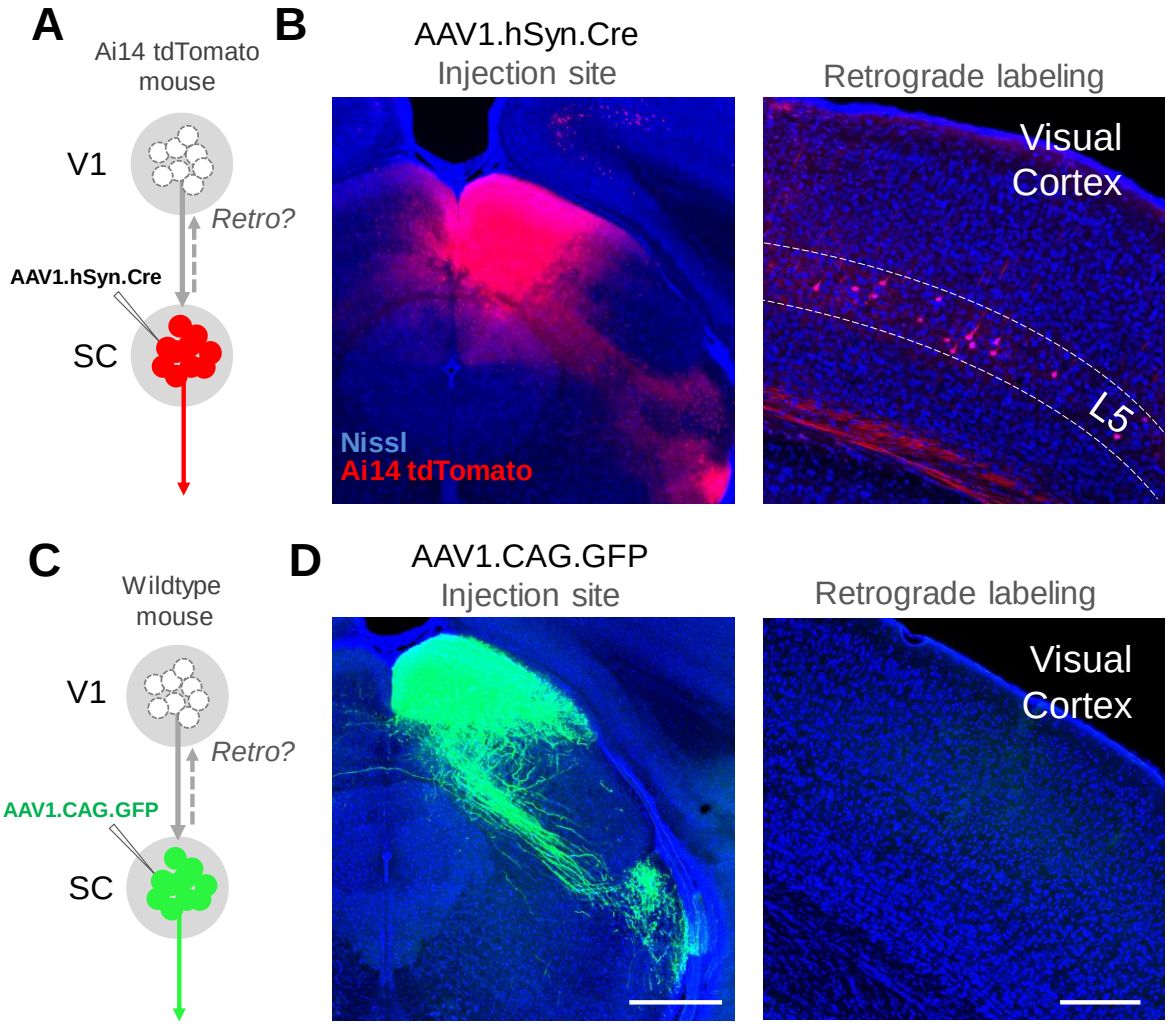

Figure S1, related to Figure 2. Retrograde transport of AAV1.
(A) Schematic diagram of AAV1-hSyn-Cre injection in SC of Ai14 tdTomato mouse.
(B) Raw images showing injection site in SC (left panel, red) and neurons in V1 (right panel). Numerous retrogradely labeled cells were observed in layer 5 (L5) of visual cortex.
(C) Schematic diagram of injection. AAV1-CAG-GFP was injected into SC of wildtype mice and V1 was examined for retrogradely labeled cell bodies.
(D) Raw images showing injection site in SC (left panel, green) and V1 (right panel). No GFP+ cell bodies were observed in V1. Scale bars, 1 mm (left panel) and 250 µm (right panel).

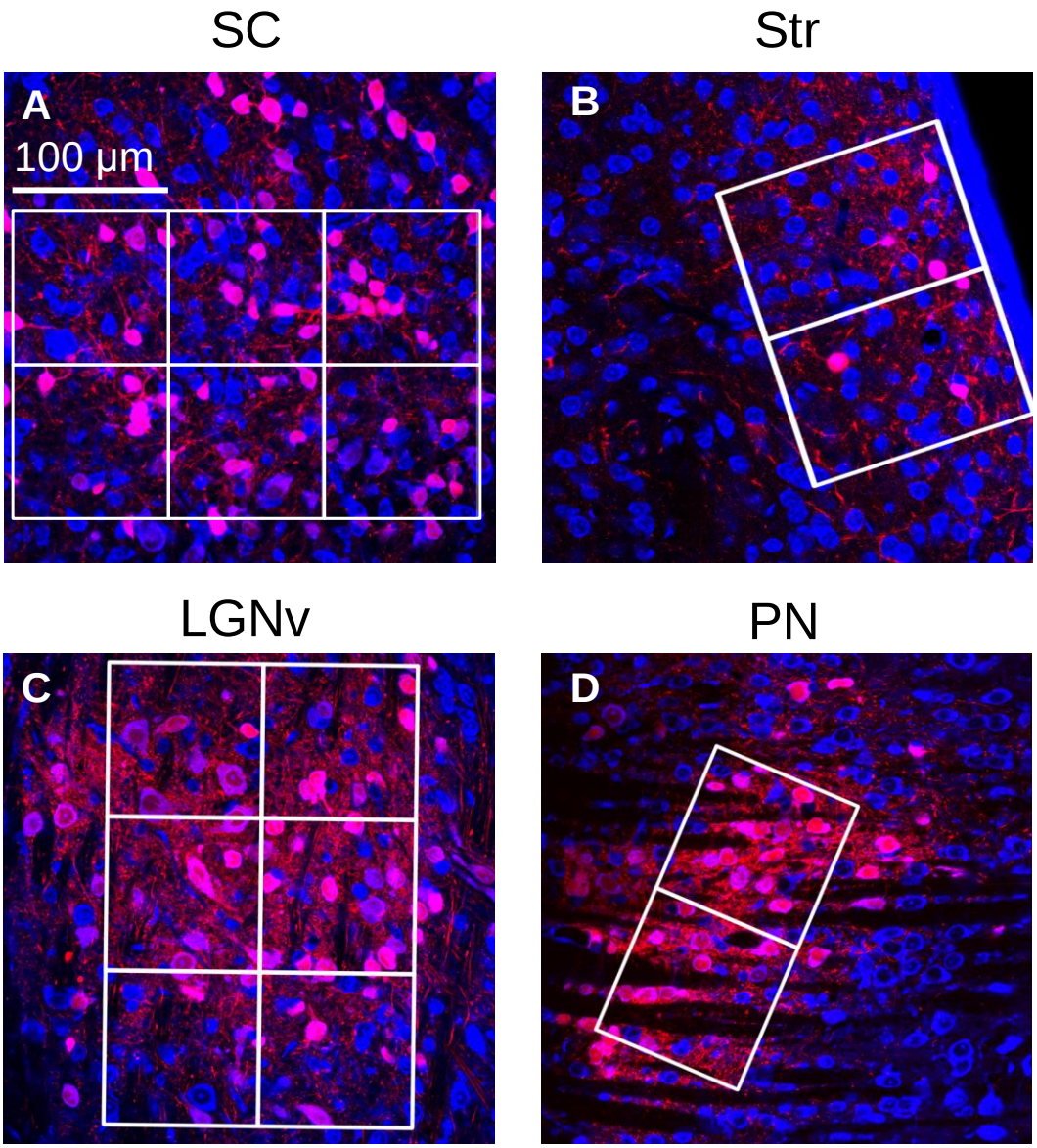

Figure S2, related to Figure 2. Quantification of labeling density in targets downstream of V1 following injection of AAV1-hSyn-Cre in Ai14 mice.
(A) Cre+/Tom+ cells (red) and Nissl stained cells (blue) were quantified within a fixed 200 x 300 µm sample space (white grid) centered on regions with Tom+ labeling across multiple sections of SC to generate an estimate of average density of cell labeling. One example image is shown.
(B) An example image for quantifying density of Tom+ cells within a 150 x 200 µm sample region in striatum (Str).
(C) Example image of ventral lateral geniculate nucleus (LGNv) quantification using a 200 x 300 µm sample region.
(D) Example image of pontine nucleus (PN) quantification using a 100 x 200 µm sample region.

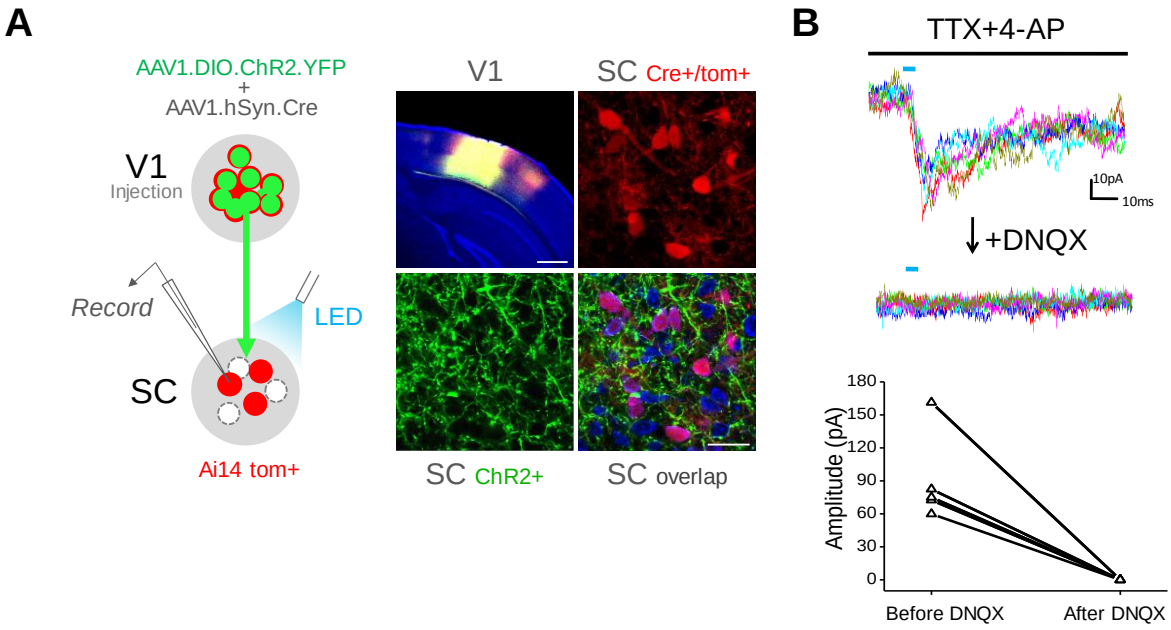

Figure S3, related to Figure 4. Pre-synaptic specificity of LED evoked currents recorded in slice preparation.

(A) Schematic diagram of injection and recording strategy. AAV1-DIO-ChR2-YFP and AAV1-hSyn-Cre were co-injected into V1 of Ai14 mice to label downstream neurons in SC (red) and SC-projecting axons with ChR2 (green). Tom+ cells in SC were then targeted for recording in slice preparation. Raw images of the injection site and labeling in SC are shown. No ChR2-YFP+ cell bodies were observed in SC (bottom panel). Blue, Nissl stain. Scale bars, 500 µm, top left panel, 25 µm, bottom right panel.

(B) Averaged LED evoked excitatory synaptic responses recorded from 5 Tom+ SC neurons in the presence of TTX and 4-AP. Responses were eliminated after adding glutamate receptor antagonist DNQX, suggesting no ChR2 expression within the recorded cells. Blue bar indicates 5 ms LED pulse. Graph below summarizes amplitudes of LED evoked responses before and after addition of DNQX. Data points for the same cell are connected with a line.

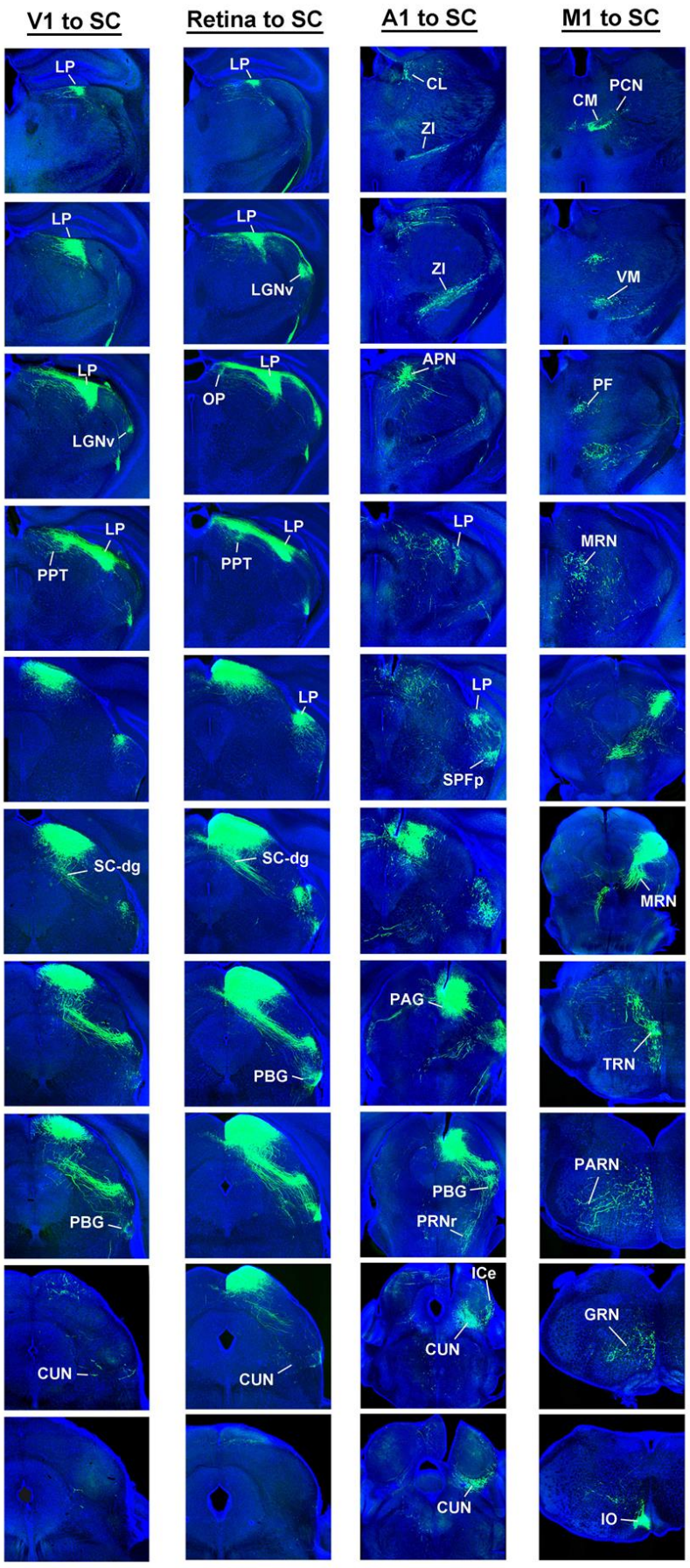

Figure S4, related to Figure 5.

Figure S4, related to Figure 5. Axonal projection patterns for different input-defined subpopulations in SC. Following two-step injection of AAV1-hSyn-Cre in either visual cortex (V1), retina, auditory cortex (A1), or primary motor cortex (M1) and AAV1-CAG-FLEX-GFP in SC, distinct patterns of axon labeling were observed throughout the brain. Abbreviations: CL, central lateral nucleus of thalamus; PCN, paracentral nucleus; ZI, zona incerta; VM, ventral medial nucleus of thalamus; CM, central medial nucleus of thalamus; APN, anterior pretectal nucleus; OP, olivary pretectal nucleus; PF, parafascicular nucleus; PPT, posterior pretectal nucleus; SPFm and SPFp, subparafascicular nucleus, magnocellular and parvicellular; MRN, midbrain reticular nucleus; PAG, periaqueductal gray; PBG, parabigeminal nucleus; PRN, pontine reticular nucleus; TRN, tegmental reticular nucleus; ICd and ICe, inferior colliculus, dorsal and external; PARN, parvicellular reticular nucleus; GRN, gigantocellular reticular nucleus; IO, inferior olivary complex.

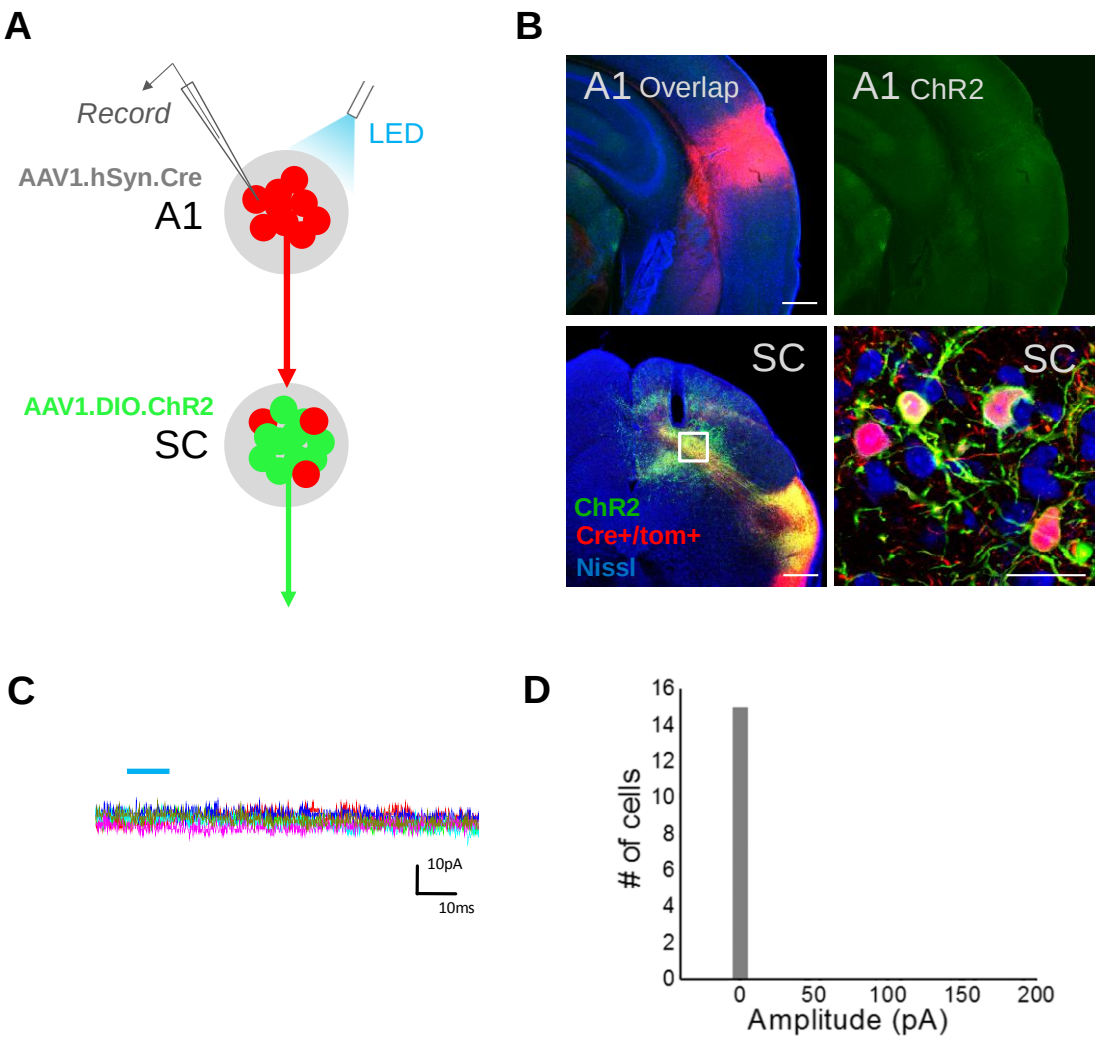

Figure S5, related to Figure 6. Lack of retrograde ChR2 expression in cortex in animals used for behavior studies.

(A) Schematic diagram showing injection and recording strategy. To test if ChR2 is retrogradely expressed in Cre+ cortical neurons, AAV1-hSyn-Cre was injected into A1 of Ai14 mice. Anterograde transneuronally labeled cells in SC (red) were then targeted for Cre-dependent expression of ChR2 with a second injection of AAV1-EF1a-DIO-ChR2-YFP. Following 4 weeks expression time, Cre+/Tom+ neurons in layer 5 (L5) of A1 were targeted for slice recording.

(B) Raw images of a representative injection. No ChR2-YFP+ cell bodies were observed in A1 (top right panel). Strong expression of ChR2 was observed in numerous Cre+/Tom+ cell bodies in A1-recipient SC neurons (bottom panels). Scale bars, 500 µm, top left panel and bottom left panel, 25 µm, bottom right panel.

(C) Superimposed average current traces for 15 Tom+ L5 neurons in A1. Blue bar indicates 10 ms LED pulse. No light evoked currents were observed in any of the recorded cells.

(D) Summary of LED evoked response amplitude for all 15 cells.

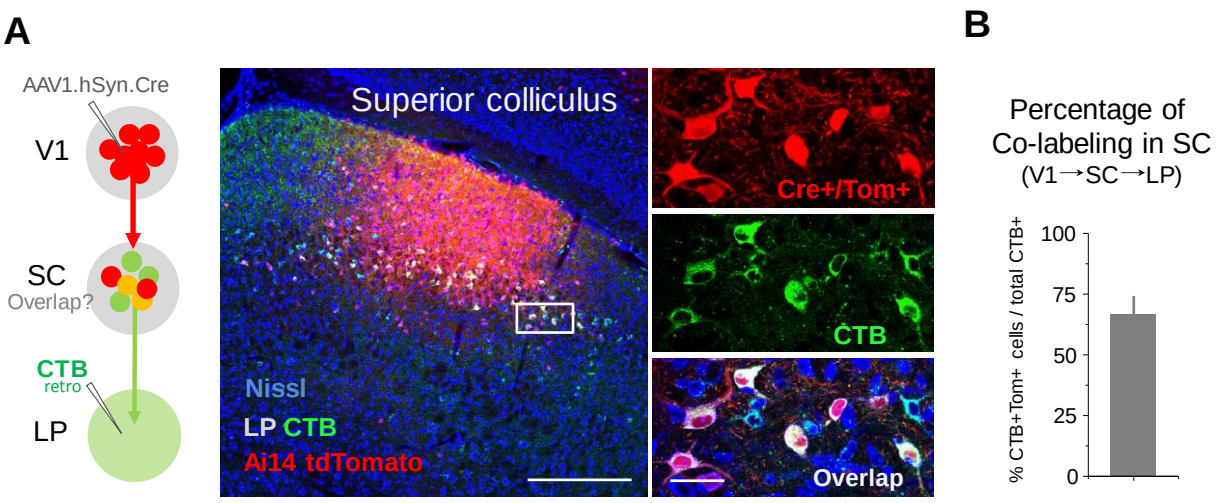

Figure S6, related to Figure 7. Efficiency of anterograde transneuronal labeling within the V1-SC-LP pathway.

(A) Schematic diagram of injections. AAV1-hSyn-Cre was injected into V1 in Ai14 tdTomato mice and fluorescently conjugated cholera toxin subunit b (CTB-488, green) was injected into LP to retrogradely label cell bodies in SC that project to LP. Numerous LP-projecting cell bodies were observed in the deepest part of superficial SC (middle and right panel, green) co-mingled with Cre+/Tom+ V1-recipient SC neurons (red). At 40X magnification, extensive co-labeling was observed. Scale bars, 250 µm, left panel, 25 µm, bottom right panel.

(B) Quantification of CTB co-labeled cells within the region of anterograde transneuronal labeling. An average of 66% of CTB+ cells were co-labeled with tdTomato (n =3 mice).

Supplemental Experimental Procedures

Animal preparation and stereotaxic surgery

All experimental procedures used in this study were approved by the Animal Care and Use Committee at the University of Southern California. Male and female C57BL/6J and Ai14 (Cre-dependent tdTomato reporter) mice (Jackson Laboratories) aged 2-6 months were used in this study. Mice were group housed in a light controlled (12 hr light: 12 hr dark cycle) environment with ad libitum access to food and water.

Stereotaxic injection of viruses was carried out as we previously described (Ibrahim et al., 2016; Liang et al., 2015; Xiong et al. 2015). Mice were anesthetized initially in an induction chamber containing 5% isoflurane mixed with oxygen and then transferred to a stereotaxic frame equipped with a heating pad. Anesthesia was maintained throughout the procedure using continuous delivery of 2% isoflurane through a nose cone at a rate of 1.5 liters/min. The scalp was shaved and a small incision was made along the midline to expose the skull. After leveling the head relative to the stereotaxic frame, injection coordinates based on the Allen Reference Atlas (Dong, 2007) were used to mark the location on the skull directly above the target area and a small hole (0.5mm diameter) was drilled. Viruses were delivered through pulled glass micropipettes with a beveled tip (inner diameter of tip: ~20 μm) using pressure injection via a micropump (World Precision Instruments). Total injection volume was 50 to 100 nl, at 15 nl/min. Following injection, the micropipette was left in place for approximately 5 mins to minimize diffusion of virus into the pipette path. After withdrawing the micropipette, the scalp was sutured closed and animals were administered ketofen (5mg/kg) to minimize inflammation and discomfort. Animals were recovered from anesthesia on a heating pad and then returned to their home cage.

For virus injection into the retina, animals were anesthetized and positioned in a stereotaxic frame as described above. Using a pulled glass micropipette with a beveled tip (40 μm inner tip diameter),

800 nl of virus was injected into the left eye at a rate of 20 nl/min at a depth of 0.7 mm from the surface of the lateral most curvature of the eye using a 40º angle of approach relative to the optic axis.

Injection of viruses for anterograde transneuronal labeling

To demonstrate the anterograde transneuronal properties of AAV, AAV2/1-hSyn-Cre-WPRE-hGH (UPenn Vector Core, 2.5 x 10^{13} GC/ml) was injected into either V1 (60 nl total volume; 3.9 mm posterior and 2.6 mm lateral to bregma and 0.5 mm ventral from the cortical surface) or retina (800 nl total volume) of Ai14 mice using the pressure injection method. Animals were euthanized 4 weeks following injection to allow time for viral transport and transgene expression. To characterize the temporal progress of the transport, mice were also euthanized at 2-day, 5-day, 2-week and 3-month post-injection time points.

To examine the potential anterograde transneuronal properties of other viruses, AAV2/1-CMV-Pl-Cre-rBG (UPenn Vector Core, 2.7 x 10^{13} GC/ml), AAV2/5-CMV-Pl-Cre-rBG (UPenn Vector Core, 2.8 x 10^{13} GC/ml), AAV2/6-CMV-Pl-Cre-rBG (UPenn Vector Core, 3.5 x 10^{13} GC/ml), AAV2/8-CMV-Pl-Cre-rBG (UPenn Vector Core, 4.4 x 10^{13} GC/ml), AAV2/9-CMV-Pl-Cre-rBG (UPenn Vector Core, 1.6 x 10^{14} GC/ml), AAV2/1-CB7-Cl-eGFP-WPRE-rBG (UPenn Vector Core, 4.2 x 10^{13} GC/ml), or CAV2-CMV-Cre (Montpellier Vector Core, 1.3 x 10^{12} GC/ml) was injected into V1 (60 nl total volume) of Ai14 mice (for Cre-expressing viruses) or wild-type C57BL/6J mice (for GFP-expressing virus). Original titers were used, while for testing dependence on titer AA1-CMV-Cre was also diluted (10X or 20X). Note that the original titer was lowest for AA1 among tested AAV serotypes all expressing CMV-driven Cre. Animals were euthanized 4 weeks following injection and postsynaptic structures were examined for the presence of cell body labeling.

To test for potential retrograde transport of AAV, injections of either AAV2/1-hSyn-Cre-WPRE-hGH (60 nl) or AAV2/1-CB7-Cl-eGFP-WPRE-rBG (60 nl) were made into SC (3.9 mm posterior and

0.8 mm lateral to bregma and 1.5 mm ventral from the cortical surface) of either Ai14 or wild-type C57BL/6J mice, respectively. Animals were euthanized 4 weeks following injection.

For mapping the axonal outputs of subpopulations of neurons in SC, AAV2/1-hSyn-Cre-WPRE-hGH was injected into V1, contralateral retina, A1 (3.1 mm posterior and 4.5 mm lateral to bregma and 0.75 mm ventral from the cortical surface) or M1 (0.5 mm anterior and 1.5 mm lateral to bregma and 0.5 mm ventral from the cortical surface) of Ai14 mice (60 nl total volume). Following 2 to 7 days, a second injection of AAV2/1-CAG-FLEX-eGFP-WPRE-bGH (UPenn vector core, 1.7 x 10^{13} GC/ml, originally created by Allen Institute) was made into the ipsilateral SC (3.9 mm posterior and 0.8 mm lateral to bregma and 1.5 mm ventral from the cortical surface; 60 nl total volume). The spacing of the two injections over several days was selected to allow sufficient time for the clearance of any residual AAV-Cre virus that may have spread across the pial surface in an effort to eliminate any local contamination of the Cre-dependent virus injection site. Animals were allowed to recover for at least 4 weeks following the second injection.

For behavioral testing, AAV2/1-hSyn-Cre-WPRE-hGH was injected into V1, A1, or SC, as described above. Following 2 to 7 days, a second injection of AAV2/1-EF1a-DIO-hChR2-eYFP (UPenn vector core, 1.6 x 10^{13} GC/ml) was made into SC (for A1 and V1 injections of AAV-Cre) or LP (for SC injections of AAV-Cre; 2.4 mm posterior and 1.6 mm lateral to bregma and 2.6 mm ventral to the cortical surface). Animals were then prepared for optogenetic testing 4 weeks after the second injection. To estimate the fraction of LP-projecting SC cells that are labeled following V1 injections of AAV-Cre, AAV2/1-hSyn-Cre-WPRE-hGH was injected into V1 of Ai14 tdTomato mice as described above, and LP was injected with cholera toxin subunit B, Alexa 488 (CTB-488, 100 nl injection volume, 0.5% solution in PBS, ThermoFisher) using coordinates described above. Animals were euthanized 4 weeks after injection.

For labeling glutamatergic and GABAergic SC neuronal populations receiving V1 input, AAV1-EF1a-DIO-Flp (1.5 x 10^{14} GC/ml, custom design, ViGene Biosciences) was injected into V1 (60 nl

total volume) of Vglut2-Cre (Slc17a6$^{tm2(cre)Lowl}$, Jackson Laboratories) or GAD2-Cre (Gad2$^{tm2(cre)Zjh}$, Jackson Laboratories) mice crossed with Ai14. Following 2-7 days, a second injection of AAVDJ-EF1a-fDIO-YFP (UNC Vector Core, 1.6 x 10^{13} GC/ml, originally created by Karl Deisseroth) was injected into SC (60 nl total volume). Animals were euthanized 4 weeks following the second injection.

Histology

Following desired post-injection survival time, animals were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. Brains were extracted and post-fixed for 24 hours at 4˚C in 4% paraformaldehyde and then sliced into 150 µm sections using a vibratome (Leica, VT1000s). The sections were serially mounted onto glass slides and coverslipped. For some experiments, a fluorescent Nissl stain was added (Neurotrace 640, Life Technologies) to reveal cell body location and cytoarchitectural information. To label astrocytes, brain sections were blocked with 10% donkey serum in PBS containing 0.1% Triton-X100 (PBST) for 1 hour, followed by overnight incubation at 4˚C in PBST with rabbit anti-GFAP antibody (Chemicon, 1:1000 dilution). Sections were then washed with PBS 3 times and incubated in PBST with donkey anti-rabbit Alexa Fluor 488 conjugated secondary antibody (Life Technologies, 1:200) for 2 hours at room temperature. To enhance Ai14 tdTomato signal in Vglut2-Cre+ neurons (Figure 8B), brain sections were treated as described above with rabbit anti-RFP antibody (Rockland, 1:500), followed by donkey anti-rabbit Alexa Fluor 555 conjugated secondary antibody (Life Technologies, 1:200).

Imaging and quantification

All images were generated using a confocal microscope (Olympus FluoView FV1000). To quantify the total number of cell bodies labeled in structures downstream of V1 or retina, serial sections across the whole brain were collected and examined. Regions with labeled cells were imaged at 10X magnification across the depth of the tissue (150 µm thickness, 15 µm z-stack interval) over all

sections containing labeling. TdTomato+ cell bodies that co-localized with fluorescent Nissl stain were manually identified and counted. The total number of tdTomato+ cells were counted across sections for each structure of interest. To estimate the percentage of tdTomato+ cells in innervated regions, 40X magnification images were taken across multiple sections for SC, LGNv, Str, and PN. A region of interest of fixed size was defined for each structure (Str = 150 x 200 μm, PN = 100 x 200 μm, and SC and LGNv = 200 x 300 μm area) and the total number of Tomato+ cells and Nissl+ cells were quantified within the region (see Figure S2). We chose to focus this quantification on restricted local regions with Tomato labeling, rather than the entire structure, as only a small portion of each target structure is innervated by a given V1 injection due to the topographic nature of its output. We defined the central regions of labeling in each structure to capture the majority of labeled neurons for this estimation. To quantify the percentage of YFP+/Tomato+ GABAergic or glutamatergic cells in SC (Figure 8E), 40X magnification images were collected and a 200 x 300 μm defined region of interest centered on the region with YFP labeling was assigned to each image. All YFP+/Tomato+ cells were manually counted and compared to the total number of Cre+/Tomato+ cells found within each region of interest. To quantify the percentage of LP-projecting SC cells labeled anterograde transneuronally with AAV-Cre injections in V1 (Figure S6), 40X magnification images were taken across all sections of SC containing Tomato+ cells. All CTB+ and CTB+/Tomato+ cells were quantified manually within the local region of Tomato+ labeling.

Slice preparation and recording

To confirm synaptic connectivity to anterogradely labeled cells in the striatum (Figure 4B) and SC (Figure S3), a 1:1 mixture of AAV2/1-hSyn-Cre-WPRE-hGH and AAV2/1-EF1a-DIO-hChR2-eYFP was injected into V1 of Ai14 mice (80 nl total volume). Following a 4 week post-injection survival time, acute brain slices containing striatum or SC were prepared. Following urethane anesthesia, the animal was decapitated and the brain was rapidly removed and immersed in an ice-cold dissection

buffer (composition: 60 mM NaCl, 3mM KCl, 1.25 mM NaH_2PO4, 25 mM $NaHCO_3$, 115 mM sucrose, 10 mM glucose, 7 mM $MgCl_2$, 0.5 mM $CaCl_2$; saturated with 95% O_2 and 5% CO_2; pH= 7.4). Brain slices of 350 μm thickness containing the rostral striatum were cut in a coronal plane using a vibrating microtome (Leica VT1000s). Slices were allowed to recover for 30 min in a submersion chamber filled with the warmed (35 °C) ACSF and then to cool gradually to the room temperature until recording. The spatial expression pattern of ChR2-EYFP in each slice was examined under a fluorescence microscope before recording. Striatal and SC neurons were visualized with IR-DIC and fluorescence microscopy (Olympus BX51 WI) for specific targeting of tdTomato+ neurons surrounded by EYFP+ fluorescent fibers. Patch pipettes (Kimax) with ~4-5 MΩ impedance were used for whole-cell recordings. Recording pipettes contained: 130 mM K-gluconate, 4 mM KCl, 2 mM NaCl, 10 mM HEPES, 0.2 mM EGTA, 4 mM ATP, 0.3 mM GTP, and 14 mM phosphocreatine (pH, 7.25; 290mOsm). Signals were recorded with an Axopatch 200B amplifier (Molecular Devices) under voltage clamp mode at a holding voltage of –70 mV for excitatory currents or 0 mV for inhibitory currents, filtered at 2 kHz and sampled at 10 kHz. 1 μM tetrodotoxin (TTX) and 1 mM 4-aminopyridine (4-AP) was added to the external solution for recording only monosynaptic responses (Petreanu et al. 2009) to blue light stimulation (3-10 ms pulse, 3 mW power, 10-30 trials, delivered via a mercury Arc lamp gated with an electronic shutter). To test if ChR2 was expressed in connected SC neurons, glutamate receptor antagonist DNQX (20 μM, Sigma-Aldrich) was added to the bath following demonstration of LED evoked synaptic responses in patched neurons. LED pulses were then delivered as before to test for non-synaptic, light evoked currents.

In vivo optogenetic preparation and stimulation

To examine whether SC neuron subgroups mediate different behavioral responses, mice were implanted with an optical fiber (200 μm diameter, Thorlabs) three weeks after secondary injection of AAV2/1-EF1a-DIO-hChR2-eYFP in SC, following our previous study (Xiong et al. 2015). Briefly,

mice were anesthetized with isoflurane and mounted into a stereotaxic apparatus. A small hole (~500 µm diameter) was drilled in the skull directly above the targeted region and the optical fiber was lowered to the desired depth and fixed in place using dental cement. For activating ChR2-expressing neurons in superficial SC, the fiber was positioned 3.9 mm posterior and 0.6 mm lateral to bregma, and 0.9 mm ventral from the cortical surface. For activating SC neurons in deep layers, the fiber was positioned as above, but lowered to a depth of 1.6 mm below the cortical surface. For activating the SC axonal projection to LP, or LP neurons directly, the optical fiber was positioned at 2.4 mm posterior and 1.6 mm lateral to bregma and 2.1 mm ventral to the cortical surface. Animals were allowed to recover for 5-7 days prior to behavioral testing. During test sessions, the implanted optical fiber was connected to a patch cord fiber secured with a plastic sleeve (Thorlabs, 200 µm Core, 0.22 NA (numerical aperture)). The latter fiber was equipped with an integrated rotary joint (Thorlabs) and was supported from above to allow the animal to move freely in the chamber without being hindered. To activate ChR2-expressing neurons or axons, optical stimulation was delivered using a blue LED source (470 nm, 5 mW, Thorlabs) at a rate of 20 Hz (20 ms pulse) for a duration of 5 seconds. Following testing sessions, animals were euthanized and each brain was sectioned and imaged to verify the specificity of ChR2 expression and location of the implanted fiber.

Behavioral testing and quantification

Escape behaviors were tested in a box containing two chambers connected by a small opening, as we previously described (Xiong, et al., 2015). Mice (n = 7 mice for A1-SC, n = 5 mice for V1-SC) were allowed to acclimate to one chamber for 10 minutes prior to behavioral testing. During this time, the opening connecting the two chambers was blocked with a removable door. After 10 mins the door was removed and the mouse was free to explore the adjacent, novel chamber. Following complete entry into the novel chamber, 5-s 20-Hz blue LED stimulation or 5-s 70 dB SPL white noise was applied. The animal behavior was recorded with a camera mounted above the box. If the mouse went back to

the home chamber within 5 s of noise or LED stimulation, it was considered as a successful escape trial. If not, it was a failure trial. Test process was repeated for 4 to 6 times depending on the willingness of the animal to enter the novel chamber after the door removal. Between trials, animals were allowed to rest for at least 5 mins. Each animal was tested for 2 sessions separated by one day. Escape rate was calculated as the fraction of total trials for each animal that evoked escape.

Freezing behavior was tested in a single or double chamber box with a camera on top recording the whole process (n = 5 mice for V1-SC, n = 6 mice for SC-LP). After the animal explored the single chamber or the novel chamber for 5 minutes, 5-s 20-Hz blue LED stimulation was applied. Each session contained 4-6 trials, with an interval of at least 5 mins. Each animal was tested for 2 sessions separated by one day. The trial number depended on the activity level of the mouse, and a session would end if the animal stayed unmoved in the corner for more than 1 min. If the animal stayed motionless for more than 1.5 s after the onset of stimulation, it was considered as a successful freezing response (Shang et al., 2015; Tovote et al., 2016; Wei et al., 2015; Wolff et al., 2014). Freezing time was quantified for each animal as the fraction of time spent freezing during the optogenetic stimulation (total freezing time/ total LED stimulation time). Freezing rate was calculated as the fraction of total trials that evoked freezing.

Control subjects for each behavioral test (sham, n = 5 each) received a sham injection of AAV2/1-CAG-FLEX-GFP, and were then implanted with an optical fiber and tested with the same stimulation parameters. In this study, the optogenetic stimulation was unilateral, but was sufficient for inducing defense behaviors such as flight and freezing, which is consistent with previous studies (Comoli et al., 2012; Dean et al., 1988; Liang et al., 2015; Sahibzada et al, 1986). For escape responses, we did not find a correlation between the direction in which the body turned and the side of SC that was stimulated (data not shown).