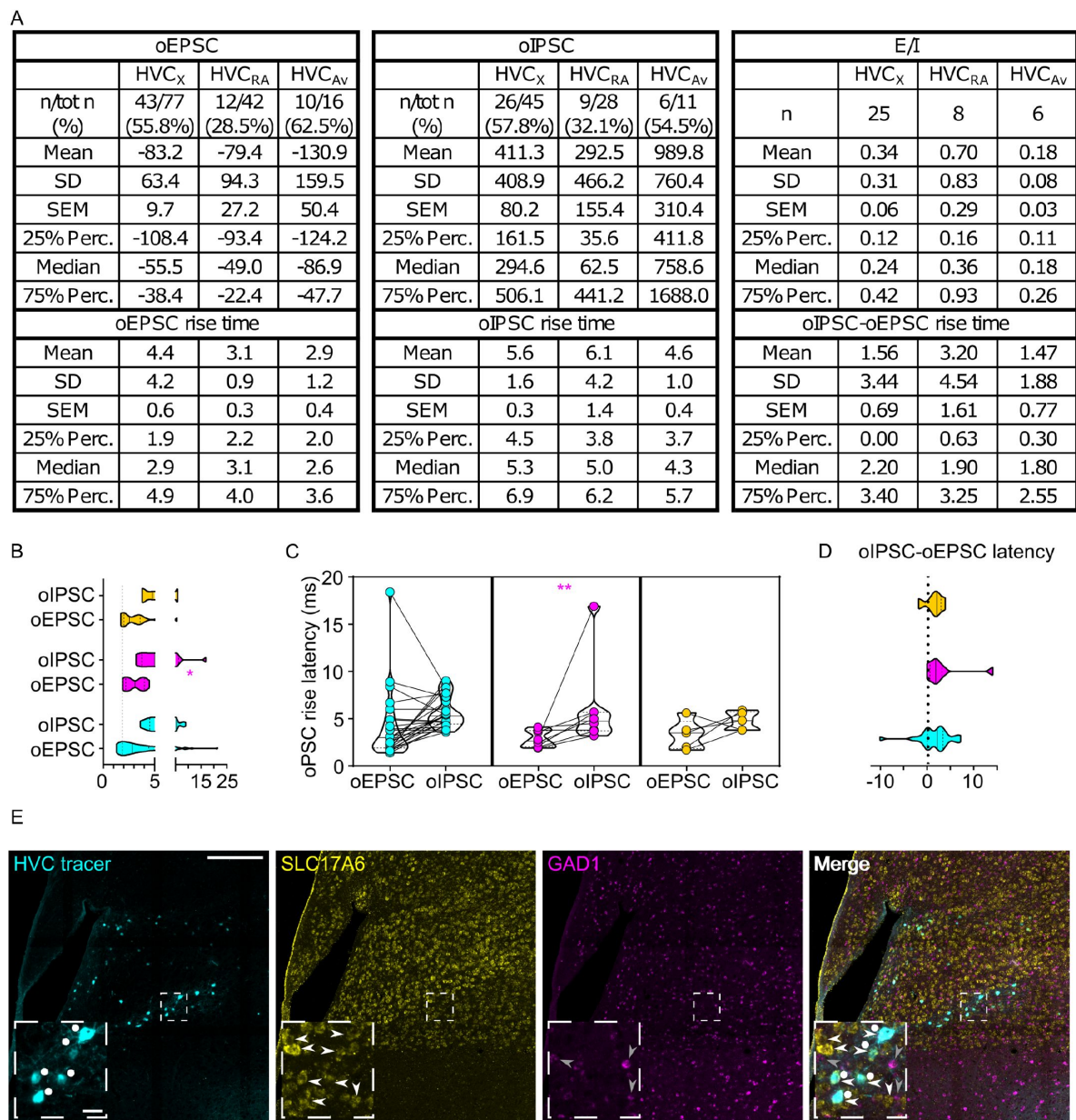


Fig. 5

mMAN inputs elicit polysynaptic oIPSCs and oEPSCs in all three HVC-PN classes and monosynaptically excite HVC_X and HVC_{Av} neurons.

A (left) Schematic of the experimental timeline, illustrating injection of AAV-eGtACR1 in MMAN, followed by retrograde tracer injections in HVC efferent areas and whole-cell patch-clamp recording in acute brain slices; (right) sample image of eGtACR1-mScarlet expression in MMAN (scalebar 200μm). **B** (top) pie charts representing the likelihood of observing oEPSCs in HVC_X (cyan), HVC_{RA} (magenta) or HVC_{Av} (yellow) (Fisher's exact test, $P=0.0088$). Numbers in the pie fragments represent the number of cells in which current (colored) or no current (white) was found. Numbers next to the pie charts represent the number of animals from which the data is obtained. (bottom) bar chart representing the number of cells where both oEPSCs and oIPSCs could be elicited (black), only oEPSCs but no oIPSCs (grey lines), only oIPSCs but no oEPSCs (grey checkers), or neither (white), for subsets of cells from the HVC-PN classes' pie charts aligned above (Fisher's exact test, $P=0.1054$). **C** violin and scatter plot and sample traces reporting average measured oEPSC amplitude of each cell, by cell class (Kruskal-Wallis test $H(2)=2.659$, $P=0.2646$; n = cells (animals); red lines represent light stimuli, 1ms; scalebars, 100ms, 100pA). **D** violin and scatter plot and sample traces of average measured oIPSC amplitude of each cell, by cell class ($H(2)=8.598$, $P=0.0136$, HVC_{RA} vs. HVC_{Av} $P=0.0102$). **E** violin and scatter plot of the ratio of oEPSC and oIPSC peak amplitude of each cell where both are measured and $\neq 0$, per cell class ($H(2)=2.443$, $P=0.2759$). **F** (top) sample traces and plot representing the amplitude of post-synaptic currents evoked by lightly-driven release of neurotransmitter from MMAN axonal terminals in HVC; oEPSCs amplitudes are reported before (HVC_X cyan, HVC_{RA} magenta, HVC_{Av} yellow) and after bath application of TTX (black) and 4AP (grey, green outline indicates polysynaptic oEPSC, see methods), (n = cells (animals); blue lines represent light stimuli, 1ms; scalebars, 100ms, 100pA) (bottom) bar charts representing the likelihood of observing polysynaptic oEPSCs in HVC_X (cyan), HVC_{RA} (magenta) or HVC_{Av} (yellow) (data from panel B), and (grey) likelihood of a subset of the corresponding oEPSCs to be monosynaptic.



Suppl. Fig 3 (related to Fig. 5).

A) Table reporting descriptive statistics relative to oEPSCs and oIPSCs as reported in [Fig. 5](#). **B**) violin and scatter plots representing oPSC rise latencies reported in [fig. 5](#) (gray line marks the minimal latency (1_{st} quartile) to oPSC rise across the cell types, for ease of comparison (Mixed-effects analysis, oEPSC vs. oIPSC $F(1,35)=8.439$, $P=0.0063$, HVC_{RA} $P=0.0198$; HVC_{PN} $F(2,64)=0.7545$, $P=0.4744$); **C**) oPSCs rise latency for cells where both oEPSC and oIPSC were measured (2W ANOVA, oEPSC vs. oIPSC $F(1,35)=8.294$, $P=0.0067$, HVC_{RA} $P=0.0470$); **D**) relative delay of oIPSC compared to oEPSC across the cells reported in **C**) (Kruskal-Wallis test, $H=0.2028$, $P=0.9036$). **E**) Sample images reporting retrogradely labeled HVC-projecting neurons in mMAN (cyan, white circles) together with in-situ labeling of glutamatergic (SLC17A6, yellow, white arrowheads) and GABAergic (GAD1, magenta, gray arrowheads) markers transcripts (scalebar 200µm, inset 20µm).

Lastly, we mapped the synaptic inputs from Av onto HVC-PNs. **6A**). Avalanche lacks clear anatomical boundaries, and our viral expression did spread into surrounding brain regions, but only brain hemispheres in which we could verify a lack of expression in Nif were included in this study (**Fig. 6A**). Whole-cell patch-clamp recordings from identified HVC-PN classes in brain slices revealed glutamatergic oEPSCs in all 3 cell classes, yet most of the cells were not excited by Av terminals (39.18% probability; Av vs. Nif oEPSC probability: Fisher's exact test $P < 0.001$). However, the probability of evoking oEPSCs was similar among HVC-PN classes (**Fig. 6B**, **Suppl. Fig. 4**). We tested the presence of oIPSCs in a subset of cells and found GABAergic currents in all three projection subtypes (43.24% probability, **Suppl. Fig. 4**). As in other pathways already described, we observed oIPSCs mainly in HVC-PNs also showing oEPSCs (oIPSC contingent with oEPSC: HVC_X 100.0% HVC_{RA} 90.0%, HVC_{AV} 84.6%; **Fig. 6B**).

While oEPSCs amplitudes were broadly similar among HVC-PN classes (**Fig. 6C**), oIPSCs had significantly smaller amplitude in HVC_{RA} compared to those evoked in HVC_X neurons (**Fig. 6D**, **Suppl. Fig. 4**). Consistently, this was also reflected in a significantly higher E/I ratio in HVC_{RA} compared to HVC_X. Similar to the other afferents, Av axon terminal stimulation elicited stronger GABAergic currents than glutamatergic currents across HVC-PN classes (**Fig. 6E**, **Suppl. Fig. 4**).

When examining which HVC-PN neuron classes received monosynaptic input from Av, we consistently observed monosynaptic transmission onto HVC_X neurons (HVC_X 4/5 cells monosynaptically contacted). Monosynaptic transmission onto HVC_{RA} and HVC_{AV} was less common (HVC_{RA} 2/5, HVC_{AV} 2/6; **Fig. 6F**). Previous reports indicated that Av neurons projecting to HVC are excitatory²⁷. Consistent with this, we didn't observe any monosynaptic oIPSC across HVC-PNs (monosynaptic oIPSCs: HVC_X 0/4, HVC_{RA} 0/3, HVC_{AV} 0/3, data not shown) and further confirmed that Av_{HVC} neurons are glutamatergic by in-situ hybridization labeling of retrogradely identified neurons (**Suppl. Fig. 4**). Our data shows that Av provides monosynaptic excitatory input to HVC_X neurons with high frequency and makes monosynaptic connections with HVC_{RA} and HVC_{AV} neurons less frequently.

Taken together, this research provides a rich dataset mapping the polysynaptic and monosynaptic connectivity of the input and output pathways of HVC - the best studied portions of the avian song circuitry – as well as a newly discovered pathway between DVR circuits that are important for vocal learning (**Fig. 7**).

Discussion

Speech and language are controlled by interconnected neocortical and subcortical networks, including dorsal and ventral cortical pathways for articulation and lexical processing, and pathways looping through the thalamus, basal ganglia, and cerebellum^{6,66-68}. The synaptic connectivity between specific populations of neurons among these circuits is going to be difficult to map. Yet, this will ultimately be necessary for understanding the neuroanatomical basis and circuit computations associated with speech and language. Although the avian DVR and mammalian neocortex have distinct developmental origins (ventral and dorsal pallium, respectively), HVC PN neurons have similar gene expression and connectivity patterns to the intratelencephalic neurons described in layers 2-6 of the mammalian neocortex³², suggesting evolutionary convergence in circuit computations needed for complex behaviors like vocal imitation. Thus, there is much to be gained by mapping the connectivity between the specific populations of neurons known to be essential in the sensory and sensorimotor process of song imitation.

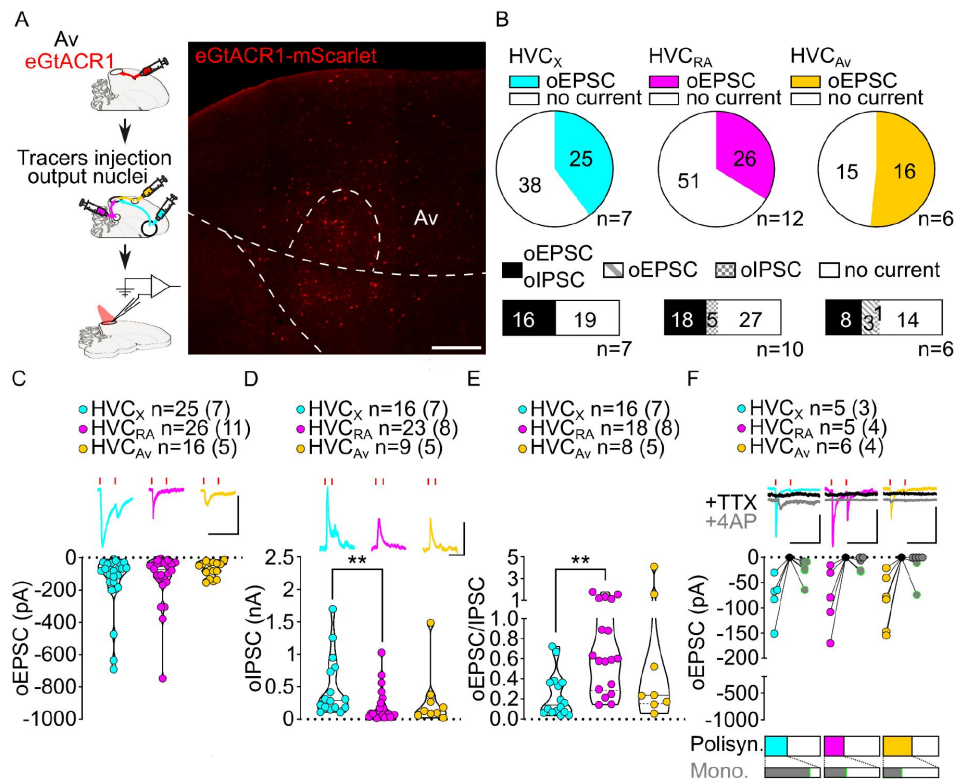
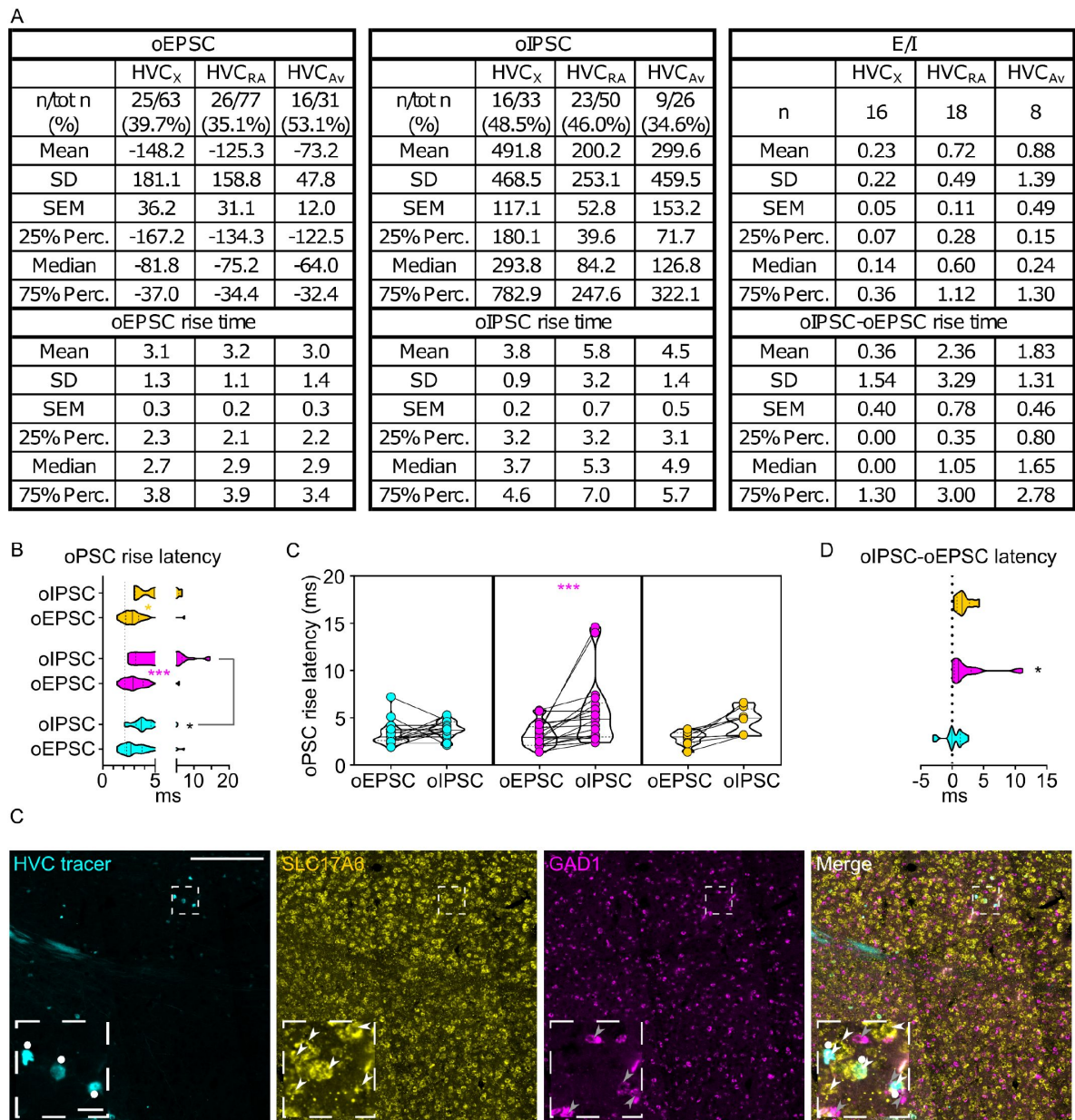


Fig. 6

Av inputs elicit polysynaptic oIPSCs and oEPSCs in all three HVC-PN classes and monosynaptically excite mainly HVC_x neurons.

A (left) Schematic of the experimental timeline, illustrating injection of AAV-eGtACR1 in Av, followed by retrograde tracer injections in HVC efferent areas and whole-cell patch-clamp recording in acute brain slices; (right) sample image of eGtACR1-mScarlet expression in Av (scalebar 200 μm). **B** (top) pie charts representing the likelihood of observing oEPSCs in HVC_x (cyan), HVC_{RA} (magenta) or HVC_{Av} (yellow) (Fisher's exact test, $P=0.2243$). Numbers in the pie fragments represent the number of cells in which current (colored) or no current (white) was found. Numbers next to the pie charts represent the number of animals from which the data is obtained. (bottom) bar chart representing the number of cells where both oEPSCs and oIPSCs could be elicited (black), only oEPSCs but no oIPSCs (grey lines), only oIPSCs but no oEPSCs (grey checkers), or neither (white), for subsets of cells from the HVC-PN classes' pie charts aligned above (Fisher's exact test, $P=0.0550$). **C** violin and scatter plot and sample traces reporting average measured oEPSC amplitude of each cell, by cell class (Kruskal-Wallis test $H(2)=1.103$, $P=0.5760$; n = cells (animals); red lines represent light stimuli, 1 ms; scalebars, 100 ms, 100 pA). **D** violin and scatter plot and sample traces of average measured oIPSC amplitude of each cell, by cell class ($H(2)=10.09$, $P=0.0064$, HVC_x vs. HVC_{RA} $P=0.0047$). **E** violin and scatter plot of the ratio of oEPSC and oIPSC peak amplitude of each cell where both are measured and ≠ 0, per cell class ($H(2)=10.64$, $P=0.0049$, HVC_x vs. HVC_{RA} $P=0.0033$). **F** (top) sample traces and plot representing the amplitude of post-synaptic currents evoked by lightly-driven release of neurotransmitter from Av axonal terminals in HVC; oEPSCs amplitudes are reported before (HVC_x cyan, HVC_{RA} magenta, HVC_{Av} yellow) and after bath application of TTX (black) and 4AP (grey, green outline indicates polysynaptic oEPSC, see methods), (n = cells (animals); blue lines represent light stimuli, 1 ms; scalebars, 100 ms, 100 pA) (bottom) bar charts representing the likelihood of observing polysynaptic oEPSCs in HVC_x (cyan), HVC_{RA} (magenta) or HVC_{Av} (yellow) (data from panel B), and (grey) likelihood of a subset of the corresponding oEPSCs to be monosynaptic.



Suppl. Fig. 4 (related to Fig. 6).

A) Table reporting descriptive statistics relative to oEPSCs and oIPSCs as reported in **Fig. 6**. **B**) violin and scatter plots representing oPSC rise latencies reported in **fig. 6** (gray line marks the minimal latency (1_{st} quartile) to oPSC rise across the cell types, for ease of comparison (Mixed-effects analysis, oEPSC vs. oIPSC $F(1,39)=21.78$, $P<0.001$, HVC_{RA} $P=0.0377$; HVC_{PN} $F(2,70)=2.764$, $P=0.0699$); **C**) oPSCs rise latency for cells where both oEPSC and oIPSC were measured (2W ANOVA, oEPSC vs. oIPSC $F(1,39)=14.26$, $P<0.001$, HVC_{RA} $P<0.001$); **D**) relative delay of oIPSC compared to oEPSC across the cells reported in **C**) (Kruskal-Wallis test, $H=6.403$, $P=0.0407$). **E**) Sample images reporting retrogradely labeled HVC-projecting neurons in Av (cyan, white circles) together with in-situ labeling of glutamatergic (SLC17A6, yellow, white arrowheads) and GABAergic (GAD1, magenta, gray arrowheads) markers transcripts (scalebar 200µm, inset 20µm).

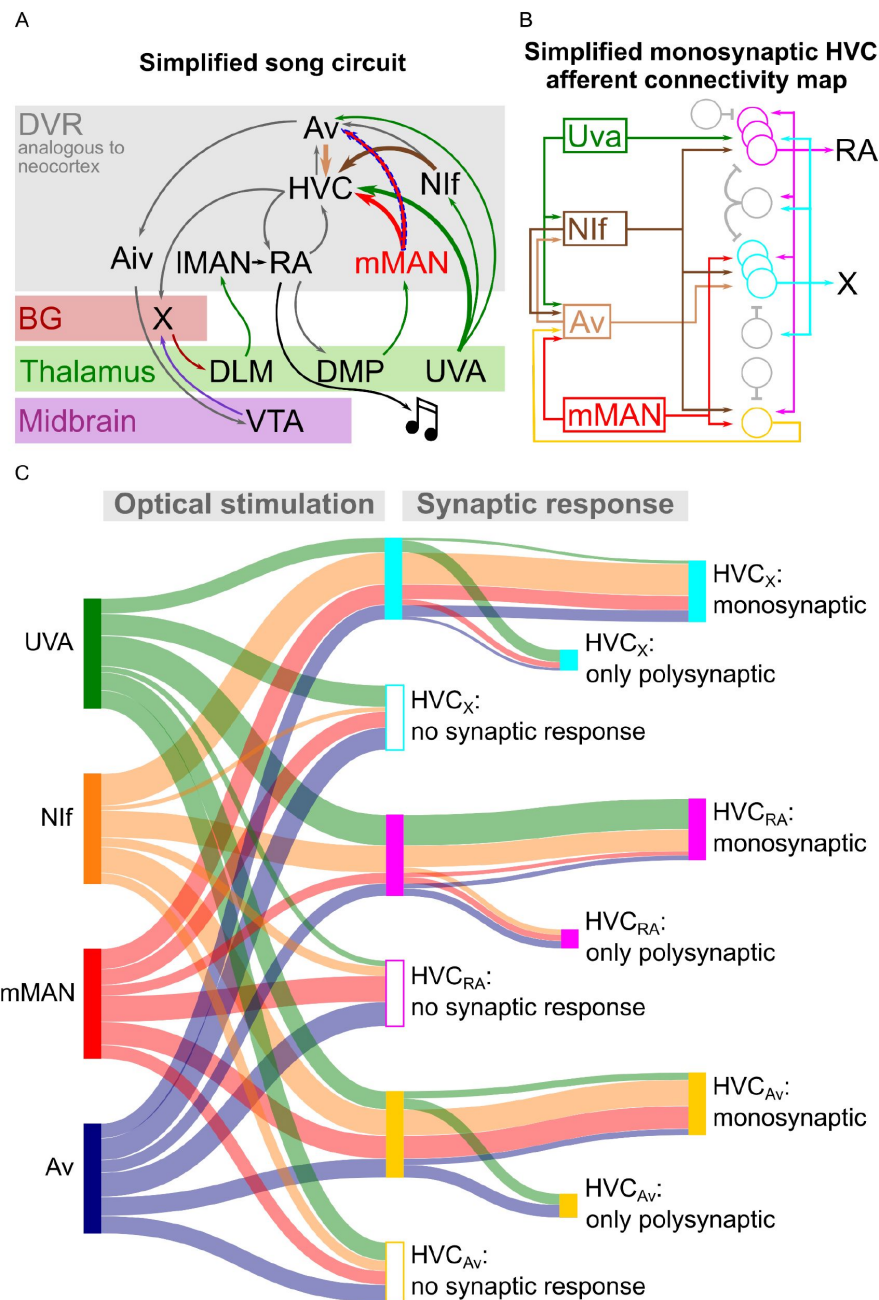


Fig. 7

Song circuitry summary and monosynaptic connectivity map of afferents to HVC-PNs.

A) Schematic representing a simplified version of the long-range connectivity map for song. Colors represent the anatomical location of each projection: gray: DVR, red: basal ganglia, green: thalamus, purple: midbrain. HVC afferents described in this manuscript are highlighted by large arrows. The novel mMAN-Av projection is highlighted by a dashed outline. **B)** Schematic of the HVC afferent connectivity map resulting from the present work, complemented with projections between HVC afferent areas (on the left) and between HVC projection neurons as per previous reports (on the right). For conceptualization purposes, afferent connectivity to HVC-PNs is shown only when the rate of monosynaptic connectivity reaches 50% of neurons examined: Nif monosynaptically contacts all 3 HVC-PNs, while Uva is preferentially monosynaptically connected to HVC_{RA}, mMAN to HVC_X and HVC_{AV}, and Av to HVC_X. **C)** Sankey diagram displaying the prevalence of connectivity for each input and cell subtype combination, based on polysynaptic and monosynaptic connectivity rates described in **figures 2-3** and **5-6**.

Using large-scale, multi-pathway, and long-range functional synaptic circuit mapping, we report cell type specific synaptic connectivity across forebrain sensorimotor networks necessary for learning and producing learned vocalizations. The songbird motor nucleus HVC is crucial for song production. Its partial or complete lesion results in song disruption and permanent loss, respectively^{28,69,70}. Electrical stimulation of HVC can halt song⁷¹⁻⁷³. Further, HVC is essential in juvenile song learning^{27,36,46,74,75}. We show that long-range sensory pathways to HVC are excitatory and contact HVC intratelencephalic PNs. We find that these connections are not stochastic but are instead strongly biased to make synapses with premotor (HVC_{RA}), auditory (HVC_{AV}), or basal ganglia (HVC_X) projection pathways. Lastly, we find a remarkable correspondence in the probability of finding oEPSCs and oIPSCs in the same postsynaptic neurons, indicative of highly interconnected cell assemblies at postsynaptic targets of long-range connections within the DVR.

In addition to finding widespread polysynaptic transmission upon stimulation of any of the afferents, we endeavored to reveal the wiring diagram of HVC inputs by isolating the monosynaptic components of these currents (**Fig. 7**). We found surprisingly specific and compartmentalized monosynaptic neurotransmission between each input and HVC-PN classes. Nif terminals monosynaptically contacted all three HVC-PN classes. Uva predominantly makes monosynaptic connections with HVC_{RA} neurons, while mMAN most frequently makes monosynaptic connections with HVC_{AV} and HVC_X PNs but appears to only sparsely project to HVC_{RA} neurons. Lastly, Av predominantly makes monosynaptic connections with HVC_X neurons and connects less frequently with HVC_{RA} and HVC_{AV} neurons. When combined with our discovery of a new pathway from mMAN to Av, our data draws a scenario of multiple intermingled loops coexisting across HVC input and output circuits.

Thalamic inputs from Uva may directly affect the song motor pathway through monosynaptic projections onto HVC_{RA} neurons. The potential relevance of this specific connection has been recently studied^{29,45}. Additionally, the seldom yet significant direct inputs to HVC_{AV} neurons we identify are interesting considering the proposed role of HVC_{AV} neurons in providing a forward model of song to the auditory system²⁷. Uva also is known to project to both Nif and Av. Uva's monosynaptic connections to classes of HVC PNs may therefore directly integrate with propagation of forward models of song timing to the auditory system, particularly during learning of syllable transitions²⁷.

mMAN has recently been found to contribute to the ordering of song syllables⁷⁶. This could be attributable to the strong connections identified here between mMAN and HVC_{AV} PNs and from mMAN directly to Av. HVC_{AV} neurons are important for learning the timing of elements within song. Lesions of HVC_{AV} neurons in juvenile birds disrupts learning of syllable syntax yet does not have a strong effect on learning the spectral features of syllables²⁷. Further, lesions block deafening-induced degradation of songs' temporal features and recovery of song timing following song-contingent feedback perturbations²⁷. Thus, our analysis of synaptic connectivity provides important anatomical connections for a circuit critical for temporal ordering of vocalizations during learning and in adulthood.

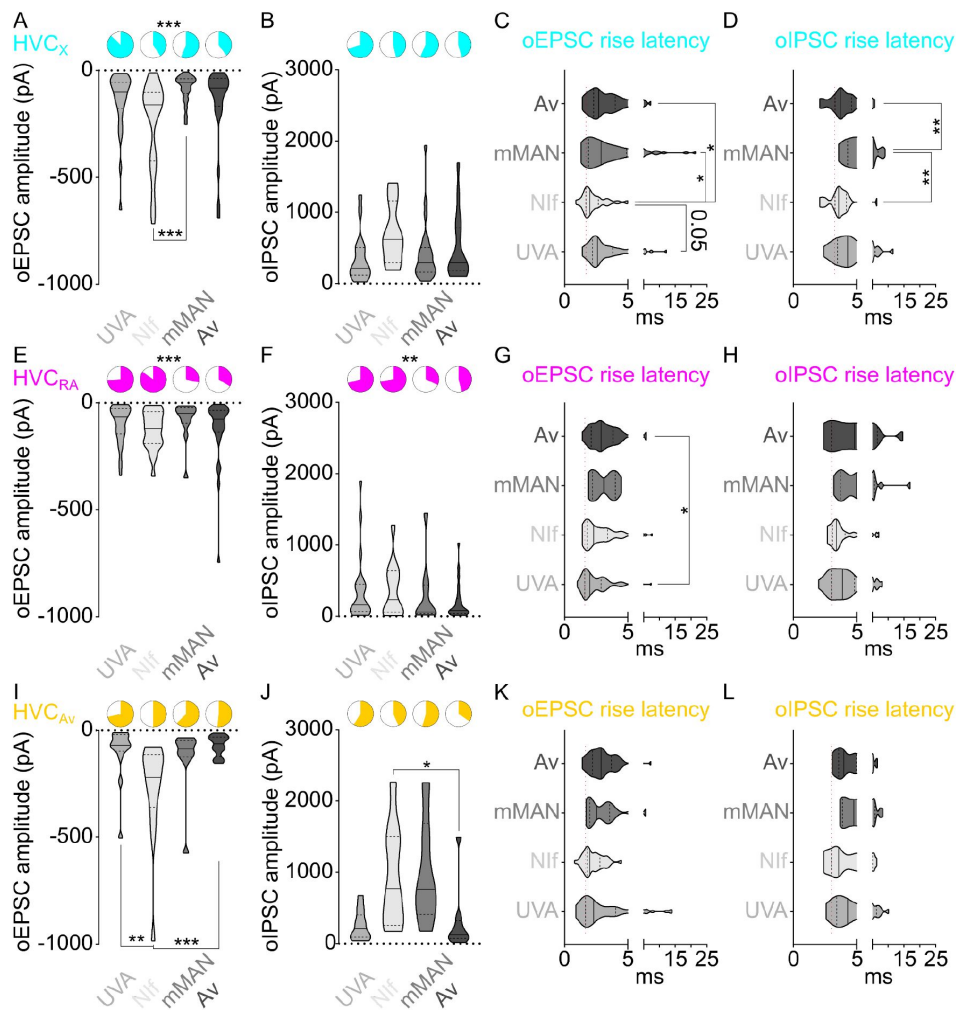
mMAN and Av's preferential connection to HVC_X PNs may create a bridge linking the thalamus, the auditory system, and the basal ganglia circuits important for song plasticity. mMAN receives projections from the nucleus DMP of the thalamus, which itself receives projections from RA. mMAN is therefore positioned to receive a copy of descending vocal motor commands transmitted through DMP while Av may relay integrated information about auditory feedback and their correspondence with motor commands for song that it receives from mMAN and from HVC_{AV} neurons. Our findings place mMAN and Av in an ideal position to relay those signals to Area X, potentially contributing to song plasticity.

In the data shown in **Figs. 2**, **3**, **5**-**6** we examined the probability and strength of oPSCs within each HVC-PNs class. We found that projection neurons in Nif, Uva, mMAN and Av consistently establish polysynaptic contact with all three HVC-PN classes. We reliably observed both glutamatergic and GABAergic polysynaptic neurotransmission upon afferent axonal terminal stimulation. Organizing our data by HVC PN types (**Suppl. Fig. 5**) reveals a bias in afferent inputs onto these different neuronal pathways. We find that HVC_X and HVC_{AV} neurons are most frequently excited by Nif afferents, while HVC_{RA} are most frequently excited by Uva, and unlikely to be excited by mMAN terminal stimulation. HVC_X and HVC_{AV} neurons are most strongly driven by Nif, while HVC_{RA} neurons receive relatively uniform amplitude oEPSCs from all 4 input pathways examined. Lastly, stimulation of Nif and mMAN terminals resulted in higher amplitude inhibition onto HVC_{AV} neurons than upon Uva or Av terminals stimulation (**Suppl. Fig. 5**).

This data points towards the existence of a rich interneuronal inhibitory network that is recruited by stimulating HVC's afferents, evoking GABAergic transmission onto all HVC-PN classes. This is in line with previous reports that found HVC-PNs activity to be tightly regulated by interneurons^{77,79}. Interestingly, across all inputs studied, the likelihood of finding oIPSCs was tightly dependent on finding oEPSCs in the same cell. Previous studies have indicated that HVC PN are disynaptically inhibited by other HVC-PNs, a feature of synaptic connectivity that is likely fundamental to the propagation of excitation in the network during song production and in the song learning process^{77,79}. When afferents cause excitation of HVC PN this would therefore cause an immediate rise in the levels of disynaptic inhibition in the surrounding HVC PN. Similar feedback inhibition dynamics have been extensively reported in cortical circuits, where this mechanism serves a crucial role of controlling the excitatory-inhibitory balance of local principal neurons⁸⁰⁻⁸³. Afferents that excite HVC PN may also contact neighboring interneurons. A similar feedforward inhibition has also been reported in the cortex, where both pyramidal neurons and interneurons projecting to them are recruited by long-range pathways^{84,85}. While feedback inhibition is poised to monitor and modulate the output of local principal neurons, feedforward inhibition is proposed to work as a coincidence detector improving the sensitivity of the network by filtering asynchronous inputs^{80,86-90}.

Distinguishing among these potential feedback and feedforward circuit motifs, as well as fully characterizing the role of afferents for HVC local circuit dynamics, will require monosynaptic connectivity mapping of afferents onto HVC interneurons. The ability to do this systematically in HVC is limited by the lack of genetic methods for labeling interneurons⁹¹. Colquitt et al.³² have recently identified multiple GABAergic cell subtypes in HVC, and a molecular handle is needed to experimentally approach the study of their synaptic connectivity. Nonetheless, our data suggests the existence of compartmentalized neuronal ensembles including both PN and interneurons projecting to those PN.

The synaptic organization of excitatory-inhibitory connections in HVC seem to allow the integration of afferent inputs within hyper-local networks or modules. This type of connectivity may make the circuit more robust to perturbations⁹². A potential caveat is that we performed all our recordings in 230µm thick sagittal brain slices and it is thought that HVC-PNs exhibit sequential activity that is organized in the rostrocaudal axis, while interneurons are speculated to be coordinated in the medio-lateral axis⁹³. Our resection of this mediolateral organization may account for the lack of lateral inhibition found in our data. For example, our optogenetic stimulation elicits oIPSCs mostly in the same neurons where oEPSCs are observed, but not in the others, which may have received inhibition from interneurons sitting in adjacent brain slices. Lateral inhibition is normally observed in circuits displaying feedback inhibition, and previous reports indicate that HVC-PNs disynaptically inhibit other HVC-PNs^{77,79}. It is however possible that differences between neocortical and HVC microcircuitry may reduce the significance of lateral inhibition, explaining why we only seldomly observed it.



Suppl. Fig. 5

Cross-comparison of afferent polysynaptic connectivity within cell types (data from Fig. 2, 3, 5, 6).

A) Pie charts representing the likelihood of evoking oEPSCs in HVC_x , and violin plots representing the oEPSCs amplitude, when stimulating afferents from Nif, UVA, mMAN or Av (probability: Fisher's exact test, $P < 0.001$; amplitude: Kruskal-Wallis test, $H(3) = 17.18$, $P < 0.001$, Nif vs. UVA $P = 0.1695$, Nif vs. mMAN, $P < 0.001$, Nif vs. Av $P = 0.0993$). **B)** Same as A) for oIPSCs (probability: Fisher's exact test, $P = 0.2828$; amplitude: Kruskal-Wallis test, $H(3) = 5.008$, $P = 0.1712$). **C)** Violin plots reporting the oEPSC rise latency in HVC_x upon stimulation of the 4 afferents (Kruskal-Wallis test, $H(3) = 11.91$, $P = 0.0077$, Nif vs. UVA $P = 0.0534$, Nif vs. mMAN, $P = 0.0119$, Nif vs. Av $P = 0.0345$). **D)** same as C) for oIPSCs (Kruskal-Wallis test, $H(3) = 19.23$, $P < 0.001$, Nif vs. UVA $P = 0.5428$, Nif vs. mMAN, $P = 0.0016$, Nif vs. Av $P > 0.9999$, mMAN vs. Av $P = 0.0028$).

E-H) Same as A-D) but for HVC_{RA} : **E)** (probability: Fisher's exact test, $P < 0.001$; amplitude: Kruskal-Wallis test, $H(3) = 3.594$, $P = 0.3088$). **F)** (probability: Fisher's exact test, $P = 0.0075$; amplitude: Kruskal-Wallis test, $H(3) = 2.132$, $P = 0.5454$). **G)** (Kruskal-Wallis test, $H(3) = 12.44$, $P = 0.0060$, Nif vs. UVA $P > 0.999$, Nif vs. mMAN, $P = 0.6258$, Nif vs. Av $P = 0.3175$, UVA vs. Av $P = 0.0128$). **H)** (Kruskal-Wallis test, $H(3) = 6.622$, $P = 0.0850$).

I-L) Same as A-D) but for HVC_{av} : **I)** (probability: Fisher's exact test, $P = 0.3150$; amplitude: Kruskal-Wallis test, $H(3) = 18.46$, $P < 0.001$, Nif vs. UVA $P = 0.0016$, Nif vs. mMAN, $P = 0.0929$, Nif vs. Av $P = 0.0010$). **J)** (probability: Fisher's exact test, $P = 0.1869$; amplitude: Kruskal-Wallis test, $H(3) = 12.52$, $P = 0.0058$, Nif vs. UVA $P = 0.1043$, Nif vs. mMAN, $P > 0.999$, Nif vs. Av $P = 0.0405$). **K)** (Kruskal-Wallis test, $H(3) = 6.441$, $P = 0.0920$). **L)** (Kruskal-Wallis test, $H(3) = 7.653$, $P = 0.0538$).

Our measurements of oPSCs onset delays from the light stimulation offer some insight into the possible wiring of afferents and intra-HVC connectivity. HVC_{RA} neurons displayed the fastest oEPSCs latency upon Uva axon terminal stimulation, consistent with their preferential monosynaptic connectivity. Likewise, HVC_X neurons display the fastest latency upon Nif axon terminal stimulation. However, cytoarchitecture, axonal transmission latency and synaptic delays may all play roles in determining this timing and other monosynaptic connections are not readily reflected through polysynaptic oPSCs latency measurements. This highlights the importance of using opsin-assisted monosynaptic mapping rather than timing to identify the details of synaptic connectivity.

While our opsin-assisted circuit mapping provides us with a new level of insight into HVC synaptic circuitry, there are limitations to this research that should be considered. All circuit mapping in this study was carried out in brain slices from adult male zebra finches. Future studies will be needed to examine how this adult connectivity pattern relates to patterns of connectivity in juveniles during sensory or sensorimotor phases of vocal learning and connectivity patterns in female birds. Ex-vivo brain slices have historically been demonstrated to be a reliable window into neuronal and circuit function. However intrinsic activity patterns of some neuronal subtypes are known to change in acute slices⁹⁴. Moreover, the slicing procedure severs both neuromodulatory and neurotransmitter afferents, potentially attenuating or removing synaptic inputs to some circuits⁹⁵. Our axonal terminal optical manipulation recruits all the fibers expressing eGtACR1 under the cone of light of the microscope objective. Therefore, our estimates of postsynaptic responses and likelihood of finding responses are based on the simultaneous release of neurotransmitter from all the afferent terminals expressing eGtACR1, and they should be understood in this context. Future studies will need to employ conditions of minimal stimulation that may further characterize the specific properties of monosynaptic and polysynaptic neurotransmission in the examined inputs. Lastly, the amplitude and likelihood of recording oPSCs directly depends on the expression rate and tropism of our viruses, and this may differ across the 4 afferent pathways studied here. We report low levels of viral expression in Av, which may skew our results. However, by testing oPSCs in all three HVC_{PN} classes in each bird helps ensure that the relative contribution Av afferent inputs onto different classes of HVC neurons is not simply an artifact associated with efficiency of viral transduction.

Although a complete description of HVC circuitry will require the examination of other potential inputs (i.e. RA_{HVC} PNs, NCM, A11 glutamatergic neurons^{40,96}) and a characterization of interneuron synaptic connectivity, here we provide a map of the synaptic connections between the 4 best described afferents to HVC and its 3 populations of projection neurons in adult birds. These results provide a view into the synaptic underpinnings of circuits fundamental for the learning and production of vocalizations. Moreover, they reveal essential connectivity patterns that can underlie song motor control and learning of syllable order. This picture of input-output organization spanning sensory, thalamic, and premotor areas may thus offer insights into the circuits for human language learning and production. Lastly, the opsin-assisted circuit mapping strategy we used provides an approach to fully characterize other long range and local circuitry in the songbird brain, which will be critical for understanding and modelling the remarkable vocal imitation abilities of songbirds.

Materials and Methods

Animals

Experiments described in this study were conducted using adult male zebra finches (130-500 days post hatch). All procedures were performed in accordance with protocols approved by Animal Care and Use Committee at UT Southwestern Medical Center.

Viral Vectors

The following adeno-associated viral vectors were used in these experiments: rAAV2/9/CBh-FLP and rAAV-CBh-eGtACR1-mScarlet (IDDRC Neuroconnectivity Core at Baylor College of Medicine or UT Southwestern AAV Viral Vector Core). All viral vectors were aliquoted and stored at -80°C until use.

Constructs

CBh-eGtACR1-mScarlet was generated by inserting KpnI and AgeI restriction sites flanking EF1a in the pAAV-EF1a-F-FLEX-mNaChBac-T2A-tdTomato (a gift from Dr. Massimo Scanziani; Addgene plasmid # 60658) through pcr cloning. The CBh promoter was isolated from pX330-U6-Chimeric_BB-CBh-hSpCas9 (a gift from Dr. Feng Zhang; Addgene plasmid # 42230) by digestion of the KpnI and AgeI restriction sites and inserted into the KpnI/AgeI restriction sites of the pAAV-EF1a-F-FLEX-mNaChBac-T2A-tdTomato backbone. Next, we isolated mScarlet from pmScarlet_C1 (a gift from Dr. Dorus Gadella; Addgene plasmid # 85042) through pcr amplification, inserting a BamHI restriction site into the 5' end of the ORF. We digested the amplified fragments with BamHI and BsrGI, inserting the digested fragments into BamHI/BsrGI restriction sites of the pAAV-CBh-F-FLEX-mNaChBac-T2A-tdTomato backbone. eGtACR1 was then amplified from pAAV-CAG-DIO-NLS-mRuby3-IRES-eGtACR1-ST (a gift from Dr. Hillel Adesnik; Addgene plasmid # 109048) with a BmtI restriction site on the 5' end and a AflII restriction site on the 3' end, avoiding amplification of the soma-targeting sequence in the original plasmid. We then digested the amplified fragment with BmtI and AflII, inserting the fragment into the BmtI/AflII sites of the pAAV-CBh-F-FLEX-mNaChBac-T2A-mScarlet.

For CBh-Flpo, we amplified the FLP ORF from pCAG-Flpo (a gift from Dr. Massimo Scanziani; Addgene plasmid # 60662) with a AgeI restriction site on the 5' end. We then digested the amplified fragment with AgeI and EcoRI, inserting the fragment into the AgeI/EcoRI sites of the CBh-eGtACR1-mScarlet.

Constructs were verified with Sanger sequencing.

Stereotaxic Surgery

All surgical procedures were performed under aseptic conditions. Birds were anaesthetized using isoflurane inhalation (0.8-1.5%) and placed in a stereotaxic surgical apparatus. The centers of HVC, Nif, mMAN and RA were identified with electrophysiological recordings, and Area X, Av and Uva were identified using stereotaxic coordinates (approximate stereotaxic coordinates relative to interaural zero and the brain surface: head angle, rostral-caudal, medial-lateral, dorsal-ventral (in mm). HVC: 45°, AP 0, ML $\pm$ 2.4, DV -0.2-0.6; Nif: 45°, AP 1.75, ML $\pm$ 1.75, DV -2.4 -1.8; mMAN: 20°, AP 5.1, ML $\pm$ 0.6, DV -2.1 -1.6; RA: 70°, AP -1.5, ML $\pm$ 2.5, DV -2.4 -1.8; X: 45°, AP 4.6, ML $\pm$ 1.6, DV -3.3 -2.7; Av: 45°, AP 1.65, ML $\pm$ 2.0, DV -0.9; UVA: 20°, AP 2.8, ML $\pm$ 1.6, DV -4.8 -4.2).

Viral injections were performed using previously described procedures^{27,46}. Briefly, a cocktail of adeno-associated viral vectors (1:2 of rAAV-CBh-FLP and rAAV-DIO-CBh-eGtACR1, respectively) were injected into Nif, Uva, mMAN or Av (1nl/s, for a total of 1.5 μ l/hemisphere). A minimum of 4 weeks after viral injections fluorophore-conjugated retrograde tracers (Dextran 10,000MW, AlexaFluor 488 and 568, Invitrogen; FastBlue, Polysciences) were injected bilaterally into Area X, Av, and RA. Tracer injections (160nl, 5 $\times$ 32nl, 32nl/s every 30 s) were performed using previously described procedures^{27,46,97}.

***In-vivo* extracellular recordings**

To test the functional expression of eGtACR1 in HVC afferent pathways, we performed extracellular recording of HVC activity in anesthetized birds previously injected with rAAV-DIO-CBh-eGtACR1 and rAAV-CBh-FLP (2:1). We performed the recordings under light isoflurane anesthesia (0.8%) with Carbostar carbon electrodes (impedance: 1670 microhms/cm; Kation Scientific). During the recordings, we lowered a 400µm multimodal optical fiber to the brain surface overlaying HVC and delivered light stimulation (470nm, ≈20mW, 1s). Signals were acquired at 10KHz, and filtered (high-pass 300Hz, low-pass 20KHz). We used Spike2 to analyze the spike rate (binned every 10ms) and build a peri-stimulus time histogram to evaluate the effect of light stimulation across trials (5-15 trials/hemisphere). We sampled a minimum of 1 site and a maximum of 3 HVC sites/hemisphere. Birds with weak or no optically evoked responses were excluded from further experiments.

***Ex vivo* physiology**

Slice preparation

Zebra finches were deeply anesthetized with isoflurane and decapitated. The brain was removed from the skull and submerged in cold (1–4°C) oxygenated dissection buffer. Acute sagittal 230 µm brain slices were cut in ice-cold carbogenated (95% O₂/5% CO₂) solution, containing (in mM) 110 choline chloride, 25 glucose, 25 NaHCO₃, 7 MgCl₂, 11.6 ascorbic acid, 3.1 sodium pyruvate, 2.5 KCl, 1.25 NaH₂PO₄, 0.5 CaCl₂; 320-330 mOsm. Individual slices were incubated in a custom-made holding chamber filled with artificial cerebrospinal fluid (aCSF), containing (in mM): 126 NaCl, 3 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 D-(+)-glucose, 2 MgSO₄, 2 CaCl₂, 310mOsm, pH 7.3–7.4, aerated with a 95% O₂/5% CO₂ mix. Slices were incubated at 36 °C for 20 minutes, and then kept at RT for a minimum of 45 minutes before recordings.

Slice electrophysiological recording

Slices were constantly perfused in a submersion chamber with 32°C oxygenated normal aCSF. Patch pipettes were pulled to a final resistance of 3-5 MΩ from filamented borosilicate glass on a Sutter P-1000 horizontal puller. HVC-PN classes, as identified by retrograde tracers, were visualized by epifluorescence imaging using a water immersion objective (×40, 0.8 numerical aperture) on an upright Olympus BX51 WI microscope, with video-assisted infrared CCD camera (Q-Imaging Rolera). Data were low-pass filtered at 10 kHz and acquired at 10 kHz with an Axon MultiClamp 700B amplifier and an Axon Digidata 1550B Data Acquisition system under the control of Clampex 10.6 (Molecular Devices).

For voltage clamp whole-cell recordings, the internal solution contained (in mM): 120 cesium methanesulfonate, 10 CsCl, 10 HEPES, 10 EGTA, 5 Creatine Phosphate, 4 ATP-Mg, 0.4 GTP-Na (adjusted to pH 7.3-7.4 with CsOH).

Optically evoked post-synaptic currents (oPSCs) were measured by delivering 2 light pulses (1 ms, spaced 50ms, generated by a CoolLED pE300) focused on the sample through the 40X immersion objective. Sweeps were delivered every 10 s. Synaptic responses were monitored while holding the membrane voltage at -70mV (for excitatory oPSCs (oEPSCs)) and +10mV (for inhibitory oPSCs (oIPSCs)). The light stimulation mostly elicited excitatory currents with amplitude in the range of ~50-600pA. We calibrated the light stimulation intensity to ~50% of the maximum amplitude. If the amplitude exceeded 600pA, we further reduced the light intensity. In cases with currents below 50pA, we set 20pA as the minimum cut-off current amplitude in order to proceed with experiments. When both oIPSC and oEPSC were measured, the measurement was conducted at the same light stimulation intensity to allow direct comparison. . Access resistance (10–30 MΩ) was

monitored throughout the experiment, and for reliability of amplitude measurement recordings where it changed more than 20% were discarded from further analysis. We calculated the paired-pulse ratio (PPR) as the amplitude of the second peak divided by the amplitude of the first peak elicited by the twin stimuli, however due to slow kinetics of eGtACR1 the results would be difficult to interpret, and therefore we are not currently reporting them. The excitatory-inhibitory (E/I) ratio was calculated by dividing the amplitude of the oEPSC at -70mV by the amplitude of the oIPSC at +10mV, while stimulating at identical light intensities. To validate inhibitory and excitatory oPSCs as GABAergic and glutamatergic, respectively, we bath applied the GABA_A receptor antagonist SR 95531 hydrobromide (gabazine, μ M) while holding the cell at +10mV, or the AMPA receptor antagonist 6,7-Dinitroquinoxaline-2,3-dione (DNQX, μ M) while holding the cell at -70mV. In another subset of cells, once the baseline measures were established, we tested for monosynaptic connectivity. To isolate monosynaptic currents driven by optogenetic stimulation we bath applied 1 μ M Tetrodotoxin (TTX), followed by 100 μ M 4-Aminopyridine (4AP) and measured the amplitude of oPSCs returning following 4-AP application. Currents under 5pA were considered not reliable, based on the signal to noise of the recordings, and were assigned value 0 for further analysis. Therefore, average amplitudes and plots relative to oPSCs amplitudes (panels C, D of [figures 2, 3, 5, 6](#)) report only cells whose values surpass this 5pA threshold. oPSCs = 0pA were considered to calculate the rate of success in evoking oPSCs and to evaluate whether currents are monosynaptic following TTX and 4AP application. For sake of classification, any current rescued by 4AP application with an amplitude lower than 10pA was considered non-monosynaptic. Birds where no oPSC was recorded in any HVC_{PN} throughout the experimental day were excluded from further analysis.

Histology

After electrophysiological recordings, the slices were incubated in 4% PFA in PBS. Sections were then washed in PBS, mounted on glass slides with Fluoromount-G (eBioscience, CA, USA), and visualized under an LSM 880 laser-scanning confocal microscope (Carl Zeiss, Germany).

In-situ hybridization

We used the hairpin chain reaction system from Molecular Instruments as previously described (Ben Tov et al., 2023). Briefly, 5 days after having injected HVC with the retrograde tracer Fast Blue (150nl/HVC, Polysciences) the brain was perfused with 4% paraformaldehyde (PFA), post-fixed at 4 °C for 12 hours and dehydrated in 30% sucrose/4% PFA at 4 °C for 24 hours. The dehydrated brains were sagittally cyrosectioned at 40 μ m and collected into ice-cold 4% PFA. Sections were then incubated twice in PBS for 3 minutes, in 5%SDS/PBS for 45 minutes, three times in 2X SSCT for 15 minutes, in Hybridization Buffer for 5 minutes, and finally in 10nM each probe/Hybridization Buffer at 37 °C for 24 hours. The sections were then washed four times in Probe Wash Buffer for 15 minutes at 37 °C, three times in 2X SSCT for 15 minutes at room temperature, then incubated in Amplification Buffer for 30 minutes at room temperature. Each Alexa fluor-conjugated hairpin was denatured in separated PCR tubes at 95 °C for 90 seconds and cooled down to room temperature at 2 °C/s. Sections were incubated in solutions containing 36nM of each hairpin in Amplification Buffer at room temperature for 24 hours. The sections were then washed four times with 2X SSCT for 15 minutes and mounted with mounting medium.

Experimental Design and Analysis

Electrophysiological data were analyzed with Clampfit (Molecular Devices). All data were tested for normality using the Shapiro-Wilk Test. Parametric and non-parametric statistical tests were used as appropriate. One-way Anova or Kruskal-Wallis test was performed when comparisons were made across more than two conditions. Two-Way Anova or Mixed-effect analysis were used to compare multiple conditions across different groups. Fisher's exact tests were used to compare the probability of finding optically evoked responses. Statistical significance refers to * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

The Sankey diagram in **Fig. 7C** [↗](#) was drawn with SankeyMATIC (www.sankeymatic.com [↗](#)) setting ribbons proportional to the percentage of interrogated synaptic connections between each afferent area and HVCPN class displaying excitatory currents or lack thereof. From the hubs reporting existing synaptic connection (center), ribbons are scaled based on the percentage of monosynaptic and polysynaptic connectivity for each combination.

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Additional information

Author contribution

MT and TFR conceived the project. TFR supervised the research. MT and TFR designed the experiments and wrote the manuscript. MT collected and analyzed the data. ZZ conducted the in-situ hybridization staining. DHA designed and optimized viral tools used in this research. ESM and MZI performed the tracer injections and quantification of mMAN cells. All authors read and commented on the manuscript. Authors declare no competing interests. All data is available in the main text or the supplementary materials.

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Reviewer #1 (Public review):

Summary:

This work has created the map of synaptic connectivity between the inputs and outputs of song premotor nucleus, HVC in zebra finches to understand how sensory (auditory) to motor circuit interact to coordinate song production and learning. The authors optimized the optogenetic technique via AAV to manipulate auditory inputs from a specific auditory area one-by-one and recorded synaptic activity from a neuron in HVC with whole-cell recording from slice preparation with identification of projection area by retrograde neuronal tracing. These thorough and detailed analysis provide compelling evidence of synaptic connections between 4 major auditory inputs (3 forebrain and 1 thalamic regions) within three projection neurons in the HVC; all areas give monosynaptic excitatory inputs and polysynaptic inhibitory inputs, but proportions of projection to each projection neuron varied. They also find specific reciprocal connections between mMAN and Av. Taken together the authors provide the map of synaptic connection between intercortical sensory to motor areas which is suggested to be involved in zebra finch song production and learning.

Strengths:

The authors optimized optogenetical tools with eGtACR1 by using AAV which allow them to manipulate synaptic inputs in a projection-specific manner in zebra finches. They also identify HVC cell type based on projection area. With their technical advance and thorough experiments, they provided detailed map synaptic connection and gave insights into the neuronal circuit for auditory guided vocal (motor) learning.

Weaknesses:

As this study is in adult brain slices, there might be a gap to the functions in developmental song learning.

<https://doi.org/10.7554/eLife.104609.2.sa3>

Reviewer #2 (Public review):

Summary:

The manuscript describes synaptic connectivity in Songbird cortex four main classes of sensory neurons afferents onto three known classes of projection neurons of the pre-motor cortical region HVC. HVC is a region associated with the generation of learned bird song. Investigators here use all male zebra finches to examine the functional anatomy of this region using patch clamp methods combined with optogenetic activation of select neuronal groups.

Strengths:

The quality of the recordings is extremely high and the quantity of data is on a very significant scale, this will certainly aid the field.

Weaknesses:

Could make the figures a little easier to navigate by having some atlas drawings.

Comments on revisions:

The authors have addressed the minor concerns and suggestions

<https://doi.org/10.7554/eLife.104609.2.sa2>

Reviewer #3 (Public review):

Nucleus HVC is critical both for song production as well as learning and arguably, sitting at the top of the song control system, is the most critical node in this circuit receiving a multitude of inputs and sending precisely timed commands that determine the temporal structure of song. The complexity of this structure and its underlying organization seem to become more apparent with each experimental manipulation, and yet our understanding of the underlying circuit organization remains relatively poorly understood. In this study, Trusel and Roberts use classic whole-cell patch clamp techniques in brain slices coupled with optogenetic stimulation of select inputs to provide a careful characterization and quantification of synaptic inputs into HVC. By identifying individual projections neurons using retrograde tracer injections combined with pharmacological manipulations, they classify monosynaptic inputs onto each of the three main classes of glutamatergic projection neurons in HVC (RA-, Area X- and Av-projecting neurons). This study is remarkable in the amount of information that it generates, and the tremendous labor involved for each experiment, from the expression of opsins in each of the target inputs (Uva, Nif, mMAN and Av), the retrograde labelling of each type of projection neuron, and ultimately the optical stimulation of infected axons while recording from identified projection neurons. Taken together, this study makes an important contribution to increasing our identification, and ultimately understanding, of the basic synaptic elements that make up the circuit organization of HVC, and how external inputs, which we know to be critical for song production and learning, contribute to the intrinsic computations within this critic circuit.

This study is impressive in its scope, rigorous in its implementation and thoughtful regarding its limitations. The manuscript is well written, and I appreciate the clarity with which the authors use our latest understanding of the evolutionary origins of this circuit to place these studies within a larger context and their relevance to the study of vocal control, including human speech. My comments are minor and primarily about legibility, clarification of certain manipulations and organization of some of the summary figures.

Comments on revisions:

The authors have done a very nice job addressing the reviewers' comments.

<https://doi.org/10.7554/eLife.104609.2.sa1>

Author response:

The following is the authors' response to the original reviews

Public Reviews:

Reviewer #1 (Public review):

Summary:

This work tried to map the synaptic connectivity between the inputs and outputs of the song premotor nucleus, HVC in zebra finches to understand how sensory (auditory) to motor circuits interact to coordinate song production and learning. The authors optimized the optogenetic technique via AAV to manipulate auditory inputs from a specific auditory area one-by-one and recorded synaptic activity from a neuron with whole-cell recording from slice preparation with identification of the projection area by retrograde neuronal tracing. This thorough and detailed analysis provides compelling evidence of synaptic connections between 4 major auditory inputs (3 forebrain and 1 thalamic region) within three projection neurons in the HVC; all areas give monosynaptic excitatory inputs and polysynaptic inhibitory inputs, but proportions of projection to each projection neuron varied. They also find specific reciprocal connections between mMAN and Av. Taken together the authors provide the map of the synaptic connection between intercortical sensory to motor areas which is suggested to be involved in zebra finch song production and learning.

Strengths:

The authors optimized optogenetic tools with eGtACR1 by using AAV which allow them to manipulate synaptic inputs in a projection-specific manner in zebra finches. They also identify HVC cell types based on projection area. With their technical advance and thorough experiments, they provided detailed map synaptic connections.

Weaknesses:

As it is the study in brain slice, the functional implication of synaptic connectivity is limited. Especially as all the experiments were done in the adult preparation, there could be a gap in discussing the functions of developmental song learning.

We thank the reviewer for their appreciation of our work. Although we agree that there can be limitations to brain slice preparations, the approaches used here for synaptic connectivity mapping are well-designed to identify long-range synaptic connectivity patterns. Optogenetic stimulation of axon terminals in brain slices does not require intact axons and works well when axons are cut, allowing identification of all inputs expressing optogenetic channels

from aXerent regions. Terminal stimulation in slices yields stable post-synaptic responses for hours without rundown, assuring that polysynaptic and monosynaptic connections can be reliably identified in our brain slices. Additionally, conducting similar types of experiments in vivo can run into important limitations. First, the extent of TTX and 4-AP diXusion, which is necessary for identification of long-range monosynaptic connections, can be diXicult to verify in vivo - potentially confounding identification of monosynaptic connectivity. Second, conducting whole-cell patch-clamp experiments in vivo, particularly in deeper brain regions, is technically challenging, and would limit the number of cells that can be patched and increase the number of animals needed.

We agree that there may well be important diXerences between adult connectivity and connectivity patterns in the juvenile brain. Indeed, learning and experience during development almost certainly shape connectivity patterns and these patterns of connectivity may change incrementally and/or dynamically during development. Ultimately, adult connectivity patterns are the result of changes in the brain that accrue over development. Given that this is the first study mapping long-range connectivity of HVC input-output pathways, we reasoned that the adult connectivity would provide a critical reference allowing future studies to map diXerent stages of juvenile connectivity and the changes in connectivity driven by milestones like forming a tutor song memory, sensorimotor learning, and song crystallization.

In this revision we worked to better highlight the points raised above and thank the reviewer for their comments.

Reviewer #2 (Public review):

Summary:

The manuscript describes synaptic connectivity in the Songbird cortex's four main classes of sensory neuron aXerents onto three known classes of projection neurons of the pre-motor cortical region HVC. HVC is a region associated with the generation of learned bird songs. Investigators here use all male zebra finches to examine the functional anatomy of this region using patch clamp methods combined with optogenetic activation of select neuronal groups.

Strengths:

The quality of the recordings is extremely high and the quantity of data is on a very significant scale, this will certainly aid the field.

Weaknesses:

The authors could make the figures a little easier to navigate. Most of the figures use actual anatomical images but it would be nice to have this linked with a zebra finch atlas in more of a cartoon format that accompanied each fluro image. Additionally, for the most part, figures showing the labeling lack scale bar values (in um). These should be added not just shown in the legends.

The authors could make it clear in the abstract that this is all male zebra finches - perhaps this is obvious given the bird song focus, but it should be stated. The number of recordings from each neuron class and the overall number of birds employed should be clearly stated in the methods (this is in the figures, but it should say n=birds or cells as appropriate).

The authors should consider sharing the actual electrophysiology records as data.

We thank the reviewer for their assessment of our research and suggestions. We have implemented many of these suggestions and provide details in our response to their specific Recommendations. Additionally, we are organizing our data and will make it publicly available with the version of record.

Reviewer #3 (Public review):

Nucleus HVC is critical both for song production as well as learning and arguably, sitting at the top of the song control system, is the most critical node in this circuit receiving a multitude of inputs and sending precisely timed commands that determine the temporal structure of song. The complexity of this structure and its underlying organization seem to become more apparent with each experimental manipulation, and yet our understanding of the underlying circuit organization remains relatively poorly understood. In this study, Trusel and Roberts use classic whole-cell patch clamp techniques in brain slices coupled with optogenetic stimulation of select inputs to provide a careful characterization and quantification of synaptic inputs into HVC. By identifying individual projection neurons using retrograde tracer injections combined with pharmacological manipulations, they classify monosynaptic inputs onto each of the three main classes of glutamatergic projection neurons in HVC (RA-, Area X- and Av-projecting neurons). This study is remarkable in the amount of information that it generates, and the tremendous labor involved for each experiment, from the expression of opsins in each of the target inputs (Uva, NIf, mMAN, and Av), the retrograde labelling of each type of projection neuron, and ultimately the optical stimulation of infected axons while recording from identified projection neurons. Taken together, this study makes an important contribution to increasing our identification, and ultimately understanding, of the basic synaptic elements that make up the circuit organization of HVC, and how external inputs, which we know to be critical for song production and learning, contribute to the intrinsic computations within this critic circuit.

This study is impressive in its scope, rigorous in its implementation, and thoughtful regarding its limitations. The manuscript is well-written, and I appreciate the clarity with which the authors use our latest understanding of the evolutionary origins of this circuit to place these studies within a larger context and their relevance to the study of vocal control, including human speech. My comments are minor and primarily about legibility, clarification of certain manipulations, and organization of some of the summary figures.

We thank the reviewer for their thoughtful assessment of our research.

Recommendations for the authors:

The following recommendations were considered by all reviewers to be important to incorporate for improving this paper:

(1) Clarify the site of viral injection and the possibility of labeling other structures a) Show images of viral injection sites.

We provide a representative image of viral expression for each pathway studied in this manuscript. Please see panel A in Figures 2-3 and 5-6 showing our viral expression in Uva, NIf, mMAN, and Av respectively.

b) Include in discussion caveats that the virus may spread beyond the boundaries of structures (e.g. especially injections into NIF could spread into Field L).

For each HVC aXerent nucleus we have now included a sentence describing the possible spread of viral infection in surrounding structures in the Results. We also now expanded the

image from the Av section to include NIf, to showcase lack of viral expression in NIf (see Fig. 6A).

(2) Clarify the logic and precise methods of the TTX and 4-AP experiments

a) Please see the detailed issue raised by Reviewer 3, Major Point 1 below.

The TTX and 4AP application is the gold-standard of opsin-assisted synaptic circuit interrogation, pioneered by the Svoboda lab in 2009 (Petreanu, Mao et al. 2009) and widely used to assess monosynaptic connectivity in multiple brain circuits, as summarized in a recent review (Linders, Supiot et al. 2022). We now better describe the logic of this approach in the second paragraph of the Results section and cite the first description of this method from the Svoboda lab and a recent review weighing this method with other optogenetic methods for tracing synaptic connections in the brain.

(3) Include caveats in discussion

a) Note that there may be other inputs to HVC that were not examined in this study (e.g. CMM, Field L)

In our original manuscript we did state “Although a complete description of HVC circuitry will require the examination of other potential inputs (i.e. RA_{HVC} PNs, A11 glutamatergic neurons (Roberts, Klein et al. 2008, Ben-Tov, Duarte et al. 2023)) and a characterization of interneuron synaptic connectivity, here we provide a map of the synaptic connections between the 4 best described aParents to HVC and its 3 populations of projection neurons” in the last paragraph of the Discussion. We have now edited this sentence to include the projection from NCM to HVC and cited Louder et al., 2024.

We have extensively mapped input pathways to HVC, and consistent with Vates (Vates, Broome et al. 1996) we have not found evidence that Field L projects to HVC. Rather that it projects to the shelf region outside of HVC. Consistent with this, we do not see retrogradely labeled neurons in Field L following tracer injections confined to HVC (see Fig. 3G). Additionally, we find that CM projections to HVC arise from the nucleus Avalanche (Roberts, Hisey et al. 2017) which we specifically examine in this study. We do not dispute that there may be other pathways projecting to HVC that will need to be examined in the future, including known projections from neuromodulatory regions and RA, from developmentally restricted pathway(s) like NCM (Louder, Kuroda et al. 2024), and from yet unidentified pathways.

b) Also note that birds in this study were adults and that some inputs to HVC likely to be important for learning may recede during development (e.g. Louder et al, 2024).

In the second to last paragraph of the Discussion we now state: While our opsin-assisted circuit mapping provides us with a new level of insight into HVC synaptic circuitry, there are limitations to this research that should be considered. All circuit mapping in this study was carried out in brain slices from adult male zebra finches. Future studies will be needed to examine how this adult connectivity pattern relates to patterns of connectivity in juveniles during sensory or sensorimotor phases of vocal learning and connectivity patterns in female birds.

(4) Consider cosmetic changes to figures as suggested by Reviewers 2-3 below.

We thank the reviewers for their suggestions and have implemented the changes as best we can.

Reviewer #1 (Recommendations for the authors):

I see this study is well designed to answer the author's specific question, mapping synaptic audiotormotor connections within HVC. Their experiments with advanced techniques of projection-specific optogenetic manipulation of synaptic inputs and retrograde identification of projection areas revealed input-output combination selective synaptic mapping.

As I found this study advanced our knowledge with the compelling dataset, I have only some minor comments here.

(1) One technical concern is we don't see how much the virus infection was focused on the target area and if we can ignore the effect of synaptic connectivity from surrounding areas. As the amount of virus they injected is large (1.5ul) and target areas are small, we assume the virus might spread to the surrounding area, such as field L which also projects to HVC when targeting Nif. While I think the majority of the projections were from their target areas, it would be better to mention (also the images with larger view areas) the possibilities of projections of surrounding areas.

We agree with the reviewer about the concern about specificity of viral expression. For this reason, we included sample images of the viral expression in each target area (panel A in Fig. 2,3,5,6). We have now also included a sentence at the beginning of each subsection of our Result to describe how we have ensured interpretability of the results. Uva and mMAN's surrounding areas are not known to project to HVC. Possible cross-infection is an issue for Av and NIf, and we checked each bird's injection site to ensure that eGtACR1+ cells were not visible in the unintended HVC-projecting areas.

As mentioned in our response the public comment, consistent with Vates (Vates, Broome et al. 1996) we do not see evidence that Field L projects directly to HVC (see Fig. 3G).

(2) Another concern about the technical issue is the damage to axonal projections. While I understand the authors stimulated axonal terminals axonal projections were assumed to be cut and their ability to release neurotransmitters would be reduced especially after long-term survival or repeated stimulation. Mentioning whether projection pathways were within their 230um-thick slice (probably depends on input sites) or not and the effect of axonal cut would be helpful.

We agree that slice electrophysiology has limitations. However, we disagree with the claim of reduced reliability or stability of the evoked response. We and others find that electrical and optogenetic repeated terminal stimulation in slices can yield stable post-synaptic responses for tens of minutes and even hours (Bliss and Gardner-Medwin 1973, Bliss and Lomo 1973, Liu, Kurotani et al. 2004, Pastalkova, Serrano et al. 2006, Xu, Yu et al. 2009, Trusel, Cavaccini et al. 2015, Trusel, Nuno-Perez et al. 2019). Indeed, long-term synaptic plasticity experiments in most preparations and across brain areas rely on such stability of the presynaptic machinery for synaptic release, despite axons being severed from their parent soma. Our assumption is the vast majority, if not all, connections between axon terminals and their cell body in the aXerent regions have been cut in our preparations. Nonetheless, the diversity of outcomes we report (currents returning after TTX+4AP or not, depending on the specific combination of input and HVCN class) is consistent with the robustness of the synaptic interrogation method.

(3) While I understand this study focused on 4 major input areas and the authors provide good pictures of synaptic HVC connections from those areas, HVC has been reported to receive auditory inputs from other areas as well (CMM, FieldL, etc.). It is worth

mentioning that there are other auditory inputs and would be interesting to discuss coordination with the inputs from other areas.

We have extensively mapped input pathways to HVC, and consistent with Vates (Vates, Broome et al. 1996) we have not found evidence that Field L projects to HVC. Rather that it projects to the shelf region outside of HVC. Consistent with this, we do not see retrogradely labeled neurons in Field L following tracer injections confined to HVC (see Fig. 3G). Additionally, we find that CM projections to HVC arise from the nucleus Avalanche (Roberts, Hisey et al. 2017) which we specifically examine in this study. We do not dispute that there may be other pathways projecting to HVC that will need to be examined in the future, including known projections from neuromodulatory regions and RA, from developmentally restricted pathway(s) like NCM (Louder, Kuroda et al. 2024), and from yet unidentified pathways.

(4) The HVC local neuronal connections have been reported to be modified and a recent study revealed the transient auditory inputs into HVC during song learning period. The author discusses the functions of HVC synaptic connections on song learning (also title says synaptic connection for song learning), however, the experiments were done in adults and do not discuss the possibility of different synaptic connection mapping in juveniles in the song learning period. Mentioning the neuronal activities and connectivity changes during song learning is important. Also, it would be helpful for the readers to discuss the potential differences between juveniles/adults if they want to discuss the functions of song learning.

We now mention in the Discussion that this is an important caveat of our research and that future studies will be needed to examine how these adult connectivity patterns relate to connectivity patterns in juveniles during sensory or sensorimotor phases of vocal learning and connectivity patterns in female birds. Nonetheless, the title and abstract cite song learning because it is important for the broader public to understand that at least some of these different brain regions carry an essential role in song learning (Foster and Bottjer 2001, Roberts, Gobes et al. 2012, Roberts, Hisey et al. 2017, Zhao, Garcia-Oscos et al. 2019, Koparkar, Warren et al. 2024).

Reviewer #2 (Recommendations for the authors):

The work is very detailed and will be an important resource to those working in the field. The recordings are of a high quality and lots of information is included such as measures of response kinetics amplitude and pharmacological confirmation of excitatory and inhibitory synaptic responses. In general, I feel the quality is extremely high and the quantity of data is on a very significant exhaustive scale that will certainly aid the field. I have come at this conclusion as a non zebra finch person but I feel the connection information shown will be of benefit given its high quality.

Figure 7 is a nice way of showing the overall organization. Optional suggestion, consider highlighting anything in Figure 7 that results in a new understanding of the song system as compared to previous work on anatomy and function.

We thank the reviewer for the kind comments about our research. We have highlighted our newly found connection between mMAN and Av and all the connections onto the HVC PN in Panel B are newly identified in this study.

Reviewer #3 (Recommendations for the authors):

Major points

(1) Clarification regarding methods for determining monosynaptic events:

One of the manipulations that I struggled the most with was those describing the use of TTX + 4AP to isolate monosynaptic events. Initially, not being as familiar with the use of optically based photostimulation of axons to release transmitter locally, I was initially confused by statements such as "we found that oEPSC returned after application of TTX+4AP". This might be clear to someone performing these manipulations, but a bit more clarification would be helpful. Should I assume that an existing monosynaptic EPSC would be masked by co-occurring polysynaptic IPSCs which disappear following application of TTX + 4AP, thereby unmasking the monosynaptic EPSC, thereby causing the EPSC to "return"? A word that I am not sure works. Continuing my confusion with these experiments, I am unsure how this cocktail of drugs is added, if it is even added as a cocktail, which is what I initially assumed. The methods and the results are not so clear if they are added in sequence and why and if traces are recorded after the addition of both drugs or if they are recorded for TTX and then again for TTX + 4AP. Finally, looking at the traces in the experimental figures (e.g. Figures 2F, 3F, 5F, and 6F), it is difficult to see what is being shown, at least for me. First, the authors need to describe better in the results why they stimulate twice in short succession and why they seem to use the response to the second pulse (unless I am mistaken) to measure the monosynaptic event. Second, I was confused by the traces (which are very small) in the presence of TTX. I would have expected to see a response if there was a monosynaptic EPSC but I only seem to see a flat line.

The confusion that I list above might be due in part to my ignorance, but it is important in these types of papers not to assume too much expertise if you want readers with a less sophisticated understanding of synaptic physiology to understand the data. In other words, a little bit more clarity and hand-holding would be welcome.

We understand the reviewer's confusion about the methodology. In Voltage clamp, the amplifier injects current through the electrode maintaining the membrane voltage to -70mV, where the equilibrium potential for Cl⁻ is near equilibrium, and therefore the only synaptic current evoked by light stimulation is due to cation influx, mainly through AMPA receptors (see Fig. 1). Therefore, cooccurring polysynaptic IPSCs wouldn't be visible. We examine those holding the membrane voltage at +10mV, see Fig. 1. TTX application suppresses V-dependent Na⁺ channels and therefore stops all neurotransmission. We show the traces upon TTX to show that currents we were recording prior to TTX application were of synaptic origin, and not due to accidental expression of opsin in the patched cell. Also, this ensures that any current visible after 4AP application is due to monosynaptic transmission and not to a failure of TTX application.

After recording and light stimulation with TTX, we then add 4AP, which is a blocker of presynaptic K⁺ channels. This prevents the repolarization of the terminals that would occur in response to opsin-mediated local depolarization. 4AP application, therefore, allows local opsin-driven depolarizations to reach the threshold for Ca²⁺-dependent vesicle docking and release. This procedure selectively reveals or unmasks the monosynaptic currents because any non-monosynaptically connected neuron would still need V-dependent Na⁺ channels to effectively produce indirect neurotransmission onto the patched cell. The TTX and 4AP application is the gold-standard of opsin-assisted synaptic circuit interrogation, pioneered by the Svoboda lab in 2009 and widely used to assess monosynaptic connectivity in multiple brain circuits, as summarized in a recent review (Linders et al., 2022). We now include 2 more sentences near the beginning of the Results to clarify this process and directly point to the Linders review for researchers wanting a deeper explanation of this technique.

The double stimulation is unrelated to our testing of monosynaptic connections. We originally conducted the experiments by delivering 2 pulses of light separated by 50ms, a

common way to examine the pair-pulse ratio (PPR) – a physiological measure which is used to probe synapses for short-term plasticity and release probability. However, through discussions with colleagues we realized that the slow decay time of eGtACR1 may complicate interpretation of the response to the second light pulse. Thus, we elected to not report these results and indicated this in the Methods section: “We calculated the paired-pulse ratio (PPR) as the amplitude of the second peak divided by the amplitude of the first peak elicited by the twin stimuli, however due to slow kinetics of eGtACR1 the results would be difficult to interpret, and therefore we are not currently reporting them.”

(2) Suggestions for improving summary figures:

Summary Figure 1a: The circuit diagram (schematic to the right of 1a) is OK but I initially found it a bit difficult to interpret. For example, it is not clear why pink RA projecting neurons don't reach as far to the right as X or Av projecting neurons, suggesting that they are not really projection neurons. Also, the big question marks in the intermediate zone are not entirely intuitive. It seems there might be a better way of representing this. It might also be worth stating in the figure legend that the interconnectivity patterns shown in the figure between PNs in HVC are based on specific prior studies.

We thank the reviewer for the constructive criticism. We have modified the figure to extend the RA projection line and mentioned in the figure legend that connectivity between PNs is based on prior studies.

Summary Figure 1a: I am not sure I love this figure. There are a few minor issues. First, there are too many browns [Nif/AV and mMAN] which makes it more challenging to clearly disambiguate the different projections. Second, it is unclear why this figure does not represent projections from RA to HVC. My biggest concern with this figure is that it oversimplifies some of the findings. From the figure, one gets the impression that Uva only projects to RA-PNs and that Av only projects to X-PNs even though the authors show connections to other PNs. With the small sample size in this current study for each projection and each PN type, one really cannot rule out that these "minority" projections are not important. I, therefore, suggest that the authors qualitatively represent the strength/probability of connections by weighting with thickness of different connections.

We assume the reviewer is commenting on our summary figure panel 7B. We agree with the referee that this is a simplified representation of our findings. We had indeed indicated in the legend that this was just a “Schematic of the HVC different connectivity map resulting from the present work” and that “For conceptualization purposes, different connectivity to HVC-PNs is shown only when the rate of monosynaptic connectivity reaches 50% of neurons examined”. We have added a title to highlight that this is but a simplification. We have now adjusted the colors to make the figure easier to follow. Based on the reviewers critique we searched for a better method for summarizing the complex connectivity patterns described in this research. We settled on a Sankey diagram of connectivity. This is now Figure 7C. In this diagram, we are able to show the proportion of connections from each input pathway onto each class of neuron and if these connections are poly or monosynaptic. We find this to a straightforward way of displaying all of the connectivity patterns identified in our figure 2-3 and 4-5 look forward to understanding if the reviewers find this a useful way of illustrating our findings.

Minor points:

(1) Line 50 - typo - song circuits.

Thank you for catching this.

(2) Line 106 - 111 - The findings suggest that 100% of Uva projections onto HVCRA neurons are monosynaptic. However, because the authors only tested 6 neurons their statements that their findings are so different from other studies, should be somewhat tempered since these other studies (e.g. Moll et al.) looked at 251 neurons in HVC and sampling bias could still somewhat explain the difference.

We observed oEPSCs in 43 of 51 (84.3%) HVC-RA neurons recorded (mean rise time = 2.4 ms) and monosynaptic connections onto 100% of the HVC-RA neurons tested (n = 6). Moll et al. combined electrical stimulation of Uva with two-photon calcium imaging (GCaMP6s) of putative HVC-RA neurons (n = 251 neurons). We should note that these are putative HVC-RA neurons because they were not visually identified using retrograde tracing or using some other molecular handle. They report that only ~16% of HVC-RA neurons showed reliable calcium responses following Uva stimulation. Although the experiments by Moll et al are technically impressive, calcium imaging is an insensitive technique for measuring post-synaptic responses, particularly subthreshold responses, when compared to whole-cell patch-clamp recordings. This approach cannot identify monosynaptic connections and is likely limited to only be sensitive suprathreshold activity that likely relies on recruitment of other polysynaptic inputs onto the neurons in HVC. Furthermore, as indicated in the Discussion, our opsin-mediated synaptic interrogation recruits any eGtACR1+ Uva terminal in the slice and therefore will have great likelihood of revealing any existing connections.

A limitation of whole-cell patch-clamp recordings is that it is a laborious low throughput technique. Future experiments using better imaging approaches, like voltage imaging, may be able to weigh in on differences between what we report here using whole-cell patch-clamp recordings from visually identified HVC-RA neurons combined with optogenetic manipulations of Uva terminals and the calcium imaging results reported by Moll. Nonetheless, whole-cell patch-clamp recordings combined with optogenetic manipulations is likely to remain the most sensitive method for identifying synaptic connectivity.

(3) Figure 2G - the significance of white circles is not clear.

The figure legend indicates that those highlight and mark the position of “retrogradely labeled HVCprojecting neurons in Uva (cyan, white circles)” to facilitate identification of colocalization with the in-situ markers.

(4) Line 135 - Cardin et al. (J. Neurophys. 2004) is the first to show that song production does not require Nif.

We thank the reviewer pointing this out and we have cited this important study.

(5) Line 183 - This is a confusing sentence because I initially thought that mMAN-mMANHVC PNs was a category!

We switched the dash with a colon.

(6) Figure 4d could use some arrows to identify what is shown. It is assumed that the box represents mMAN. Should it be assumed that Av is not in the plane of this section? If not, this should be stated in the legend. It is also unclear where the anterograde projections are. Is this the dark highway that goes from the box to the dorsal surface? If yes this should be indicated but it should also be made clear why the projections go both in the dorsal as well as the ventral directions.

The inset, as indicated by the lines around it, is a magnification of the terminal fields in Av. We added an explanation of the inset.

(7) Discussion. In the introduction, the authors mention projections from RA to HVC but never end up studying them in the current manuscript which seems like a missed opportunity and perhaps even a weakness of the study. In the discussion, it would certainly be good for the authors to at least discuss the possible significance of these projections and perhaps why they decided not to study them.

We thank the reviewer for the comment. Unfortunately, we couldn't reliably evoke interpretable currents from RA, and we elected to publish the current version of the paper with these 4 major inputs. Nonetheless, we have indicated in the Introduction and in the Discussion that more inputs (e.g. RA, A11, NCM) remain to be evaluated.

(8) Line 622 - Is this reference incomplete?

We thank the reviewer. We have corrected the reference.

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