Organization of long-range inputs and outputs of frontal cortex for top-down control

Siyu Zhang1,2, Min Xu1,2, Wei-Cheng Chang1, Chenyan Ma1, Johnny Phong Hoang Do1, Daniel Jeong1, Tiffany Lei1, Jiang Lan Fan1 & Yang Dan1

Long-range projections from the frontal cortex are known to modulate sensory processing in multiple modalities. Although the mouse has become an increasingly important animal model for studying the circuit basis of behavior, the functional organization of its frontal cortical long-range connectivity remains poorly characterized. Here we used virus-assisted circuit mapping to identify the brain networks for top-down modulation of visual, somatosensory and auditory processing. The visual cortex is reciprocally connected to the anterior cingulate area, whereas the somatosensory and auditory cortices are connected to the primary and secondary motor cortices. Anterograde and retrograde tracing identified the cortical and subcortical structures belonging to each network. Furthermore, using new viral techniques to target subpopulations of frontal neurons projecting to the visual cortex versus the superior colliculus, we identified two distinct subnetworks within the visual network. These findings provide an anatomical foundation for understanding the brain mechanisms underlying top-down control of behavior.

RESULTS
To label the long-range inputs and outputs of each cortical area with fluorescent proteins, we injected various viral vectors into the mouse brain, as detailed below. After histological sectioning and fluorescence imaging, each brain sample was aligned to the Allen mouse brain, as detailed below. After histological sectioning and fluorescence imaging, each brain sample was aligned to the Allen Mouse Brain Atlas to facilitate 3D whole-brain visualization and quantitative analyses (Fig. 1; also see Online Methods). The labeled neurons and axons were detected, and their locations were registered in the reference atlas (Table 1 and Supplementary Table 1).

To facilitate data visualization at multiple levels of detail, we also used interactive sunburst diagrams (adapted from Allen Mouse Brain Atlas, http://www.brain-map.org/api/examples/examples/sunburst/) to represent the distribution of labeled inputs and outputs in all brain structures (http://top-down-network.org/). The brain structures are arranged hierarchically from inner to outer circles in the diagram.

1Division of Neurobiology, Department of Molecular and Cell Biology, Helen Wills Neuroscience Institute, Howard Hughes Medical Institute, University of California, Berkeley, California, USA. 2These authors contributed equally to this work. Correspondence should be addressed to Y.D. (ydan@berkeley.edu).

Received 15 January; accepted 15 September; published online 17 October 2016; doi:10.1038/nn.4417
and the size of each sector represents the percentage of labeling in the corresponding structure. The numerical values can be read out by pointing the cursor, and each region of interest can be expanded with a mouse click.

**Identification of frontal regions for each sensory modality**

To identify the frontal regions directly innervating each sensory cortical area, we used rabies virus (RV)-mediated trans-synaptic retrograde tracing, which labels monosynaptic inputs to selected starter cells with high specificity\(^{16,17}\). Avian-specific retroviral receptor (TVa), enhanced green fluorescent protein (EGFP) and rabies glycoprotein (RG) were expressed by injecting two Cre-inducible adeno-associated virus (AAV) vectors (AAV2-EF1α-DIO-TVA-EGFP and AAV2-CAG-DIO-RG) into the primary visual (VIS), somatosensory (SS) or auditory (AUD) cortex of Camk2a-Cre mice (Fig. 2a). Two weeks later, we injected a modified RV expressing tdTomato (RV-ΔG-tdTomato+EnvA), which only infects cells expressing TVa and requires RG to spread retrogradely to presynaptic cells.

**Figure 2b** shows examples of starter cells (expressing both tdTomato and EGFP) in each injected sensory area. Across brain samples, the starter cells were distributed over large portions of VIS, SS and AUD (Supplementary Fig. 1), with similar laminar distributions among these areas (Supplementary Fig. 2). Trans-synaptically labeled presynaptic neurons (expressing tdTomato only) were found in multiple cortical and subcortical regions (Supplementary Fig. 3, Supplementary Table 1, Supplementary Video 1 and interactive sunburst diagram at [http://top-down-network.org/]). Within the frontal cortex, neurons trans-synaptically labeled from VIS were found primarily in the anterior cingulate area (ACA) and the medial portion of secondary motor cortex (MOS), whereas those presynaptic to SS and AUD were mainly located in the primary motor cortex (MOp) and the lateral portion of MOS (Fig. 2b–d and Supplementary Fig. 4). In all three modalities, inputs from the frontal cortex arose primarily from layers 2/3 and 5, consistent with previous studies\(^{3,5,8}\).

We next mapped the brain regions receiving direct axonal projections from each sensory area by injecting AAV expressing mCherry (AAV2-Camk2a-mCherry) into VIS, SS or AUD of wild-type mice (Fig. 2e–h, Supplementary Fig. 5, Supplementary Table 1, Supplementary Video 2 and sunburst diagram at [http://top-down-network.org/]). Within the frontal cortex, labeled axons from VIS were found mostly in ACA and medial MOS\(^{18}\), and those from SS or AUD were distributed primarily in MOp and lateral MOS (Fig. 2f–h). The spatial correspondence between the axonal projections of each sensory area (Fig. 2g,h) and its retrogradely labeled presynaptic neurons (Fig. 2c,d) indicates strong reciprocity of long-range corticocortical connections\(^{10}\). These anterograde and retrograde tracing experiments

| Abbreviation | Definition |
|--------------|------------|
| ACA          | Anterior cingulate area |
| AI           | Agranular insular area |
| AUD          | Auditory areas |
| ECT          | Ectorhinal area |
| ILA          | Infrahilimbic area |
| MO           | Somatomotor areas (MOS + MOp) |
| MOp          | Primary motor area |
| MOS          | Secondary motor area |
| ORB          | Orbital area |
| PL           | Prelimbic area |
| PTLp         | Posterior parietal association areas |
| RSP          | Retrosplenial area |
| SS           | Somatosensory areas |
| TEa          | Temporal association area |
| VIS          | Visual areas |
| AD           | Anterodorsal nucleus |
| AM           | Anteromedial nucleus |
| AV           | Anteroventral nucleus |
| LD           | Lateral dorsal nucleus |
| LGd          | Dorsal part of the lateral geniculate complex |
| LGv          | Ventral part of the lateral geniculate complex |
| LP           | Lateral posterior nucleus |
| MD           | Mediodorsal nucleus |
| MG           | Medial geniculate complex |
| RT           | Reticular nucleus |
| PF           | Parafascicular nucleus |
| PO           | Posterior complex |
| VAL          | Ventral anterior-lateral complex |
| VM           | Ventral medial nucleus |
| VP           | Ventral posterior complex |
| PAL          | Pallidum |
| SC           | Superior colliculus |
| STR          | Striatum |
also indicated a clear segregation between the frontal areas connected to the visual (ACA and medial MOs) and the somatosensory and auditory cortices (MOp and lateral MOs).

**Other long-range connections of ACA and MO**

In addition to sensory cortices, the frontal areas also project to many other brain structures. To label these projections, we injected AAV

---

**Figure 2** Mapping connections between sensory and frontal cortices. (a) Viral vectors and injection procedure for RV-mediated trans-synaptic retrograde tracing from sensory cortices. (b) Upper panel, injection sites in VIS, SS and AUD (scale bars, 1 mm). Inset, enlarged view of region in white box; starter cells, yellow (scale bars, 200 µm). Lower panel, fluorescence images of ACA and MO (yellow box in coronal diagram) showing RV-labeled input neurons (red) to each sensory area (scale bars, 200 µm). Inset, enlarged view of region in white box (scale bars, 40 µm). Green, EGFP; red, tdTomato; blue, DAPI. (c) Percentages of input neurons in ACA, MOs, MOp retrogradely labeled from VIS (blue, n = 3 mice), SS (red, n = 3), AUD (green, n = 3). Each circle represents one mouse. Error bars, ± s.e.m. (d) Summary of RV-labeled neurons in all samples of each group (scale bars, 1 mm). Dots, detected neurons; white masks, injection sites excluded from analysis. (e) Viral vector and injection procedure for tracing the axonal projections from each sensory area. (f) Upper panel, injection sites in VIS, SS, AUD (scale bars, 1 mm). Lower panel, fluorescence images of ACA and MO (yellow box in coronal diagram) showing axons from each sensory area (scale bars, 200 µm). Red, mCherry; blue, DAPI. (g) Percentages of labeled axons in ACA, MOs, MOp from VIS (n = 3), SS (n = 3), AUD (n = 3). Each circle represents one mouse. Error bars, ± s.e.m. (h) Summary of axons detected in all samples of each group (scale bars, 1 mm); white masks, injection sites excluded from analysis.
expressing mCherry (AAV2-Camk2a-mCherry) into ACA or MO (Fig. 3a–d, Supplementary Fig. 6 and Supplementary Table 1). Among the sensory cortical areas, ACA projected extensively to VIS and sparsely to SS and AUD, whereas MO projected extensively to SS and moderately to AUD and VIS, consistent with retrograde tracing from these sensory areas (Fig. 2b–d). Among other cortical areas, ACA projected extensively, and MO moderately, to the posterior parietal cortex (PTLp) and the retrosplenial area (RSP) (Fig. 3c). While PTLp made reciprocal connections with VIS, SS and AUD, RSP was densely connected only with VIS (Supplementary Figs. 3 and 5), suggesting that it belongs mainly to the visual network. Within the frontal cortex, the prelimbic/infralimbic area (PL/ILA) received more input from ACA and the orbital area (ORB) received more input from MO (Fig. 3c).

Figure 3 Whole-brain distributions of axonal projections and input neurons of ACA and MO. (a) Injection procedure for tracing projections. (b) Fluorescence images of ACA and MO (red box in coronal diagram) at injection sites (scale bars, 200 µm). Red, mCherry; blue, DAPI. (c) Axons detected in all samples of each group (MO, yellow; ACA, magenta; overlap, white; scale bar, 1 mm); white masks, injection sites excluded from analysis. (d) Percentages of labeled axons in cortical and subcortical structures (MO, n = 3; ACA, n = 3). Included are cortical areas with >1% labeling and thalamic structures with >0.8% labeling. Error bars, ± s.e.m. (e) Injection procedure for RV-mediated retrograde tracing. (f) Fluorescence images of ACA and MO (red box in coronal diagram) at injection sites (scale bars, 200 µm). Inset, enlarged view of region in white box showing starter cells (yellow; scale bar, 10 µm). Green, EGFP; red, tdTomato; blue, DAPI. (g) RV-labeled neurons detected in all samples of each group (MO, yellow; ACA, magenta; scale bar, 1 mm); white masks, injection sites excluded from analysis. (h) Percentages of retrogradely labeled neurons in selected cortical and subcortical regions (MO, n = 3; ACA, n = 3). Included are areas with >2% (cortical) or >1% (thalamic) labeling. Error bars, ± s.e.m.
In the thalamus, the projections from ACA and MO were segregated mainly along the dorsal–ventral axis. For example, ACA projected more to the lateral posterior and lateral dorsal (LP/LD, combined in the analysis) thalamic nuclei, whereas MO projected more to posterior (PO), ventral posterior (VP) and ventral anterior-lateral and ventral medial (VAL/VM, combined in the analysis) complex. Both ACA and MO projected to the mediodorsal (MD) nucleus, which is densely connected to the prefrontal cortex. Other major subcortical targets of the frontal regions included the striatum (STR) and superior colliculus (SC; Fig. 3d), with partially overlapping projections from ACA and MO (Fig. 3c).

To map the inputs to these frontal areas, we injected the AAV and RV vectors for trans-synaptic retrograde tracing (as in Fig. 2a) into ACA or MO of Camk2a-Cre mice (Fig. 3e–h, Supplementary Fig. 6, Supplementary Video 4 and Supplementary Table 1). Among the sensory cortices, MO injection led to dense labeling in SS but little labeling in VIS and AUD, whereas ACA injection caused the densest labeling in VIS (Fig. 3b), consistent with the anterograde tracing results (Fig. 2f,g). Among other cortical areas, we found extensive inputs from PTLp and RSP to ACA but only sparse inputs to MO (Fig. 3g,h), further attesting to their membership in the visual network (Fig. 4). Within the frontal cortex, PL/ILA provided more input to ACA than to MO, and ORB provided similar inputs to both regions. The striking similarity between the cortical distributions of inputs to (Fig. 3g,h) and projections from (Fig. 3c,d) each frontal region again demonstrates the reciprocity of corticocortical connections.

In the thalamus, the distributions of inputs to ACA and MO also largely mirrored the distributions of their axonal projections (Fig. 3c,d). ACA received more inputs from LP/LD, whereas MO received more inputs from PO, VP and VAL/VM (Fig. 3g,h). By contrast, MO projected to both ACA and MO, as expected for these prefrontal cortical areas. Since LP/LD is reciprocally connected to both ACA and VIS (Fig. 3c,d,g,h and Supplementary Figs. 3 and 5), it forms an integral part of the visual network (Fig. 4a). PO, VP and VAL/VM are connected to both MO and SS, suggesting that they are part of the somatosensory network (Fig. 4b). Among other subcortical regions, the pallidum projected to both ACA and MO (Fig. 3g,h), consistent with the known cholinergic and noncholinergic projections from the basal forebrain to the entire cortex.

Besides excitatory neurons, several subtypes of inhibitory interneurons have been implicated in long-range corticocortical interactions. To determine whether the long-range axonal projections directly innervate these interneuron subtypes in each cortical area, we injected the AAV and RV vectors for trans-synaptic tracing into VIS, RSP, PTLp and ACA of Pvalb-, Sst- and Vip-Cre mice (Supplementary Table 1 and sunburst diagram at http://top-down-network.org/). Like excitatory neurons, all three subtypes of inhibitory neurons in VIS, PTLp and RSP received monosynaptic inputs from ACA (Supplementary Fig. 7 and Fig. 2b–d), and all of them in ACA received monosynaptic inputs from VIS, PTLp and RSP (Supplementary Fig. 8 and Fig. 3f–h). Thus, both top-down and bottom-up corticocortical projections directly recruit inhibitory interneurons in their target areas.

Outputs of VIS- and SC-projecting ACA neurons

Different projections from each cortical area in some cases originate from different subsets of neurons, but in other cases they may reflect axon collaterals of the same neurons. Distinguishing these possibilities is crucial for understanding how the frontal cortex coordinates the activity of different brain areas for top-down executive control. This issue has not been addressed systematically in previous efforts mapping mesoscale mouse brain connectivity. In particular, we wondered what other brain regions are also innervated by the frontal cortical neurons projecting to sensory cortex.

We focused this analysis on the ACA neurons projecting to VIS (ACA\textsubscript{VIS} neurons). To label these neurons and their axons, we injected the AAV vector expressing TVA (AAV2-EF1α-DIO-TVA-EGFP) into ACA of Camk2a-Cre mice, but the RV vector (RV-ΔG-tdTomato+EnvA) into VIS 2 weeks after AAV injection (Fig. 5a). This allowed RV to enter the TVA-expressing ACA axon terminals in VIS, be transported retrogradely to the ACA neurons and label all their axon collaterals with tdTomato (Fig. 5b). To enhance the visibility of labeled thin axons, we performed immunostaining for tdTomato to convert the fluorescence signal into nickel-enhanced diaminobenzidine signal.

In addition to the dense projection to VIS, we found that the ACA\textsubscript{VIS} neurons also projected extensively to PTLp and moderately to RSP (Fig. 5c,e, Supplementary Table 1 and Supplementary Video 5). This suggests that similar modulatory signals are broadcast to VIS, PTLp and RSP, pointing to a tightly coordinated ACA–PTLp–RSP–VIS network for visual processing. In contrast, the projection from the ACA\textsubscript{VIS} neurons to PL/ILA was much sparser than that from the entire ACA population (Fig. 5e). The thalamus also received very little inputs from ACA\textsubscript{VIS} neurons (Fig. 5c,e), suggesting that it is not strongly and directly influenced by the modulatory signals sent to VIS. Furthermore, while the SC received a sizable projection from ACA (Fig. 3d), we found few labeled axons from the ACA\textsubscript{VIS} neurons, suggesting that the SC projection originates from a separate ACA neuron population. This is consistent with a finding based on retrograde tracing from VIS and SC in primates. Targeting the MO\textsubscript{SS} neurons using the same technique revealed a similar level of selective axonal projections (Supplementary Fig. 9).

The SC is also known to be important in top-down attentional modulation. We thus examined the outputs of the SC-projecting ACA (ACA\textsubscript{SC} neurons) by injecting the AAV vector expressing TVA into ACA and RV vector into SC (Fig. 5a). Unlike the ACA\textsubscript{VIS} neurons, which were distributed in both layers 2/3 and 5 (Fig. 5b), the ACA\textsubscript{SC} neurons were found primarily in layer 5 (Fig. 5b). These spatial distributions are similar to those of intratelencephalic and pyramidal tract neurons in the motor cortex, which form non-overlapping populations of projection neurons with distinct roles in motor control. We found little projection from the ACA\textsubscript{SC} neurons to VIS (Fig. 5d,e). In addition, the ACA\textsubscript{VIS} but not ACA\textsubscript{SC} neurons projected to the contralateral ACA through the corpus callosum.
Figure 5  Whole-brain distributions of axonal projections from ACA_{VIS} and ACA_{SC} neurons. (a) Viral vectors and injection procedure. (b) Upper panel: left, bright field image of ACA and MO showing RV-labeled neurons from VIS (scale bars, 200 µm). Inset, enlarged view of region in red box (scale bar, 20 µm). Immunostaining for tdTomato was performed to convert fluorescence signal into nickel-enhanced diaminobenzidine signal; right, images of VIS and PTLp (red box in coronal diagram), showing RV-labeled axons of ACA_{VIS} neurons (scale bar, 100 µm). Lower panel, as in upper panel, but the ACA neurons were labeled by RV injection into SC (left), and axons from ACA_{SC} neurons are concentrated in PL/ILA and LP (right). (c) Axons detected in all ACA_{VIS} samples (scale bar, 1 mm); white masks, injection sites excluded from analysis. (d) As in c, for axons of ACA_{SC} neurons (scale bar, 1 mm). (e) Percentages of labeled axons in selected cortical and subcortical structures (ACA, n = 3; ACA_{VIS}, n = 3; ACA_{SC}, n = 3). Error bars, ± s.e.m. Data for ACA axons are the same as in Figure 2d, shown here for comparison.
Figure 6  Whole-brain distributions of inputs to ACA→VIS and ACA→SC neurons. (a) Viral vectors and injection procedure. (b) Left, fluorescence images of AAV-mCherry-IRES-WGA-Cre injection site in VIS (upper) or SC (lower) (scale bars, 1 mm). Middle, injection site of other AAVs and RV in ACA (scale bars, 1 mm). Inset, enlarged view of region in white box showing AAV/RV infected neurons (green; scale bars, 20 µm). Right, retrogradely labeled neurons (green) in VIS and PL/ILA (scale bars, 200 µm). Inset, enlarged view of region in white box (scale bars, 20 µm). Green, EGFP; red, mCherry; blue, DAPI. (c) RV-labeled neurons detected in all ACA→VIS samples (scale bar, 1 mm); white masks, injection sites excluded from analysis. (d) As in c, for inputs to ACA→SC neurons (scale bar, 1 mm). (e) Percentages of retrogradely labeled neurons in selected cortical and subcortical brain structures (ACA, n = 3 mice; ACA→VIS, n = 4; ACA→SC, n = 4). Error bars, ± s.e.m. Data for ACA inputs are the same as in Figure 2h.
We found that $\text{ACA} \rightarrow \text{VIS}$ but not $\text{ACA} \rightarrow \text{SC}$ neurons received extensive monosynaptic inputs from VIS (Fig. 6b–e). Such selective innervation of sensory cortex-projecting neurons was also found in the somatosensory $\rightarrow$ motor cortex connection (Supplementary Fig. 15), resulting in a recurrent loop between the sensory cortex and a subset of frontal cortical neurons. Among other cortical areas, inputs from PL and ILA were much more extensive for $\text{ACA} \rightarrow \text{SC}$ neurons, whereas those from PTLp were much more extensive for $\text{ACA} \rightarrow \text{VIS}$ neurons (Fig. 6c–e), matching the distributions of axonal projections of the two ACA subpopulations (Fig. 5c–e). Together, these findings suggest that the $\text{ACA} \rightarrow \text{VIS}$ neurons have enhanced reciprocal connections with the posterior sensory and association areas, whereas the $\text{ACA} \rightarrow \text{SC}$ neurons are preferentially connected to the medial prefrontal cortex (Fig. 7, Supplementary Table 1 and Supplementary Video 6). Thus, within the visual network, there are two subnetworks involving separate populations of ACA neurons.

From the thalamus, the $\text{ACA} \rightarrow \text{VIS}$ neurons received more inputs than $\text{ACA} \rightarrow \text{SC}$ neurons (Fig. 6e), opposite to the relative strengths of their projections to the thalamus (Fig. 5e). Thus the corticothalamic connections are much less reciprocal than the corticocortical connections (Fig. 7). Finally, we found inputs from the pallidum to $\text{ACA} \rightarrow \text{VIS}$ neurons but not to $\text{ACA} \rightarrow \text{SC}$ neurons.

**DISCUSSION**

Using a variety of virus-assisted circuit tracing techniques, we have mapped the long-range inputs and outputs of the frontal cortical regions that are directly connected to the visual, somatosensory and auditory cortices. Both anterograde and retrograde tracing from the sensory areas indicate a clear spatial segregation between the frontal neurons connected to the visual cortex ($\text{ACA}$) and those connected to somatosensory and auditory cortices (MO) (Fig. 2). Anterograde and retrograde tracing from $\text{ACA}$ and MO allowed us to delineate separate brain networks associated with different sensory modalities (Figs. 3 and 4). Furthermore, within the visual network, we identified two distinct subnetworks, involving subpopulations of ACA neurons that project to the visual cortex or to the superior colliculus (Figs. 5–7).

Our anterograde tracing approach is similar to that used for generating the Allen Mouse Brain Connectivity Atlas, but we focused on the brain networks for top-down modulation of sensory processing. In addition, we complemented anterograde tracing of axonal projections with RV-mediated retrograde tracing of input neurons. The results of these different tracing strategies are highly consistent with each other. The distributions of thalamic inputs to $\text{ACA}$ and MO mapped with RV tracing (Fig. 3h) are also broadly consistent with a recent mapping study using anterograde tracing from the thalamus.

The visual and somatosensory/auditory networks we have identified using viral tracers generally correspond to the medial and somatic sensorimotor networks mapped in the Mouse Connectome Project using nonviral tracers. However, there are some noticeable differences. While Zingg et al. grouped the visual and auditory areas into the same medial network and the somatosensory cortex in a separate network, we found that MOp and lateral MOs were connected to both AUD and SS whereas ACA and medial MOs were connected mainly to VIS (Fig. 2). This difference may reflect different emphases of the two studies; while Zingg et al. performed cluster analysis of all corticocortical connections, our study focused on the frontal–sensory cortex connections. In addition, preferential labeling of different neuronal subtypes by viral vs. nonviral tracers may also contribute to the difference between the two studies. Although the somatosensory and auditory networks overlap spatially in the frontal

**Figure 7** Schematic diagram of visual subnetworks. Shown are major inputs and outputs of $\text{ACA} \rightarrow \text{VIS}$ and $\text{ACA} \rightarrow \text{SC}$ neurons. The putative unidirectional connection from $\text{ACA} \rightarrow \text{VIS}$ to $\text{ACA} \rightarrow \text{SC}$ neurons (dashed blue line) was based on previous literature. (Supplementary Video 5), further supporting the correspondence between $\text{ACA} \rightarrow \text{VIS}$/$\text{ACA} \rightarrow \text{SC}$ and intratelencephalic/pyramidal tract neurons. Compared to the $\text{ACA} \rightarrow \text{VIS}$ neurons, the $\text{ACA} \rightarrow \text{SC}$ neurons projected much less to PTLp but more to PL/ILA (Fig. 5d). Thus, the two subpopulations of ACA neurons show distinct cortical projection patterns.

For the thalamic nuclei innervated by ACA, the inputs from $\text{ACA} \rightarrow \text{SC}$ neurons were much more extensive than those from $\text{ACA} \rightarrow \text{VIS}$ neurons (Fig. 5c–e). In contrast, the striatum received extensive projections from both $\text{ACA} \rightarrow \text{VIS}$ and $\text{ACA} \rightarrow \text{SC}$ neurons, and their spatial distributions largely overlapped. This is reminiscent of the striatal projections from both intratelencephalic and pyramidal tract neurons in the ipsilateral cortex.

To assess whether $\text{ACA} \rightarrow \text{VIS}$ and $\text{ACA} \rightarrow \text{SC}$ neurons form synapses in the identified areas, we expressed membrane-bound GFP (mGFP, for labeling axons) and synaptophysin-mRuby (SYP-mRuby, for labeling putative presynaptic sites) in these neurons. CAV-FLEXloxP-Flp was injected into VIS or SC and AAV-hSyn1-FLExFRT-mGFP-2A-synaptophysin-mRuby was injected into ACA of Camk2a-Cre mice for Flp-dependent expression of mGFP and SYP-mRuby (Supplementary Fig. 10). We found synaptophysin-mRuby labeling in all the major cortical and subcortical areas identified above (Fig. 5e), indicating synaptic innervation of those areas.

**Inputs to VIS- and SC-projecting ACA neurons**

The top-down signals from $\text{ACA} \rightarrow \text{VIS}$ and $\text{ACA} \rightarrow \text{SC}$ neurons to their distinct postsynaptic targets are determined by their respective inputs. To map the monosynaptic inputs to each subpopulation, we injected AAV expressing the transcellular tracer protein wheat germ agglutinin (WGA) fused with Cre recombinase (AAV2-EF1α-mCherry-IRESCamK2-Cre) into VIS or SC of wild-type mice, and AAV vectors with Cre-inducible expression of TVA and RG (as in Fig. 2a) into their ACA. Four weeks after these AAV injections, RV expressing EGFP was injected into ACA (Fig. 6a). This viral strategy ensured that TVA and RG were expressed only in the $\text{ACA} \rightarrow \text{VIS}$ or $\text{ACA} \rightarrow \text{SC}$ neurons retrogradely labeled with Cre recombinase, thus restricting RV labeling to their presynaptic inputs (Supplementary Figs. 11–14).
cortex, they are largely separate in other brain regions. For example, in the thalamus AUD received extensive inputs from MG but SS mainly from VP, VAL and VM. Even in the frontal cortex, the neurons connected to AUD are likely to be distinct from those connected to SS at the level of single cells. Of course, it is also important to note that, in addition to their distinct connections, the visual, somatosensory and auditory networks also receive shared inputs and project to common targets in both the cortex (for example, PTlp and ORB) and thalamus (for example, MD), allowing cross-talk between the different sensory modalities.

While ACA→VIS neurons make dense reciprocal connections with sensory and association areas, ACA→SC neurons are preferentially connected to PL and ILA (Figs. 5 and 6). These medial prefrontal areas are known to be important for the control of actions27,36, and the SC is crucial for controlling saccadic eye movement37,38. Thus the two subnetworks may be specialized in different functions, one for sensory perception (RSP&PTlp ↔ ACA ↔ VIS ↔ RSP&PTlp, the ‘perception subnetwork’) and the other for action control (RSP&PL/ILA ↔ ACA → SC, the ‘action subnetwork’). Notably, we found inputs from the pallidum to ACA→VIS but not ACA→SC neurons (Fig. 6e). Cholinergic and noncholinergic pallidal neurons project widely to the cortex and are important to brain-state-dependent modulation of sensory processing39–43. Their selective innervation of ACA→VIS neurons should allow preferential regulation of the perception subnetwork in a brain-state-dependent manner. In contrast, RSP provides dense inputs to and thus may regulate the activity of both subnetworks.

In both frontal and sensory cortices, intratelencephalic (IT) neurons provide extensive inputs to, but receive little innervation from, pyramidal tract (PT) neurons24,32,34–46, suggesting a nonreciprocal connection from ACA→VIS to ACA→SC neurons. IT and PT neurons also differ in other cellular properties, with, for example, greater hyperpolarization-activated current (Ih) and faster action potentials in PT than IT neurons24. Notably, ACA→SC neurons provide much more projections to the thalamus (Fig. 5e), whereas ACA→VIS neurons receive more thalamic inputs (Fig. 6e). This points to a largely unidirectional thalamocortical loop for the interactions between ACA→VIS and ACA→SC neurons and between the two subnetworks (Fig. 7). Note that the mouse LP is densely connected to VIS (Supplementary Figs. 3 and 5) and thought to be functionally analogous to the primate pulvinar10, which powerfully controls the responses in visual cortex47. The ACA→SC → LP projection (Fig. 5d) may thus provide an additional pathway for top-down modulation of visual cortical processing. In the somatosensory network, the MO → PO → SS pathway (Fig. 3d and Supplementary Fig. 3d,f) may serve a similar function. However, deep-layer MO → AUD neurons were found to project to thalamus and brainstem motor regions3, suggesting a different organization of the auditory network.

The long-range projections from ACA to both VIS and SC suggest a strong analogy between the mouse ACA and the primate frontal eye field13. Optogenetic activation of ACA markedly enhances visual performance of the mouse and neuronal responses in VIS3, similarly to the effect of frontal eye field stimulation on attentional modulation in primates48,49. In the rat, a frontal orienting field has also been identified, whose activation can bias the orientating response, and presumably attention, to the contralateral side50. Our findings indicate that the mouse ACA is a point of convergence between the visual perception and action subnetworks. Such anatomical characterization provides a blueprint for future physiological investigation of how each subnetwork contributes to top-down attentional modulation and behavioral control.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank B. Kim, S. Zhu and P. Kim for technical help. We thank L. Luo, K. Beier, B. Lim and E. Callaway for viral vectors and cell lines. We thank T. Kamigaki for discussion. This work was supported by NIH R01 EY018861.

AUTHOR CONTRIBUTIONS

S.Z. and Y.D. conceived and designed the experiments. S.Z. performed and organized all the experiments. M.X. wrote the software for data analyses and analyzed the data. W.-C.C., C.M. and J.P.H.D. prepared AAV and RV vectors for rabies-virus-based retrograde tracing. W.-C.C. also performed some viral injections. T.L. and D.J. performed the detection for labeled cells and axons in some brain samples. J.L.F. and D.J. performed the brain tissue sectioning with a cryostat. S.Z., M.X. and Y.D. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Xu, N.L. et al. Nonlinear dendritic integration of sensory and motor input during an active sensing task. Nature 492, 247–251 (2012).
2. Lee, S., Kruglikov, I., Huang, Z.J., Fishell, G. & Rudy, B. A disinhibitory circuit mediates motor integration in the somatosensory cortex. Nat. Neurosci. 16, 1662–1670 (2013).
3. Nelson, A. et al. A circuit for motor cortical modulation of auditory cortical activity. J. Neurosci. 33, 14342–14353 (2013).
4. Schneider, D.M., Nelson, A. & Mooney, R. A synaptic and circuit basis for corollary discharge in the auditory cortex. Nature 513, 189–194 (2014).
5. Zhang, S. et al. Selective attention. Long-range and local circuits for top-down modulation of visual cortex processing. Science 345, 660–665 (2014).
6. Desimone, R. & Duncan, J. Neural mechanisms of selective visual attention. Annu. Rev. Neurosci. 18, 193–222 (1995).
7. Fuster, J.M. The Prefrontal Cortex: Anatomy, Physiology, and Neuropsychology of the Frontal Lobe (Lippincott-Raven, Philadelphia, 1997).
8. Mao, T. et al. Long-range neuronal circuits underlying the interaction between sensory and motor cortex. Neuron 72, 111–123 (2011).
9. Noudoost, B., Chang, M.H., Steinmetz, N.A. & Moore, T. Top-down control of visual attention. Curr. Opin. Neurobiol. 20, 183–190 (2010).
10. Oh, S.W. et al. A mesoscale connectome of the mouse brain. Nature 508, 207–214 (2014).
11. Petersen, C.C. Cortical control of whisker movement. Annu. Rev. Neurosci. 37, 183–203 (2014).
12. Stanton, G.B., Bruce, C.J. & Goldberg, M.E. Topography of projections to posterior cortical areas from the macaque frontal eye fields. J. Comp. Neurol. 353, 291–305 (1995).
13. Zingg, B. et al. Neural networks of the mouse neocortex. Cell 156, 1096–1111 (2014).
14. Hooks, B.M. et al. Organization of cortical and thalamic input to pyramidal neurons in mouse motor cortex. J. Neurosci. 33, 748–760 (2013).
15. Hunnicutt, B.J. et al. A comprehensive thalamocortical projection map at the mesoscopic level. Nat. Neurosci. 17, 1276–1285 (2014).
16. Miyamichi, K. et al. Cortical representations of olfactory input by trans-synaptic tracing. Nature 472, 191–196 (2011).
17. Wickersham, I.R. et al. Monosynaptic restriction of transsynaptic tracing from single, genetically targeted neurons. Neuron 53, 639–647 (2007).
18. Wang, Q., Sporns, O. & Burkhalter, A. Network analysis of corticocortical connections reveals ventral and dorsal processing streams in mouse visual cortex. J. Neurosci. 32, 4386–4399 (2012).
19. Jones, E.G. The Thalamus 2nd edn. (Cambridge University Press, 2007).
20. Wu, H., Williams, J. & Nathans, J. Complete morphologies of basal forebrain cholinergic neurons in the mouse. Elife 3, e02444 (2014).
21. Fishell, G. & Rudy, B. Mechanisms of inhibition within the telencephalon: “where the wild things are”. Annu. Rev. Neurosci. 34, 535–567 (2011).
22. Xu, X., Sporns, O. & Burkhalter, A. Network analysis of corticocortical connections reveals ventral and dorsal processing streams in mouse visual cortex. J. Neurosci. 32, 4386–4399 (2012).
23. Jones, E.G. The Thalamus 2nd edn. (Cambridge University Press, 2007).
24. Wu, H., Williams, J. & Nathans, J. Complete morphologies of basal forebrain cholinergic neurons in the mouse. Elife 3, e02444 (2014).
25. Fishell, G. & Rudy, B. Mechanisms of inhibition within the telencephalon: “where the wild things are”. Annu. Rev. Neurosci. 34, 535–567 (2011).
26. Xu, X., Sporns, O. & Callaway, E.M. Immunohistochemical characterization of inhibitory mouse cortical neurons: three chemically distinct classes of inhibitory cells. J. Comp. Neurol. 518, 389–404 (2010).
27. Lodato, S., Shetty, A.S. & Ariotta, P. Cerebral cortex assembly: generating and reprogramming projection neuron diversity. Trends Neurosci. 38, 117–125 (2015).
24. Shepherd, G.M. Corticostriatal connectivity and its role in disease. Nat. Rev. Neurosci. 14, 278–291 (2013).
25. Makino, H. & Komiya, T. Learning enhances the relative impact of top-down processing in the visual system. Nat. Neurosci. 18, 1116–1122 (2015).
26. Pouget, P. et al. Visual and motor connectivity and the distribution of calcium-binding proteins in macaque frontal eye field: implications for saccade target selection. Progr. Neurobiol. 3, 2 (2009).
27. Goldberg, M.E. & Wurtz, R.H. Activity of superior colliculus in behaving monkey. II. Effect of attention on neuronal responses. J. Neurophysiol. 35, 560–574 (1972).
28. Horwitz, G.D. & Newsome, W.T. Separate signals for target selection and movement specification in the superior colliculus. Science 284, 1188–1191 (1999).
29. Ignashchenkova, A., Dicke, P.W., Haarmeier, T. & Thier, P. Neuron-specific contribution of the superior colliculus to overt and covert shifts of attention. Nat. Neurosci. 7, 56–64 (2004).
30. Kustov, A.A. & Robinson, D.L. Shared neural control of attentional shifts and eye movements. Nature 384, 74–77 (1996).
31. Zénó, A. & Krauzlis, R.J. Attention deficits without cortical neuronal deficits. Nature 434, 437–437 (2012).
32. Kirschen, K. et al. Cortex circuit architecture of VTA dopamine neurons revealed by systematic input-output mapping. Cell 162, 622–634 (2015).
33. Euston, D.R., Gruber, A.J. & McNaughton, B.L. The role of medial prefrontal cortex in memory and decision making. Neuron 76, 1057–1070 (2012).
34. Wang, L., Liu, M., Segreves, M.A. & Cang, J. Visual experience is required for the development of eye movement maps in the mouse superior colliculus. J. Neurosci. 35, 12281–12286 (2015).
35. Fu, Y. et al. A cortical circuit for gain control by behavioral state. Cell 156, 1139–1152 (2014).
36. Goard, M. & Dan, Y. Basal forebrain activation enhances cortical coding of natural scenes. Nat. Neurosci. 12, 1444–1449 (2009).
37. Metherate, R. & Ashe, J.H. Nucleus basalis stimulation facilitates thalamocortical synaptic transmission in the rat auditory cortex. Synapse 14, 132–143 (1993).
38. Wang, L., Liu, M., Segraves, M.A. & Cang, J. Visual experience is required for the development of eye movement maps in the mouse superior colliculus. J. Neurosci. 35, 12281–12286 (2015).
39. Fu, Y. et al. A cortical circuit for gain control by behavioral state. Cell 156, 1139–1152 (2014).
40. Goard, M. & Dan, Y. Basal forebrain activation enhances cortical coding of natural scenes. Nat. Neurosci. 12, 1444–1449 (2009).
41. Metherate, R. & Ashe, J.H. Nucleus basalis stimulation facilitates thalamocortical synaptic transmission in the rat auditory cortex. Synapse 14, 132–143 (1993).
42. Pinto, L. et al. Fast modulation of visual perception by basal forebrain cholinergic neurons. Nat. Neurosci. 16, 1857–1863 (2013).
43. Nelson, A. & Mooney, R. The basal forebrain and motor cortex provide convergent yet distinct movement-related inputs to the auditory cortex. Neuron 90, 635–648 (2016).
44. Brown, S.P. & Hestrin, S. Intracortical circuits of pyramidal neurons reflect their long-range axonal targets. Nature 457, 1133–1136 (2009).
45. Kintsch, T., Wickersham, I.R., Seung, H.S. & Shepherd, G.M. Hierarchical connectivity and connection-specific dynamics in the corticospinal-corticostratial microcircuit in mouse motor cortex. J. Neurosci. 32, 4992–5001 (2012).
46. Morishima, M. & Kawaguchi, Y. Recurrent connection patterns of corticostriatal pyramidal cells in frontal cortex. J. Neurosci. 26, 4394–4405 (2006).
47. Purushothaman, G., Marion, R., Li, K. & Casagrande, V.A. Gating and control of primary visual cortex by pulvinar. Nat. Neurosci. 15, 905–912 (2012).
48. Moore, T. & Armstrong, K.M. Selective gating of visual signals by microstimulation of frontal cortex. Nature 421, 370–373 (2003).
49. Noudoost, B. & Moore, T. Control of visual cortical signals by prefrontal dopamine. Nature 474, 372–375 (2011).
50. Erlich, J.C., Blaek, M. & Brody, C.D. A cortical substrate for memory-guided orienting in the rat. Neuron 72, 330–343 (2011).
A software package was developed 7–8 weeks after the injection. The mice were anesthetized with isoflurane (5% induction and 1.5% maintenance) and placed on a stereotaxic frame. Temperature was kept at 37 °C (thermoregulation) and red Retrobeads (150 nL) were injected into VIS or SC of wild-type mice. Four weeks later, RV-AG-GFP+EYFP (500 nL) was injected into ACA (Supplementary Fig. 14).

Histology. The mice were deeply anesthetized with isoflurane and immediately perfused with chilled 0.1 M PBS followed by 4% paraformaldehyde (wt/vol) in PBS. The brain was removed and postfixed overnight at 4 °C. After fixation, the brain was placed in 30% sucrose (wt/vol) in PBS solution for 1–2 d at 4 °C. After embedding and freezing, the brain was sectioned into 50-μm coronal slices using a cryostat. For fluorescence images, brain slices were washed with PBS for 0.5 h and mounted with Vectashield mounting medium with DAPI. For immunohistochemistry for tdTomato, brain slices were washed with PBS for 0.5 h, quenched with 3% H2O2 for 0.5 h, permeabilized using PBST (0.3% Triton X-100 in PBS) for 0.5 h and then incubated with blocking solution (2% normal goat serum in PBST) for 1 h followed by primary antibody incubation overnight at 4 °C using anti-mCherry rat monoclonal antibody (M11217, Life Technologies; 1:1,000). The next day, slices were washed three times with PBS, incubated with biotinylated secondary antibody (biotin–goat anti-rat IgG, 629540, Life Technologies; 1:1,000) for 2 h and then incubated with Vectastain ABC Reagent (PK-6100, Vector labs) for ~10 min, washed with PBS again and then mounted with Vectashield mounting medium with DAPI. For immunohistochemistry for tdTomato, brain slices were washed with PBS for 0.5 h, quenched with 3% H2O2 for 0.5 h, permeabilized using PBST (0.3% Triton X-100 in PBS) for 0.5 h and then incubated with blocking solution (2% normal goat serum in PBST) for 1 h followed by primary antibody incubation overnight at 4 °C using anti-mCherry rabbit polyclonal antibody (sc-11833, Santa Cruz; 1:1,000). The next day, slices were washed three times with PBS, incubated with biotinylated secondary antibody (biotin–goat anti-rabbit IgG, sc-2005, Santa Cruz; 1:1,000) for 1 h and then incubated with Alexa Fluor 568-conjugated donkey anti-rabbit IgG (1:500, Life Technologies). After washing, slices were counterstained with DAPI (1 mg/mL) for 15 min and mounted with ProLong Gold antifade reagent (Life Technologies). For confocal images, brain slices were washed with PBS for 10 min, incubated with primary antibodies overnight at 4 °C, washed again and mounted with ProLong Gold antifade reagent (Life Technologies) under a confocal microscope (Zeiss, LSM 710).

3D reconstruction and quantification. A software package was developed in Matlab to analyze the digitized brain images. The analysis software consists of three modules: image registration, signal detection and quantification/visualization.

Registration module. The registration module is a reference point-based image alignment software used to align images of brain sections to the Allen Mouse Brain Atlas for further quantification and 3D reconstruction. First, we manually selected a set of reference points in both the atlas and the brain image. The module then applied several geometric transformations (translation, rotation and scaling) of the brain section to optimize the match between the reference points between the brain image and the atlas. Since histological sectioning can sometimes cause tissue compression, we allowed the scaling factors along the dorsal–ventral and medial–lateral axes to be optimized independently. Following the transformation, the match between the image and the atlas was inspected and further adjustments were made manually if necessary.

Detection module. The detection module has two independent submodules designed for counting RV-labeled cells and detecting axons, respectively. The cell counting module records the position of manually identified tdTomato-labeled neurons in each digitized brain section image. For axon detection, the ridge detection method was used (http://en.wikipedia.org/wiki/Ridge_detection). The following steps were taken to maximize the detection accuracy: (1) Image ridges were computed across multiple scales to extract all possible axon-like signals from each image. In the resulting binary ‘ridge image’, the number of pixels occupied...
by each detected axon depends on the length but not the thickness of the axon. In addition to valid axons, the ridge image also contains many noise pixels. (2) To remove the noise pixels due to the general background in the fluorescence image, we set a threshold based on the intensity distribution of the original image and use this as a mask to remove the noise pixels in the ridge image obtained from step (1). (3) To remove the discrete noise pixels with fluorescence intensities higher than the general background (thus not removed by step 2), we first identified pixels that are spatially contiguous in the ridge image (after a spatial convolution with a Gaussian kernel), computed the size of each contiguous region and removed the regions (of the original ridge image) below a threshold size. Steps 2 and 3 were repeated until satisfactory detection results were achieved. (4) The results were then visually inspected and the remaining noise pixels, which were mostly artifacts introduced during brain tissue processing, were rejected manually.

Quantification and visualization module. After detection and registration, signals were quantified across the whole brain and projected to the 3D reference atlas for better visualization. The 3D viewer plug-in of the ImageJ software was used to animate the final 3D model. The atlas, 3D reference mouse brain, quantification ontology and layouts for sunburst plot were obtained from the open online resource of Allen Institute for Brain Science, licensed under the Apache License (Version 2.0). The input from each region was quantified by dividing the number of labeled neurons in that region by the total number of labeled neurons detected in the whole brain (excluding the injection site). The output (axon projection) to each region was quantified as the number of pixels occupied by detected axons in the cleaned ridge image (see “Detection module” above) divided by the total number of axon-occupied pixels in the entire brain (excluding the injection site and locations with known major fiber tracks). In addition, the normalized density of labeled neurons and axons (number of neurons/length of axons divided by volume, normalized by the total number of neurons/total length of axons in the whole brain) in each structure was computed (Supplementary Table 1).

To assess the data variability, we computed the correlation coefficients (CCs) between individual brain samples for both input and output distributions. The mean CC between individual samples of the same group was 0.90 ± 0.02 (s.e.m.). For the same brain sample, the CC between the whole-brain distributions of axons detected by two different observers was 0.96 and the CC between the whole-brain distributions of RV-labeled cells detected by two observers was >0.99.

Code availability. The atlas, 3D reference mouse brain, quantification ontology and layouts for sunburst plot are freely available in the open online resource of Allen Institute for Brain Science, licensed under the Apache License (Version 2.0). The other computer code packages used to generate the findings of this study are available from the corresponding author upon request.

Statistical analyses. A Supplementary Methods Checklist is available summarizing statistical tests and results. Data collection and analysis were not performed blind to the conditions of the experiments. Data randomization was not applicable to our study, and no statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications.16,17

Data availability. The data that support the findings of this study are available from the corresponding author upon request.

51. Maheshri, N., Koerber, J.T., Kaspar, B.K. & Schaffer, D.V. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. Nat. Biotechnol. 24, 198–204 (2006).
52. Weissbourd, B. et al. Presynaptic partners of dorsal raphe serotonergic and GABAergic neurons. Neuron 83, 645–662 (2014).
53. Lim, B.K., Huang, K.W., Grueter, B.A., Rothwell, P.E. & Malenka, R.C. Anhedonia requires MC4R-mediated synaptic adaptations in nucleus accumbens. Nature 487, 183–189 (2012).
54. Wall, N.R., Wickersham, I.R., Cetin, A., De La Parra, M. & Callaway, E.M. Monosynaptic circuit tracing in vivo through Cre-dependent targeting and complementation of modified rabies virus. Proc. Natl. Acad. Sci. USA 107, 21848–21853 (2010).
55. Osakada, F. & Callaway, E.M. Design and generation of recombinant rabies virus vectors. Nat. Protoc. 8, 1583–1601 (2013).