

1 [The organization of intracortical connections by layer and cell class in the mouse brain](#)

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10 [Abstract](#)

11 The mammalian cortex is a laminar structure composed of many cell types densely interconnected in  
12 complex ways. Recent systematic efforts to map the mouse mesoscale connectome provide  
13 comprehensive projection data on inter-areal connections, but not at the level of specific cell classes or  
14 layers within cortical areas. We present here a significant expansion of the Allen Mouse Brain  
15 Connectivity Atlas, with ~1000 new axonal projection mapping experiments across nearly all isocortical  
16 areas in 50 Cre driver lines. Using 13 lines most selective for cortical layer and/or projection neuron  
17 class we identify the differential contribution of each layer/class to the overall intracortical connectivity  
18 patterns. We find that layer 5 (L5) projection neurons account for essentially all intracortical outputs.  
19 L2/3, L4, and L6 neurons contact a subset of the L5 cortical targets. We describe the most common  
20 axon lamination patterns in target regions, and their relationships to source layer/class. Most patterns  
21 were consistent with previous anatomical rules used to determine hierarchical position between  
22 cortical areas (feedforward, feedback), with notable exceptions. We observe a diversity of target  
23 patterns arising from every source layer/class, but supragranular (L2/3 and upper L4) neurons are  
24 most associated with feedforward type patterns, whereas infragranular (L5 and L6) neurons have both  
25 feedforward and feedback. Network analyses revealed a modular organization of the intracortical  
26 connectome. Using the cell class-based target lamination patterns, we labeled all connections and  
27 intermodule connections as feed-forward or -back, and finally present an integrated view of the  
28 intracortical connectome as a hierarchical network.

29

## 30 Introduction

31 Cognitive processes and voluntary control of behavior originates in the isocortex. Anatomical circuitry  
32 constrains the routes by which information can travel within the brain, so understanding how incoming  
33 sensory information is processed, integrated with past experiences and current states to generate outputs  
34 to motor systems downstream requires knowledge about the patterns and rules of connectivity between  
35 cortical areas. Connectomes, complete descriptions of the wiring in a brain<sup>1</sup>, are described across spatial  
36 scales (micro-, meso-, and macro-scale), each with the potential to reveal different principles of brain  
37 organization. At the mesoscale<sup>2</sup>, connectivity is described between brain areas at the level of cell  
38 populations, classes, or types. Recently, several mesoscale cortical connectomes have been produced,  
39 either through new systematic data generation<sup>3-5</sup>, or via expert collation of historical tract tracing  
40 literature<sup>6-8</sup>. Common organizational features of cortical connectivity in both rodent and macaque have  
41 been distilled from these datasets, often using graph theoretical approaches to describe network  
42 architecture<sup>9,10</sup>. For example, areas have unique patterns of connections (*i.e.*, a “fingerprint”), connection  
43 strengths follow a log-normal distribution spanning > 4 orders of magnitude<sup>4,5</sup>, and display high clustering  
44 coefficients, with some nodes (“hubs”) that are very highly connected<sup>4</sup>. Finally, the organization of cortical  
45 areas is modular, with distinct modules corresponding to specific functions<sup>3,11</sup>.

46 Organizational schemes other than modular networks have previously been applied to cortical  
47 connections to explain information flow and processing. Specifically, the concept of a cortical  
48 hierarchy<sup>12,13</sup> has been very useful for understanding computational and architectural properties of the  
49 cortex and has inspired the development of neuronal network and methods in machine vision<sup>14</sup>. These  
50 schemes (modular and hierarchical) are not mutually exclusive, and the actual organization of the cortex  
51 may involve both types.

52 So far, the available mesoscale datasets are not clearly differentiated from macroscale connectivity in that  
53 the experiments and analyses focus on connections between areas, treating them as more or less  
54 homogenous regions. Data are often generated using tracers that cover entire cortical columns. In the  
55 first phase of the Allen Mouse Connectivity Atlas, injections were intentionally placed at multiple depths  
56 within a cortical area in one experiment to infect all neurons across the cortical layers<sup>4</sup>. However, each  
57 cortical region is composed of many cell types. Perhaps the most characteristic feature of the isocortex is  
58 its organization into six layers. Within these layers, distinct cell types exist that can be further  
59 differentiated based on morphological, electrophysiological, and transcriptional properties<sup>15-17</sup>. Specific  
60 long-distance connectivity patterns have already been associated with excitatory cell populations or  
61 genetically-identified types in each layer<sup>16,18</sup>. Long-distance axon projections are commonly used to  
62 classify excitatory neurons into three main classes; intratelencephalic (IT), pyramidal tract (PT), and  
63 corticothalamic (CT). Axons from IT neurons project to both ipsilateral and contralateral cortex and  
64 striatum. PT neurons target subcortical structures, including those in the spinal cord, medulla, pons, and  
65 midbrain, and can send branches to ipsilateral cortex, striatum, and thalamus. CT neurons project to  
66 ipsilateral thalamus. IT neurons are found across all layers containing excitatory cells (L2/3, L4, L5, L6),  
67 while PT neurons locate to L5 and CT neurons to L6<sup>19,20</sup>. Projections from these major classes to different  
68 target regions suggests that they play distinct roles in information processing.

69 Experimental access to cortical cells in different layers and classes is feasible due to the generation and  
70 characterization of large collections of Cre driver transgenic mouse lines<sup>21-24</sup>. By taking advantage of  
71 these driver lines, we significantly expanded upon our previously published Allen Mouse Brain  
72 Connectivity Atlas (<http://connectivity.brain-map.org><sup>4,25</sup>) adding ~ 1000 new experiments in the cortex.  
73 Here we present this enhanced online resource of projection data from cortical cell classes defined by  
74 laminar position and brain-wide projection patterns. We first describe macroscale organization of cortical  
75 connectivity into modules and the unique patterns of intracortical connectivity from each source. Then, we  
76 show the contribution of projection neurons within each mapped layer and cell class to the complete  
77 intracortical projection pattern for a given region. We observe diverse axon lamination patterns in cortical  
78 targets related to the layer of origin in the source and the projection neuron class labeled by each Cre  
79 line. These patterns are both similar to and different from previous anatomical patterns derived from

80 anterograde tracing to define hierarchical position (feedforward and feedback) in the visual cortex of  
81 primates<sup>12</sup> and rodents<sup>26</sup>. Using the cell layer/class-based projection patterns, we ordered 40 cortical  
82 areas into hierarchical positions. We also ordered the 6 network modules; from bottom to top:  
83 somatomotor, temporal, visual, medial, anterolateral, and prefrontal cortex.

## 84 Results

### 85 *Data generation and characterization of Cre driver lines for layer- and cell class-selective mapping*

86 Our pre-existing pipeline for generation and quantification of projection mapping data across the entire  
87 brain<sup>4</sup> was used in this study, with some modifications. In brief, in the first study<sup>4</sup>, we used only wild-type  
88 C57Bl/6J mice injected with a Cre-independent tracer, rAAV2/1.hSyn.EGFP.WPRE. Here, we used  
89 transgenic Cre driver mice. Most Cre mice were injected with a Cre-dependent rAAV tracer,  
90 rAAV2/1.pCAG.FLEX.EGFP.WPRE. A subset had a duplicate injection of an rAAV with a synaptophysin-  
91 EGFP fusion transgene in place of the cytoplasmic EGFP, rAAV2/1.pCAG.FLEX.SypEGFP.WPRE (see  
92 **Supplemental Figure 1** for detailed comparisons). Following tracer injections, brains were imaged using  
93 serial 2-photon tomography (STP) at high resolution ( $x,y,z = 0.35 \mu\text{m} \times 0.35 \mu\text{m} \times 100 \mu\text{m}$ ), after which  
94 the images underwent QC and manual annotation of injection sites, followed by signal detection and  
95 registration to the 3D Allen Mouse Brain reference atlas for subsequent data analyses, data visualization,  
96 and public release to our web portal<sup>27</sup>.

97 Our goal for expanding on the Allen Mouse Connectivity Atlas (<http://connectivity.brain-map.org>) was to  
98 create a comprehensive map of all inter-areal (long-distance) projections originating from neurons in  
99 different cortical layers and, when possible, from different cell classes within a given source area. Forty-  
100 nine Cre driver lines (**Supplemental Table 1**) entered the pipeline for cortical projection mapping after  
101 initial anatomical characterization of transgene expression across the brain using *in situ* hybridization<sup>23</sup>  
102 (<http://connectivity.brain-map.org/transgenic>). These driver lines have either pan-layer Cre expression  
103 (e.g. Emx1-IRES-Cre), layer-selective Cre expression, or Cre expression driven by inhibitory neuron-  
104 specific promoters. Layer-selectivity data for the 15 lines are summarized in the second row ("Layer") of  
105 **Figure 1a**. Data used for determining layer-selectivity can be found in **Supplemental Figure 2**. Many Cre  
106 lines showed relatively even distribution of expression across the entire isocortex, but we also saw  
107 gradients and area-restricted expression patterns which were used to choose appropriate locations for  
108 tracer injections.

109 Across the 50 mouse lines (49 Cre + 1 wild type), we generated a total of 1082 experiments  
110 (**Supplemental Tables 1 and 2**). Most of these experiments ( $n=850$ ) used 15 out of 50 lines (14 Cre + 1  
111 wild type, see below). **Figure 1a** shows the 850 tracer experiments by line and cortical area. Mouse  
112 isocortex is parcellated into 43 areas in the Allen 3D reference atlas, shown here in two ways. A top-down  
113 view (**Figure 1k**), and a flattened view of the entire right hemisphere of cortex, to visualize all cortical  
114 areas in one 2D image (**Figure 1l**). Locations of the injections' centroids ( $x,y,z$  voxel coordinate after  
115 registration to the Allen 3D reference atlas) for all 1082 experiments are plotted onto the cortical surface  
116 flat map in **Figure 1m**, color coded by layer-selectivity as in **Figure 1a**. Left side injections were plotted  
117 here on the right hemisphere to visualize completeness of areal coverage. All subsequent analyses refer  
118 to "ipsilateral" and "contralateral" relative to the side of injection. Of the 43 cortical areas, only 10 had 5 or  
119 less experiments. These areas (FRP, Alv, SSp-un, Alp, GU, VISC, AUDv, TEa, PERI, and ECT) were  
120 generally harder to target due to their size (SSp-un) or more often their location in very ventral or lateral  
121 regions (see **Figure 1l**). Abbreviations for isocortex regions used throughout are listed in **Supplemental**  
122 **information**.

123 Data from all 1000+ experiments in the isocortex are publicly available at the Allen Mouse Connectivity  
124 Atlas data portal (<http://connectivity.brain-map.org/>). Individual experimental IDs and associated metadata  
125 are listed in **Supplemental Table 2**.

126 We visually inspected the brain-wide axonal projection patterns and classified all 1000+ experiments  
127 based on the principles described above for defining IT, PT, and CT neuron classes. Each experiment

128 was manually assigned to one of five groups (**Supplemental Figure 3a-e**); (a) IT PT CT, when labeled  
129 axons were observed in all regions of interest (ipsilateral and contralateral cortex and striatum, and  
130 subcortical and thalamic projections), (b) IT, when labeled axons were restricted to ipsilateral and  
131 contralateral cortex and striatum, (c) PT, when labeled axons were ipsilateral and subcortically-projecting,  
132 (d) CT, when labeled axons projected almost exclusively to thalamus, and (e) local, when no (or few)  
133 long-distance (*i.e.*, outside of the source area) axons were seen. The consensus of results across all  
134 sources for each mouse line is shown in the “projection class row” of **Figure 1a**, and more detail can be  
135 found in **Supplemental Figure 3f**, which shows both the consensus projection neuron class for each Cre  
136 line and the class per source mapped within a Cre line. A subset of Cre lines are highly selective for IT,  
137 PT, or CT neurons, consistent with previous characterizations<sup>22,28,29</sup>. Most lines label neurons of the  
138 same projection class independent of the source area, but there are interesting exceptions. For example,  
139 L5 cells expressing Cre in the *Chrna2-Cre\_OE25* line are of the PT class in 14 of the 19 sources tested,  
140 but only locally projecting neurons are labeled in other sources in this line (e.g. VISp).

141 To validate the manual assignment of experiments and Cre lines to projection neuron classes, we also  
142 performed unsupervised hierarchical clustering using spearman rank correlations on the fraction of  
143 informatically-derived projection volumes in each relevant major brain division to the whole brain  
144 projection volume (ipsilateral and contralateral isocortex, caudoputamen, thalamus, the combined signal  
145 in the medulla, pons, and midbrain, and the primary injection source). The resulting dendrogram was cut  
146 into 5 clusters (dotted line in **Figure 1b**), which corresponded strongly with the manual projection class  
147 assignments (**Figure 1c-h**, and **i,j**), described in more detail in **Supplementary information**.

148 Together, the characterization of layer- and projection neuron class-selectivity for each Cre line enabled  
149 us to choose a core set of the best lines for comprehensively mapping connectivity from known classes of  
150 projection neurons in each cortical layer. These 13 lines, together with experiments in wild type mice  
151 (C57Bl/6J) and the pan-layer *Emx1-IRES-Cre* line were used to identify all intracortical projections. These  
152 lines include L2/3 IT (*Cux2-IRES-Cre* and *Sepw1-Cre\_NP39*), L4 IT (*Nr5a1-Cre*, *Scnn1a-Tg3-Cre*, and  
153 *Rorb-IRES-Cre*), L5 IT (*Tlx3-Cre\_PL56*), L5 PT (*A93-Tg1-Cre*, *Chrna2-Cre\_OE25*, *Efr3a-Cre\_NO108*,  
154 *Sim1-Cre\_KJ18*), L5-all classes (*Rbp4-Cre\_KL100*), and L6 CT (*Ntsr1-Cre\_GN220*, *Syt6-Cre\_K1148*).  
155 One class for which we did not identify a suitable Cre line is L6 IT<sup>16</sup>.

#### 156 *Intracortical connections are organized into modules*

157 Cortical areas have distinct patterns (targets and weights) of intracortical projections revealed through  
158 anterograde tracing in wild type mice<sup>3,4</sup> (**Figure 2**). However, similarities between output patterns of some  
159 areas are also obvious when viewing the anatomical data spatially (**Figure 2a**) or the connection  
160 strengths in matrix form (**Figure 2b**). The matrix shows the output of a newly constructed model, which,  
161 for the isocortex, was based on the 122 injections in wild type mice (Knox et al., submitted). This model  
162 differs from our previously published model<sup>4</sup> in that it is built through interpolation at the voxel level (100  
163  $\mu\text{m}$ ), rather than for each brain region, enabling the recovery of high spatial resolution for connectivity  
164 strengths between voxels. The voxel-based connection strengths were then unionized for every  
165 isocortical region annotated in the Allen 3D reference atlas ( $n=43$ , **Figure 1**). **Figure 2b** shows the  
166 ipsilateral intracortical connectivity matrix. Here, rows are sources, columns are targets, and the strength  
167 between every pair of areas is the modeled projection density (Log10-transformed).

168 We analyzed the network structure of this ipsilateral matrix using the Louvain algorithm from the Brain  
169 Connectivity Toolbox (<https://sites.google.com/site/bctnet/>, Rubinov and Sporns, 2010). This algorithm  
170 maximizes a modularity metric ( $Q$ , range [-1,1]) to identify groups of nodes (cortical areas) most densely  
171 connected to each other compared to a randomized network. To identify stable modules, we  
172 systematically varied the spatial resolution parameter,  $\gamma$ , from 0-2.5, and measured  $Q$  at each value of  $\gamma$ ,  
173 compared to  $Q$  for a shuffled network. Increasing  $\gamma$  enables the detection of more modules, each  
174 containing fewer nodes<sup>8,31</sup>. The mouse cortex showed significant modularity ( $Q > Q$  for the shuffled  
175 network) for every value of  $\gamma$  above 0.3. Between 1-14 modules were identified across this range (**Figure**  
176 **2b**, colors on left axis). For subsequent analyses, we chose to focus on the modules identified at  $\gamma=1.3$ .

177 This value of  $\gamma$  corresponds to the midpoint between no modules at all, and the value where modules  
178 contain single regions, 2.5. It is also the  $\gamma$  level where the difference between Q and Qshuffled was at its  
179 peak ( $0.2224 \pm 0.0021$ ), although this difference was relatively stable between  $\gamma = 1$  and  $\gamma = 1.8$   
180 ( $0.2187 \pm 0.0048$  at  $\gamma = 1$ ,  $0.2020 \pm 0.0009$  at  $\gamma = 1.8$ ). The network is divided into six modules at this point,  
181 containing 5-8 regions each.

182 We named the six modules based on the cortical areas assigned to each; (1) **Prefrontal**: FRP, MOs,  
183 ACAd, ACAv, PL, ILA, ORBI, ORBm, ORBvl (2), **Anterolateral**: Aid, Alv, Alp, GU, VISC, (3)  
184 **Somatomotor**: SSs, SSp-bfd, SSp-tr, SSp-ll, SSp-ul, SSp-un, SSp-n, SSp-m, MOp, (4) **Visual**: VISal,  
185 VISrl, VISl, VISp, VISpl, VISli, VISpor, (5) **Medial**: RSPagl, RSPd, RSPv, VISa, VISam, VISpm, and (6)  
186 **Temporal**: AUDd, AUDp, AUDpo, AUDv, TEa, PERI, and ECT. Although we use these six modules for  
187 subsequent analyses, and to provide a description of the organization of cortical areas based on  
188 connections, it should be emphasized that there are other, equally valid, levels of organization that could  
189 be chosen for future analyses. For example, there is a four-module solution at  $\gamma = 1.0$  that results in  
190 prefrontal, somatomotor, visual, and temporal modules, in which the anterolateral areas are not split from  
191 somatomotor areas, and medial regions are still grouped with the visual areas. The spatial relationships  
192 between areas in these six modules are shown in the 3-D renderings of brain areas in the Allen 3D  
193 reference atlas in **Figure 2c**. There is a clear spatial component to the module assignment, in that nearby  
194 areas often belong to the same module. This is perhaps not surprising given that connectivity strengths  
195 drop as a function of distance<sup>4,32</sup>, but does not negate the likely importance of long-distance inter-module  
196 connections for integration and information flow throughout cortical circuits. The network of connections  
197 within and between all modules and nodes was also visualized using a force-directed layout algorithm<sup>33</sup>  
198 which highlights the overall high density, and variation in connection strengths across the cortex (**Figure**  
199 **2d**).

#### 200 *Interareal patterns of connectivity by output layer and class*

201 Network analysis of the intracortical connectivity matrix revealed a modular organization based on the  
202 total output of a given cortical area. While this provides an important framework for understanding  
203 macroscale rules of cortical connectivity, the contributions of distinct cell classes within each area to the  
204 overall pattern are still unknown. To begin to explore this, we first compiled groups of spatially-matched  
205 experiments. These experiments were pulled from the 850 listed in **Figure 1a**, using up to 15 mouse lines  
206 for coverage of layer/class within a given source. Each group was “anchored” by one of 90 Rbp4-  
207 Cre\_KL100 tracer experiments (green triangles, **Figure 1m**). Rbp4-Cre\_KL100 is a L5 selective line  
208 which labels all classes of projection neurons in L5. This line was chosen as the anchor because the  
209 largest number of sources were injected of all the Cre lines (33 of 43 cortical areas had at least 1  
210 experiment). Potential group members for each anchor were defined as experiments where the distance  
211 between the Rbp4 and other experiment injection centroids was  $< 500 \mu\text{m}$ . An experiment was only used  
212 once, even if it was within  $500 \mu\text{m}$  of two Rbp4 anchors. To be considered a complete group, at least one  
213 experiment from a Cre line representing L2/3 IT, L4 IT, L5 IT, L5 PT, L6 CT, and a wild type or Emx1-  
214 IRES-Cre dataset had to be present. Within a group, the median distance from the Rbp4-Cre\_KL100  
215 anchor was  $296 \mu\text{m}$ . For some anchors, when a specific Cre line was otherwise missing, the injection  
216 centroid distance exceeded  $500 \mu\text{m}$  (range  $502\text{-}616 \mu\text{m}$ ,  $24/332$  total experiments). All experiments within  
217 a group utilized the same tracer (*i.e.*, SypEGFP experiments were not grouped with EGFP experiments).  
218 In this way, we identified 43 anchor groups composed of unique sets of experiments ( $n=364$  total),  
219 representing 25 of 43 potential source areas.

220 The locations of all anchor groups and individual experiments are shown mapped onto the flat cortical  
221 surface view in **Figure 3a**. Five examples are shown to illustrate layer selectivity, spatial matching of  
222 injections into different lines, and cortical projection patterns by line, including sources from the prefrontal  
223 cortex module (ACAd and MOs, **Figure 3b,c**), somatomotor module (SSp-m, **Figure 3d**), visual module  
224 (VISp, **Figure 3e**), and medial module (VISam, **Figure 3f**). 2D overlays of the injection sites confirmed the  
225 expected layer selectivity and relative size or proportion of cells labeled in these different experiments

226 (**Figure 3b'-f'**, and see **Supplemental Figure 4** for individual panels). For example, prefrontal areas  
227 ACAd and MOs are both agranular structures (lacking L4), and the corresponding injections into the  
228 predominantly L4 IT Cre lines Scnn1a-Tg-3 or Nr5a1-Cre (colored in magenta) result in much sparser  
229 labeling than for areas with a large L4, such as primary somatosensory and visual areas (compare **Figure**  
230 **3b'-c'** with **d'-e'**). The distribution and density of cells labeled after viral infection of Cre-dependent  
231 reporter in each injection also closely matches expectations based on ISH characterization of tdTomato  
232 expression in Cre x Ai14 reporter lines for these regions<sup>23</sup> (<http://connectivity.brain-map.org/transgenic>).

233 From any given source, cortical projections labeled in the L5 Rbp4-Cre\_KL100 line (middle column in  
234 **Figure 3b-f**) appear to be more extensive than from any other line or layer. However, it is also visually  
235 obvious that the projections labeled in different Cre lines originating from the same location had very  
236 similar projection patterns overall (looking across rows). Indeed, it appears that the projections from every  
237 other spatially-matched Cre line are a subset of the L5 outputs. Of note, L5 PT and L6 CT lines (3  
238 columns on the right) also have varying amounts of intracortical projections, still a subset of those  
239 mapped from L5 IT and pan Cre lines, although most of their projections are subcortical (see **Figure 1b**).

240 To quantitatively explore how similar, or different, cortical projection patterns are across layers and cell  
241 classes from the same location, we first manually curated the complete anchor group dataset (n=43  
242 anchors, 364 experiments). This was accomplished by careful visual inspection of the corresponding  
243 high-resolution 2D images for each of the possible cortical targets to identify true positive and true  
244 negative connections for each experiment (43 ipsilateral and 43 contralateral, for a total of 31,304  
245 connections manually checked). We also noted when a target contained only fibers of passage, and  
246 considered it as a true negative for subsequent binarization of the matrix. Using the output of our  
247 automated segmentation and registration algorithms we generated multiple weighted connectivity  
248 matrices, one for each Cre line, and applied the binary mask to remove all true negative weights (*i.e.*,  
249 segmentation artifacts). As mentioned above, only 25 different cortical areas were represented in the 43  
250 anchor groups. This was due to both denser spatial sampling within a larger structure (e.g. we targeted 6  
251 retinotopic locations within primary visual cortex, 3 sub-regions in secondary motor and 2 in the ventral  
252 part of anterior cingulate), and the replication of experiments in several visual locations with the SypEGFP  
253 virus. To avoid biases related to differences between source areas, we selected only one anchor group  
254 per cortical region, if there was a significant, positive correlation between Rbp4-Cre\_KL100 replicates  
255 (Spearman  $r > 0.8$ ). Following this selection, we present the results from 27 of the anchor groups,  
256 consisting of 25 unique areas and two locations in MOs and SSs. Eight of the lines with the most  
257 experiments, representative of each layer/class, in these 27 groups are shown in **Figure 4a**. However, all  
258 underlying data from these lines and the other 6 lines (Nr5a1-Cre, Rorb-IRES-Cre, Chrna2-Cre\_OE25,  
259 Sim1-Cre\_KJ18, Efr3a-Cre\_NO108, and Sepw1-Cre\_NP39) are available upon request. Of note, we  
260 merged the data from C57Bl6/J and Emx1-IRES-Cre experiments into one matrix as these both account  
261 for outputs of all projection neurons across layers in a cortical region. In support of this compilation, we  
262 also found that cortical projection patterns between pairs of spatially-matched Emx1-IRES-Cre and wild  
263 type experiments in 3 different regions were highly correlated, even given locations in opposite  
264 hemispheres (Spearman  $r = 0.88$  for VISp, 0.90 for VISI, and 0.97 for VISam, **Supplemental Figure 5**).

265 Overall, these matrices reveal several similar and unique features of layer- and projection class-specific  
266 connectivity between areas in terms of number, strength, and specificity of connections. First, we  
267 quantified the number of output connections ("out-degree") for each experiment. For all lines, this varied  
268 across source areas (**Supplemental Figure 6a**). In wild type and Emx1-IRES-Cre mice, out-degree  
269 ranged from 15–43 on the ipsilateral side and 10–42 contralaterally. The three areas with the largest  
270 number of outputs are in the prefrontal module; MOs (-2), ORBI, and ORBvl. The range was equally large  
271 in both hemispheres for the L5 lines, Rbp4-Cre\_KL100 (20–43 ipsi, 12–43 contra) and Tlx3-Cre\_PL56 (13–  
272 42 ipsi, 9–40 contra), but obviously different overall for the L2/3 and L4 IT lines as well as the L5 and L6  
273 lines, particularly in the contralateral hemisphere. We then calculated the average out-degree per line in  
274 both hemispheres (**Figure 4b**) to assess for differences across lines. Overall, we find a significant effect  
275 of both Cre line and hemisphere (but not the interaction) on the number of connections (2-way ANOVA,

276  $p < 0.0001$ ). The mean numbers of C57Bl6/Emx1-IRES-Cre ipsilateral and contralateral connections are  
277 not significantly different from Rbp4-Cre\_KL100 or Tlx3-Cre\_PL56 in either hemisphere, but are  
278 significantly higher than all other Cre lines (Tukey's multiple comparison test,  $p < 0.0001$ ), except for Rorb-  
279 IRES-Cre on the ipsilateral side. Similarly, Rbp4-Cre\_KL100 also had significantly more connections on  
280 both ipsilateral and contralateral hemispheres compared to every other line, except for Tlx3-Cre\_PL56 on  
281 the contralateral side. As also seen in the matrices, the L5 PT and L6 CT lines have the fewest number of  
282 connections to regions in both hemispheres, followed by the L2/3, L4, and L5 IT lines. **Figure 4b** also  
283 shows that for every line, there are fewer contralateral connections compared to ipsilateral connections.  
284 The difference between the number of connections per hemisphere is shown in the center panel, ranging  
285 from 6-14 more connections made to ipsilateral targets. Cux2-IRES-Cre had the largest difference (14),  
286 significantly more than C57Bl6/Emx1-IRES-Cre, Rbp4-Cre\_KL100, and Tlx3-Cre\_PL56 (1-way ANOVA,  
287 effect of Cre line,  $p = 0.0006$ , and Tukey test,  $p = 0.02$ ,  $0.02$ , and  $0.0003$ , respectively).

288 **Figure 4a,b** shows that L5 Rbp4-Cre\_KL100 labeled cells project most widely, and are most like wild type  
289 experiments for any given source. **Figure 3b-f** also shows that the connections from each line appear to  
290 be a subset of the Rbp4-Cre\_KL100 patterns, as opposed to a different set of target regions. So, next we  
291 determined how much overlap there was between the specific targets contacted by each experiment and  
292 the Rbp4 anchor within the spatially-matched groups (**Figure 4c**). C57Bl6/Emx1-IRES-Cre and Rbp4-  
293 Cre\_KL100 shared, on average, 80% of the targets from any given source. A roughly equal number of  
294 targets are unique to either Rbp4-Cre\_KL100 or C57Bl6/Emx1-IRES-Cre (12.7%, 7%) which may be due  
295 to differences in sensitivities of the viral tracers or the homozygosity of the Emx1-IRES-Cre line. For every  
296 other Cre line, all target connections were a subset of the L5 Rbp4 targets (**Figure 4c** white bars,  $< 5\%$   
297 of the targets are unique to any Cre line). L5 IT cells (Tlx3-Cre\_PL56) project to 71% of the Rbp4 target set,  
298 L2/3 IT cells project to 54% (Cux2-IRES-Cre) and 37% (Sepw1-Cre\_NP39). L4 IT lines also project to  
299  $\sim 50\%$  of the complete Rbp4 set of connections (Nr5a1=48%, Rorb-IRES2-Cre=56%, and Scnn1a=50%).  
300 L5 PT and L6 CT lines also contact a subset of the Rbp4 targets, between 20-30%. Together, it appears  
301 that L5 cells project to almost all possible targets from any given source. Within L5, the L5 IT cells have  
302 the most overlap with Rbp4-Cre\_KL100 while L5 PT cells have more limited projections within the Rbp4  
303 set, and predominantly to the ipsilateral hemisphere (**Figure 3b-f and 4a**). L2/3 (and L4) IT cells project to  
304 a subset of the same targets of L5. Fewer projections to the contralateral hemisphere appear to account  
305 for most of the differences between L2/3 and L5, suggesting that most callosal projections arise from L5  
306 in the mouse.

307 Next, we looked at the strength of connections made by the projection neurons labeled in each line. After  
308 removal of the manually verified true negative connections, individual output strengths still spanned  $\sim 5$   
309 orders of magnitude, like previously reported for both outputs and inputs in mouse and macaque brains  
310 <sup>4,34,35</sup>. Overall, we find a significant effect of Cre line and hemisphere, and the interaction of these two  
311 factors, on the strength of connections (2-way ANOVA,  $p < 0.0001$  for Cre line and hemisphere,  $p = 0.02$   
312 interaction effect). Across all lines, the average projection strengths are stronger within the ipsilateral  
313 compared to contralateral hemisphere (**Figure 4d**). This overall pattern was also observed across  
314 individual sources within each line (**Supplemental Figure 6b**). The largest disparities in strength across  
315 hemispheres is seen in the L5 PT (blue) and L6 CT lines (yellow). In other words, not only do these lines  
316 contact few targets contralaterally ( $< 5$ , **Figure 4b**), but, when axon terminals are present, they are  $\sim 1$   
317 order of magnitude weaker than the ipsilateral side connections.

318 As indicated, we used several Cre lines with the same layer/class-selectivity designation when available.  
319 These lines may label overlapping, or possibly distinct, sets of projection neurons within these larger  
320 classes. To measure the similarity in intracortical projection patterns between all the lines, even those of  
321 the same layer/class, we calculated the Spearman rank correlation coefficients between each Cre line  
322 experiment in the same anchor group, and plotted the average  $r$  in **Figure 4e**. C57Bl6/Emx1-IRES-Cre  
323 (black circle) and Rbp4-Cre\_KL100 (green circle) are most strongly correlated ( $r = 0.89$ ). Tlx3-Cre\_PL56  
324 (white circle) was also highly correlated with Rbp4-Cre\_KL100 ( $r = 0.86$ ) and C57Bl6/Emx1-IRES-Cre

325 (r=0.84). All other lines had positive correlations, but with  $r < 0.80$ , even for those within the same  
326 layer/class.

327 *Axon terminal lamination patterns and their relationship to source layer and cell class.*

328 Data in **Figure 4** demonstrate differential patterns of intracortical outputs originating from different layers  
329 or classes of projection neurons within a source region. We showed that L5 neurons make connections to  
330 essentially all the targets of that source area, and all other layers contact a subset of these targets. Next,  
331 we looked at whether differences exist in the targets at the level of layers rather than areas.

332 First, we visually inspected and described the relative densities of axon terminal labeling across layers for  
333 all targets in a subset of the Rbp4 anchor group experiments (79 of 364 experiments covering 14 source  
334 areas in all 15 lines; total=6,794 connections checked for layer patterns, true negatives then removed).  
335 Several frequently observed patterns emerged from this subset of representative experiments (**Figure 5a-  
336 j**). The four most common lamination patterns included; **(a)** columnar, with relatively equal densities  
337 across all layers (21%), **(b)** superficial and deep layers in equal densities (18%), **(c)** superficial layers only  
338 (25%), or **(d)** deep layers only (18%). The remaining patterns observed **(e-j)** together account for 19% of  
339 all true positive connections. Of note, almost all patterns involved dense terminals in L1, except for three  
340 rare patterns **(e, i, j; 2-4%)**, which were distinctive in that L1 contained relatively few labeled axons.

341 Following the qualitative assignment of axon terminals to a lamination pattern as shown, we checked  
342 whether informatically-obtained values of projection strength by layer, derived following registration to the  
343 Allen 3D reference atlas, could quantitatively capture these patterns. The inset bars in **Figure 5a-j** show  
344 the average fraction of the total projection volume per layer, scaled by the relative size of the layer in  
345 each target. We used the actual ratio of each layer's volume per target for scaling because this number  
346 varies across cortical areas (e.g. some areas have large L4, others very small). The resulting heat map in  
347 each panel visually corresponded well to the qualitative classifications of laminar patterns.

348 We next performed unsupervised hierarchical clustering, for the *complete* dataset of **Figure 1a**, to  
349 visualize laminar termination patterns from all source areas and Cre lines. In the heatmap shown in  
350 **Figure 5k**, each column is a unique combination of mouse line, source area and target. Relative density  
351 data were calculated as just described (*i.e.*, the fraction of the total projection in each layer, scaled by  
352 relative layer size). Data included for clustering had to pass three filters. (1) target connection strength  
353 ( $\log_{10}$ -transformed normalized projection volume) was greater than -1.5. This threshold was chosen  
354 based on the frequency distributions for informatically-derived normalized projection volumes of the set of  
355 manually-verified true positive and true negative connections (**Supplemental Figure 8**). At a  $\log_{10}$   
356 connection strength of -1.5, the number of true positives was first larger than true negatives. Less than  
357 3% of true negative values remain, while over 50% of true positives are still present. (2) The percentage  
358 of infection volume in the primary source was  $> 50\%$ , and (3) self-to-self projections were removed.  
359 Following these steps, if present, multiple experiments with the same source-line-target were averaged,  
360 resulting in a total of 6,469 unique source-line-target connections in **Figure 5k**.

361 We performed unsupervised hierarchical clustering on the relative density of projections in L1, L2/3, L4,  
362 L5, and L6a using Spearman rank correlations, and average linkages, to measure similarities. The first  
363 dendrogram branch point split the targets based on the density of projections to L6a (low on the left, high  
364 on the right). Then, within each of these two clusters, the next split was made by relative projection  
365 density in L1. The third split was determined by L2/3 relative projection density. At this point, 6 clusters  
366 were identified which resembled the manual categories, and we discuss each of these patterns further.  
367 The median values for each layer and the overall frequencies of these clusters are shown in **Figure 5l,m**.  
368 Clusters 1-3 had relatively weak projections to L6a compared to clusters 4-6. **(1)** Cluster 1 most  
369 resembled the superficial layer pattern (**Figure 5c**), with dense projections in L1 and L2/3 ( $n=1777$ , 27%).  
370 **(2)** Cluster 2 resembled the L1+L5 pattern (**Figure 5g**;  $n=314$ , 4.9%). **(3)** Cluster 3 resembled two of the  
371 patterns avoiding L1 and L6 (**Figure 5e,j**,  $n=499$ , 7.7%), preferentially projecting into L2/3, L4 (if present),  
372 and L5. Unlike clusters 1-3, clusters 4-6 had high projection density to L6a. **(4)** Cluster 4 was the largest



373 group (n=1982, 31.0%) and most like the superficial and deep layer pattern (**Figure 5b**). Cluster 4 is also  
374 likely to contain the targets with lamination patterns visually described as “columnar” and “polylaminar”  
375 (**Figure 5a,f**), although even for those there does appear to be stronger projection density to L1 and L6  
376 (see insets in a,f). **(5)** Cluster 5 targets were most densely innervated in L2/3 and L6a, like the pattern  
377 shown in **Figure 5i** (n=778, 12.0%). **(6)** Cluster 6 targets were most densely innervated in deep layers like  
378 in **Figure 5d** (L5 and L6a, n=1119, 17.3%). All 6 of these broad classes of lamination patterns occurred in  
379 targets on the ipsi- and contra-lateral hemispheres at similar frequencies to the overall ratio of the number  
380 of ipsi- and contra-lateral targets (68.77% ipsilateral, **Figure 5n**).

381 Next, we wanted to determine the relationships (if any) between these laminar patterns and the Cre lines  
382 which label neurons of different layers and projection classes. For each mouse line, we calculated the  
383 relative frequency of that line in each cluster, divided by the overall relative frequency of each Cre line in  
384 the entire dataset. **Figure 5o** shows that the projections labeled in each mouse line have more than one  
385 type of target layer pattern (*i.e.*, very few of the boxes are 0, or colored dark green). However, for most  
386 lines, 1-3 layer patterns were identified that occur most frequently (pink-magenta). First, projections  
387 labeled following tracer injections into C57Bl6/J and Emx1-IRES-Cre mice, which label the outputs of all  
388 layers and all classes together, are found with roughly equal frequencies in clusters 4,5, and 6 (all  
389 involving dense targeting to L6). The two L2/3 IT lines, Cux2-IRES-Cre and Sepw1-Cre\_NP39 are most  
390 associated with cluster 3, as is one of the L4 IT lines, Nr5a1-Cre. In contrast, experiments from the other  
391 two L4 IT lines, Rorb-IRES-Cre and Scnn1a-Tg3-Cre, which had some selectively for L5 as well as L4  
392 (**Supplemental Figure 2**) occur with higher than expected frequencies in clusters 1 and 2. The L5 pan-  
393 class line, Rbp4-Cre\_KL100, is most associated with clusters 4 and 5, but the L5 IT line, Tlx3-Cre\_PL56  
394 is strongly associated with cluster 1. All four L5 PT lines were associated strongly with cluster 2, and three  
395 of these were also identified at higher than expected frequencies in cluster 6 (A93-Tg1-Cre, Sim1-  
396 Cre\_KJ18, and Efr3a-Cre\_NO108). The L5 PT line Chrna2-Cre\_OE25, on the other hand, had relatively  
397 more projections of the cluster 4 type. Finally, L6 CT lines, Ntsr1-Cre\_GN220 and Syt6-Cre\_KI148, were  
398 like L5 PT lines in that they each had high relative frequencies of projections assigned to cluster 2 and 6  
399 patterns.

400 The *most common* (but not all) laminar patterns from each Cre line are schematized in **Figure 5p**. In  
401 summary, L2/3 and L4 (Nr5a1) source neurons project predominantly to the middle layers in a target  
402 (L2/3, L4, and L5), avoiding L1. Other L4 source neurons project to L1 and either L2/3 or L5, avoiding L4  
403 and L6. In L5, when both IT and PT classes are labeled, as in the Rbp4-Cre\_KL100 line, projections  
404 target L6 and *either* L1 or L2/3. L5 IT source neurons predominantly target superficial layers (L1 and  
405 L2/3). L5 PT source neurons target either deep layers only (L5 and L6) or deep layers and L1, consistent  
406 with the L5 Rbp4-patterns representing both IT and PT patterns. L6 CT source neurons project to L1 and  
407 L5 or deep layers only.

#### 408 *Anatomical rules for determining hierarchical position in mouse isocortex.*

409 Anatomical patterns of connections derived from anterograde and retrograde tracing data have been  
410 used to describe the hierarchical relationships between cortical areas for decades<sup>12,26,36,37</sup>. In the  
411 schemes based on the macaque monkey visual cortex, different lamination patterns correspond to  
412 feedforward, feedback, and lateral connections between pairs of areas. Briefly, feedforward connections  
413 were characterized by densest terminations in L4, feedback by the preferential avoidance of terminals in  
414 L4, usually with denser projections in both superficial and deep layers, and lateral connections  
415 characterized by having relatively equal density across all layers, including L4<sup>12</sup>. The fraction of  
416 retrogradely labeled cells in supragranular layers has also been used as a continuous variable index of  
417 hierarchical position in macaque cortex<sup>37</sup>.

418 Here, we observed some obvious similarities between the previously published laminar patterns derived  
419 from anterograde tracing data and our results, particularly for the feedback projection pattern. In our view,  
420 clusters 2 and 4 projection patterns (most like **Figure 5g** and **Figure 5b**, respectively) are most like the  
421 feedback rule described by Felleman and van Essen (1991). These patterns avoid L4, strongly targeting

422 L1 and either L5 or L6 and can arise from neurons in all layers and classes (**Figure 5o**). However, we did  
423 not see a pattern emerge, either through unsupervised clustering or from the manual inspection, exactly  
424 like the feedforward projection rule of Felleman and van Essen (*i.e.*, preferentially targeting L4). The most  
425 similar projection pattern to a feedforward rule is seen in cluster 3 (most like **Figure 5e,j**), which involves  
426 projections into L2/3, L4, and L5, arising most often from L2/3 and one L4 IT class (Nr5a1 only, **Figure**  
427 **5o**). The sparsity of projections into L1 with denser signal in mid layers is consistent with an index of  
428 feedforward connections recently described for mouse visual cortex<sup>38</sup>, and suggests that cluster 5 may  
429 also be a feedforward pattern in the mouse. Two patterns without an obvious match to previous literature  
430 were the superficial layer only projections in cluster 1 and the deep layer-only projections in cluster 6.  
431 Both patterns may be feedback because they do not involve mid-layers. Indeed, it was noted in Felleman  
432 and van Essen (1991) that the superficial only pattern was occasionally seen, and they grouped them  
433 with the feedback pattern because it did not involve L4. Also, of note, in the tracer experiments where all  
434 projection neuron classes were labeled (C57Bl6J/Emx1-IRES-Cre) the most common patterns were 4, 5,  
435 and 6. Based on the above descriptions, these would also support the identification of both feedforward  
436 (cluster 5) and feedback (cluster 4 and 6) types between areas when all neuron projection patterns  
437 between a given source-target are compiled.

438 To determine whether the tentative assignments of layer patterns to feedforward or feedback connections  
439 were consistent with past results and assumptions, we looked at specific pairs of connections where  
440 hierarchical relationships have previously been explored or intuited in rodents. **Figure 6** shows two  
441 examples of reciprocally connected pairs of areas within unimodal sensory regions (visual and  
442 somatosensory cortex) that are considered lower (primary) and higher (secondary) in a hierarchy. All  
443 projections out of VISp to higher visual areas are generally described as feedforward, whereas the  
444 reverse (to VISp from higher visual areas) are feedback<sup>26,39-41</sup>. We directly compared the axon projection  
445 patterns originating from neurons in different layers and classes in these examples using the spatially  
446 matched groups of experiments described in **Figures 3** and **4**. In the feedforward direction (VISp to  
447 VISal), projections from VISp terminated with different layer patterns depending on the Cre-defined cells  
448 of origin. L2/3, L4, and L5 IT projections were densest in L2/3-L5 of VISal, and relatively sparse in L1 and  
449 L6. These connections were assigned to cluster 3. Rbp4-Cre\_KL100 projections from VISp to VISal were  
450 densest in L2/3, L4 and L6, characteristic of cluster 5. The L5 PT and L6 CT cells projected to L1 and L5  
451 (cluster 2). In the opposite direction (VISal to VISp), patterns were often different from the corresponding  
452 reciprocal layer-specific projections. From VISal L2/3 IT cells, axons were distributed across all layers,  
453 with a sparser region in L5 (cluster 4). There was also a weak projection from L4 IT cells in VISal to VISp,  
454 with terminals in L1 and L5/6 (cluster 4). The projection originating from L5 IT cells ended predominantly  
455 in superficial layers (cluster 1), while the Rbp4-Cre\_KL100 labeled axons from VISal to VISp were dense  
456 in L1 and deep layers (cluster 4). Again, projections from L5 PT and L6 CT cells were sparse, but present  
457 in both L1 and L6 (cluster 4). Overall, more of the projections in the feedforward direction involved middle  
458 layers, with sparser terminations in L1.

459 In the somatomotor module (**Figure 6b**), we focused on projections between a primary (SSp-m) and  
460 secondary (SSs) area as another example of a reciprocal feedforward (SSp-m to SSs) and feedback  
461 (SSs to SSp-m) connection. Like for the visual pair, projections from L2/3 and L4 IT cells preferentially  
462 innervated L2/3-L5, with relatively sparser terminals in L1 and L6. Both L5 IT and Rbp4-Cre\_KL100  
463 projections strongly innervate L1 and upper L5, unlike the VISp to VISal feedforward connection from L5  
464 IT cells, which avoided L1. L5 PT and L6 CT cell projections were sparse, and to deep layers (cluster 6).  
465 In the reverse direction (SSs to SSp-m), the patterns looked remarkably like the layer-specific FB  
466 projections from VISal to VISp. L2/3 IT cells terminated densely in mid layers (but appeared more diffuse  
467 across the entire column), but the other lines containing IT cells all had similar projections to superficial  
468 and deep layers. One very striking result from laying out the projection patterns originating from different  
469 layers is in the Scnn1a-Tg3-Cre: L4 IT column. For both examples (visual and somatomotor), there is a  
470 very strong connection originating from L4 cells in the lower to higher area that preferentially terminate in  
471 mid-layers. This is clearly not the case in the reverse direction (higher to lower area). Two additional  
472 examples of reciprocally connected areas within different modules (medial: RSPv to VISam and

473 prefrontal: ORBI to MOs) are shown in **Supplemental Figure 9**. These generally follow the same patterns  
474 described above, including the obvious difference in strength and layer pattern in the L4 IT projection  
475 between reversed directions.

476 We next looked at the layer-specific projection patterns between reciprocally connected areas assigned to  
477 different network modules. The anterior cingulate cortex (ACA) exerts top-down control of sensory  
478 processing in VISp<sup>42,43</sup>. We thus assume that the intermodule connection from VISp to ACA is  
479 feedforward, and ACA to VISp is feedback. Layer-specific projection patterns are shown in **Figure 7a**. In  
480 contrast to the intramodule feedforward connections in **Figure 6** and **Supplemental Figure 9**, there is  
481 remarkable similarity in the target layer patterns arising from L2/3, L4, and all classes of L5 cells. These  
482 all preferentially innervate L1 in ACA (cluster 1). In the feedback direction (ACA to VISp), L2/3 cells  
483 also predominantly terminate in L1, but L5 cells project to both L1 and deep layers (L5 and L6, cluster 4),  
484 consistent with previous reports<sup>42</sup>. There may also be a sub-layer distinction in these L1 terminals. Axons  
485 from VISp to ACA seem to be relatively deeper in L1 of ACA, compared to the more superficial  
486 termination of ACA axons in L1 of VISp. In **Figure 7b**, we present images showing laminar termination  
487 patterns arising from the different cell layer/classes between primary (MOp) and secondary (MOs) motor  
488 cortex, which we assigned here to the somatomotor (MOp) and prefrontal (MOs) modules based on their  
489 overall connectivity strengths with the other cortical areas. MOp is generally considered to be at a lower  
490 hierarchical level than MOs, although MOp is also the final output of the cortex driving voluntary control of  
491 behavior. All the IT cells from MOp to MOs appear to have more characteristics in common with  
492 intramodule FF connections (superficial layers, with sparse terminations in L1), including labeled axons  
493 from the predominantly L4 line. From MOs to MOp, there is more involvement of L1 in all patterns, and  
494 the L5 IT pattern includes deep and superficial layers like the Rbp4-Cre\_KL100 experiment.

495 In summary, within a module, feedforward and feedback projections are consistent with the tentative  
496 assignments to cluster/layer pattern described above. Feedforward projections have more target patterns  
497 in clusters 3 and 5, feedback in clusters 2, 4 and 6. The relationship of cluster 1 to feedforward or  
498 feedback is less clear in these intramodule examples, although results in **Supplemental Figure 9** would  
499 support this pattern as feedforward, even with the L1 involvement. The intermodule connections from  
500 ACA to VISp and VISam further support characterization of the cluster 4 layer pattern (superficial and  
501 deep, avoiding L4) as feedback, and, somewhat of a surprise, the cluster 1 pattern (superficial layers) as  
502 feedforward. Also, in all cases, projections from L5 PT and L6 CT neurons, when present, appear to be of  
503 the feedback type regardless of the overall top/down direction.

504 Taken together, these data suggest that the assignment of “feedforward” and “feedback” to specific  
505 connections between any pair of areas should account for all the contributions from each source layer,  
506 and the overall mix of target lamination patterns for a given connection (see also **Supplemental Figure**  
507 **10** showing this concept, more detail below).

508 Based on the anatomical analyses as described, we grouped the observed layer patterns into either  
509 feedforward (clusters 1,3,5) or feedback (clusters 2,4,6). Quantification of the relative frequency of these  
510 patterns for intra- and inter-module connections showed that clusters 1 and 4 occurred more often than  
511 expected in intermodule connections; clusters 3, 5, and 6 were identified more often in intramodule  
512 connections (**Figure 8b**), consistent with data in **Figure 6** and **Figure 7**. All source modules had relatively  
513 high frequencies of the feedforward pattern described by cluster 3 for their intramodule connections  
514 (**Figure 8c**). Between modules (**Figure 8d**), visual, temporal, and somatomotor were strongly associated  
515 with the superficial lamination pattern of cluster 1, which we classified as feedforward. Prefrontal  
516 connections were relatively more frequently assigned to cluster 4 (feedback), and all other modules  
517 occurred more often than expected in cluster 5 (feedforward).

#### 518 *Unsupervised hierarchy of all cortical areas from layer termination patterns*

519 We next determined whether it was possible to use the cell class-based layer termination patterns to  
520 define a direction of information processing through the ipsilateral cortex. We defined hierarchical position

521 for a cortical source area as the number of feedback connections originating from this area minus the  
522 number of feedforward connections, divided by their sum. Each connection was also normalized by a  
523 confidence level for the Cre line in providing information about the direction (see equations 1 and 2 in the  
524 methods). Similarly, the position in the hierarchy as a target is the normalized number of feedforward  
525 minus feedback connections terminating in the target. Each of these measures is bounded between -1 to  
526 1. We used the sum of these measures as the hierarchical position (see equation 3 in the methods,  
527 possible range then becomes -2 to 2). Individual pair-wise measures for each connection, based on the  
528 clustering assignments to target lamination pattern types, are shown in **Supplemental Figure 10**. This  
529 matrix also represents the concept of a “multigraph”, in that there are many possible edges between  
530 nodes (e.g. each of the 13 Cre lines), and all information is used when searching for the optimal  
531 hierarchical positions. We searched over all possible mappings between the layer patterns (the 6 clusters  
532 above) and feedforward and feedback assignments, and checked the self-consistency for every  
533 assignment. For the whole cortex assignment, the highest self-consistency (see equation 4 in methods)  
534 was obtained when clusters 1,3 and 5 were assigned to one type, and 2, 4 and 6 to the opposite. These  
535 are the *same* classifications derived from the anatomical analyses, providing an example of how the  
536 human brain is an excellent unsupervised hierarchy discoverer.

537 Hierarchical positions of 39 (of the 43 parcellated) cortical areas are presented in **Figure 8e** (AUDv, GU,  
538 PERI, and ECT did not have data that passed thresholding). This measure shows primary visual cortex  
539 (VISp) at the bottom and the dorsal part of anterior cingulate cortex (ACAd) at the top. Areas were color  
540 coded by module assignment, which reveals a general pattern for prefrontal areas (red) to be on average  
541 higher in the hierarchy, and unimodal sensory regions (VISp, SSp, AUDp) to be closer to the bottom. For  
542 the entire cortex, the global hierarchy score is 0.126 (value “0”= chance, value “1”= two level, perfectly  
543 consistent hierarchy). Therefore, while we find a global hierarchy, there are still many connections for  
544 which a hierarchical organization may not provide a full explanation. We performed the same analyses on  
545 intramodule connections separately. Here, we observed a hierarchy score of 0.51 for the temporal, 0.33  
546 for the visual module, 0.31 for anterolateral, 0.12 for medial, 0.08 for somatomotor, and 0.03 for  
547 prefrontal, given the same pattern assignments. The range in values suggests that different schemes, or  
548 different assignments of connections to feedforward and feedback, may better describe organization of  
549 connectivity within different modules. A visual module network diagram, edge weights derived from the  
550 matrix of **Figure 2b**, is hierarchically ordered from bottom to top (VISp to VISpor) in **Figure 8h**. The  
551 intramodule hierarchy scores revealed three major levels of note within this module: 1) VISp, 2) VISI,  
552 VISrI, VISpl, VISli, VISal, and 3) VISpor.

### 553 *Hierarchy of cortical network modules*

554 We also determined the overall “feedback-ness” of each module’s ipsilateral outputs (as opposed to the  
555 individual nodes) by measuring the number of feedback minus feedforward patterns, divided by their sum  
556 for every module as a source to all other modules (**Figure 8f**). Then, we calculated the difference  
557 between these feedback fractions for every pair of modules in both directions to obtain a single measure  
558 (the intermodule hierarchy index) predicting the forward/back relationship between each pair (**Figure 8h**).  
559 From this plot, it was obvious that the prefrontal module is above all other modules (e.g., every circle  
560 plotted for prefrontal as a source on the y-axis is positive on the x-axis). Conversely, the somatomotor  
561 module is below all other modules. We thus anchored the hierarchy at the top and bottom with prefrontal  
562 and somatomotor modules. **Figure 8j** shows a network diagram with each module collapsed into a single  
563 node. Edges are the sum of all the weights between modules from **Figure 2b**. The modules were  
564 positioned from top to bottom based on the data points in **Figure 8h**. Remarkably, the order is self-  
565 consistent at all levels given the available data. We did not have enough data or connections between  
566 anterolateral and medial, and anterolateral and visual modules to confirm these positions, however these  
567 are also the weakest of the intermodule connections (thinnest lines in **Figure 8j**). The combination of  
568 layer/class-specific projection patterns between network modules thus enabled the prediction of a  
569 consistent hierarchical framework for cortical information processing, with three modules containing  
570 primary sensory regions at the bottom (somatomotor, temporal, and visual), progressing to higher levels

571 with modules containing more associational areas (medial, anterolateral) and ending in the prefrontal  
572 cortex.

### 573 Discussion

574 Here we used a genetic tracing approach, building on our previously established viral tracing, whole brain  
575 imaging, and informatics pipeline, to map projections originating from unique cell populations in the same  
576 cortical area. Two key features of our high-throughput connectivity mapping pipeline are the automated  
577 segmentation of fluorescent signal, from which we calculate measures of long-distance projection  
578 strength between areas, and the registration of every experiment to our fully annotated 3D Allen Mouse  
579 Brain Reference Atlas. These methods together enabled a comprehensive and more detailed view of  
580 mesoscale cortical wiring patterns, and the derivation of several general anatomical rules of long-range  
581 intracortical connections. Specifically, we show: (1) network analysis of intracortical connectivity patterns  
582 reveals a modular organization of cortical areas, (2) L5 neurons in any given source area make the most  
583 connections, and neurons in L2/3, L4, and L6 project to a subset of these L5 targets, (3) intracortical  
584 target lamination patterns are diverse, but at a coarse-grained level are related to layer of origin and are  
585 like previously described anatomical rules for defining feedforward/feedback connections, (4) projections  
586 originating from specific source layers/classes and target layer patterns together can define a single  
587 direction of information flow (*i.e.*, a hierarchy) between cortical areas and between network modules, with  
588 primary sensory areas and related modules at the bottom, and prefrontal areas at the top. All data are  
589 publicly available through the Allen Mouse Brain Connectivity Atlas portal ([http://connectivity.brain-  
590 map.org/](http://connectivity.brain-map.org/)).

591 Our previously generated whole brain connectome provided a comprehensive, directed, and quantitative  
592 connectivity map between areas of the mouse cortex<sup>4</sup>. In the current study, we used these data and a  
593 novel voxel-based model (Knox et al., submitted) to first provide a macroscale organizational framework  
594 for viewing cortical areas as networks of connections. Through community detection analysis of the  
595 ipsilateral intracortical connectome, we identified six modules. The first module (“prefrontal”) consisted of  
596 cortical areas in predominantly frontal, agranular regions strikingly similar to those recently proposed as  
597 the mouse prefrontal cortex<sup>44</sup>. Very broadly, the function of the prefrontal cortex is cognition, and it is  
598 likely that the connections in and out of this module enable the necessary information flow for incoming  
599 sensory and behavioral state input that influence voluntary control of behavior. Indeed, there is relatively  
600 strong output from this module to primary motor cortex (MOp, **Figure 2b**), and strong input to secondary  
601 motor cortex (MOs) from many regions in other modules. The second module (“anterolateral”) included all  
602 three agranular insular subregions, plus gustatory and visceral cortex, consistent with a role for insular  
603 cortex in integration of taste and body homeostasis/energy needs<sup>45,46</sup>. At lower levels of spatial resolution  
604 ( $\gamma < 1$ ) these areas were part of a larger sensory module together with our third module (“somatomotor”).  
605 The somatomotor module contained all the primary somatosensory regions, secondary somatosensory  
606 cortex, and MOp. All SSp divisions project to SSs and MOp, and to MOs, which is in the prefrontal  
607 module. Although the MOp is not sub-parcellated in the Allen 3D reference atlas, our projection mapping  
608 data show that projection domains are preserved in MOp when originating from the different SSp domains  
609 (*i.e.*, there are specific terminal fields in MOp for barrel field, trunk, lower limb, upper limb, nose, mouth),  
610 as previously reported<sup>3</sup>. The fourth module (“visual”) contained primary visual cortex and six of nine  
611 higher order visual areas. The remaining three visual areas (VISa, VISam, and VISpm) were grouped with  
612 the three retrosplenial cortex subregions (RSPagl, RSPd, RSPv) in the fifth module (“medial”). At lower  
613 spatial resolution, these two modules (visual and medial) consistently merged into one, reflecting the high  
614 connectivity strengths between all visual areas. However, the medial module areas are more strongly  
615 connected to prefrontal areas (particularly ACAd, ACAv, and ORBvl). The sixth module (“temporal”) contained  
616 both auditory sensory regions and associational cortical areas, perirhinal (PERI), ectorhinal  
617 (ECT), and temporal association cortex (TEa). This combination is the least intuitive of all these modules,  
618 and may be a consequence of limitations in the underlying dataset. Specifically, in only one experiment  
619 did we successfully label projections from ECT cortex (shown in bottom right panel, **Figure 2a**), and so

620 the voxel-based model had very little information to use for prediction of connection strengths (Knox et al.,  
621 submitted).

622 Within these modules and areas, we identified generalizable layer-specific intracortical projection  
623 patterns. For a given cortical source area, L2/3, L4, L5 PT, and L6 CT cells project to a subset of the  
624 target regions contacted by L5 neurons. Notably, all of the excitatory neuron classes we surveyed had  
625 intracortical projections labeled outside of the infection area, including the L4 IT and subcortical PT and  
626 CT projection neurons. This somewhat unexpected result could be caused by Cre lines with less than  
627 perfect specificity. However, we also found that when long-distance projections are present from PT or CT  
628 cells, they have distinct termination patterns compared to the IT lines. These projection patterns were  
629 consistent with characteristics of feedback pathways, even in connections that were overall feedforward.  
630 A feedback collateral arising from deep layer subcortical-projection neurons may thus be another  
631 generalizable feature of PT and CT intracortical axons<sup>47</sup>.

632 The strength and presence of projections between areas from the predominantly L4 Cre lines was also  
633 unexpected. Canonical circuits, mostly derived from primate and cat, do not include inter-areal L4  
634 excitatory neuron projections<sup>48</sup>. The three Cre lines used here had varying degrees of selectivity for L4  
635 expression (**Supplemental Figure 2**), with some expression in L5 for both the Scnn1a-Tg3-Cre and  
636 Rorb-IRES-Cre lines. These two Cre lines also contain cells classified into both L4 and L5a types based  
637 on transcriptomics; while the Nr5a1-Cre line appears most specific to L4 cell types<sup>15</sup>. Thus, it is difficult to  
638 definitively conclude that these inter-areal projections originate from L4 rather than L5. Differences in the  
639 number of connections and the specificity of these inter-areal connections suggest that if the origin is in  
640 L5, they are at least a unique subclass of projection neurons. It is also worth noting that although the  
641 prefrontal and some other areas in the cortex are considered “agranular”, *i.e.*, lacking a distinct L4, Cre  
642 expression is often still detectable, though much sparser, in the so-called L4 lines used here.

643 Classic definitions for PT and CT cell classes exclude contralateral cortical projections<sup>20</sup>, roughly  
644 consistent with our observations. However, our data also showed that for some source areas, particularly  
645 in the prefrontal module, PT and CT lines had labeled axons that crossed the callosum, terminating in a  
646 small number of contralateral cortical targets. Overall, most callosal projections were made by neurons in  
647 L5, and, to a lesser extent, L2/3 and L4 Cre lines, consistent with expectations from previous results<sup>49</sup>.  
648 The number of ipsilateral connections made by L2/3 IT cells was similar to L5 IT cells; however, they  
649 differed on the contralateral side. A recent analysis of the rat macroscale cortical connectome identified a  
650 set of general rules regarding ipsilateral (associational) and contralateral (commissural) connections that  
651 are mostly consistent with our observations; namely that all cortical areas have more associational than  
652 commissural targets<sup>8</sup>. The mesoscale connectome data we present here reveals that some of these  
653 differences can be better explained at the level of cell class.

654 Cortical areas are densely interconnected, and projections arise from all layers. Here, we first present an  
655 organizational scheme, the network, that groups cortical areas based on the strength of their connections.  
656 This kind of network view of cortical organization presents a structural view of all possible paths of  
657 information flow between areas and modules, but does not impose a direction or order on that flow.  
658 Another very influential organization scheme is the cortical hierarchy. The existence of a hierarchy implies  
659 classifying inter-areal connection types into two general classes: feedforward or feedback. From the  
660 macaque brain, studies have demonstrated that specific anatomical projection patterns between areas  
661 are characteristic of either a feedforward or feedback connection<sup>12,13</sup>. In short, feedforward is  
662 characterized as a pathway in which superficial layer neurons projected most densely to L4; feedback as  
663 originating from deep layers and avoiding L4 in the target. These primate rules were applied to build the  
664 visual cortex hierarchy that has inspired multiple computational models of cortical function<sup>12</sup>. In these  
665 models, feedforward and feedback pathways are mapped to two different functions. Some focus on deep  
666 neuronal networks mapping to visual processing<sup>14,50</sup>. While these models focus on the feedforward  
667 connections for information processing, feedback connections could be viewed as carrying a learning  
668 signal. Another popular model for cortical computations is predictive coding<sup>51</sup>. In this model, the

669 feedforward connection represent an error signal, while feedback connections represent predictions, and  
670 local circuits integrate them<sup>52</sup>.

671 Partial hierarchies of the visual cortex exist for rodents, also based on anatomical projection patterns from  
672 anterograde tracing studies<sup>26,38,53</sup>. Differences between those used in the primate were noted, and re-  
673 classified for rodent. Specifically, feedforward connections were characterized by having less dense axon  
674 terminations in L1 compared to L2/3, but axon terminals still spanned L2/3 to L5 evenly<sup>38</sup>. Feedback  
675 avoided L4 (like for the primate), terminating most densely in L1 and L6. We noted the same kinds of  
676 patterns in visual cortex feedforward and feedback connections. Whether these patterns can be extended  
677 to other sensory, motor, and associational modules (including those with “agranular” cortex<sup>54</sup>) was less  
678 clear from previous studies. Our analysis within and between modules suggests that there are several  
679 patterns associated with feedforward and feedback connections, but that every pattern can be classified  
680 into one or the other type. Within most modules, feedforward has the characteristics described above  
681 (i.e., densest in L2/3-L5), but between modules the feedforward connections were either dense in L2/3  
682 and L6, or preferentially terminated in superficial layers (L1-L2/3). Feedback patterns matched previous  
683 descriptions in that there was preferential termination in L1 + deep layers (L5 or L6), or deep layers only.  
684 One of the most unusual patterns, not reported previously to the best of our knowledge, that clearly  
685 differentiated known feedforward from feedback connections was the strong presence of axons  
686 originating from L4 and ending in L2/3-L5. This occurred only in the feedforward direction of reciprocally  
687 connected pairs, but in the reverse direction there was both fewer axons from L4 and, when present, they  
688 were associated with more feedback patterns (avoiding L4). We also observed a striking relationship  
689 between feedforward/feedback patterns with the cell layer/classes, namely, supragranular (L2/3  
690 upper 4) neurons have predominantly feedforward projections, whereas infragranular (L5 and L6)  
691 neurons have both feedforward and feedback projections. However, as we have already noted, these  
692 types and their relationship to cell classes are also dependent on the specific connection.

693 Using these rules for feedforward and feedback across all cortical areas, we observe that the global  
694 organization of cortical connections is consistent with a hierarchical organization scheme, in which a  
695 bottom-to-top direction can be well defined. However, we want to emphasize that the hierarchical position  
696 does not explain all the connections of all the cell classes within each area, as the pathways between  
697 cortical areas are complex. We obtained a global hierarchy score of 0.126 for the entire cortex (with 0  
698 being chance and upper bounded by 1), thus, it is actually quite far from a complete explanation. The  
699 model used for **Figure 8e** shows results of an optimized hierarchy, but it is not the only possible solution,  
700 akin to the differences described for determining hierarchical order in primate visual cortex using the  
701 fraction of supragranular projection neurons (SLN), instead of discrete levels as in the Felleman and Van  
702 Essen diagram<sup>12,37</sup>. It is very interesting that there is also a range of scores at the level of modules, from  
703 the highest end of 0.51 for temporal and 0.33 for visual modules, to the low end of 0.03 for the prefrontal  
704 module, suggesting that primary sensory areas like the visual cortex, representing the initial and most  
705 long-standing cortical hierarchy, also best fit into this organizational scheme.

706 Given the number of different connection types arising from a single area, we believe that new  
707 computational models, containing more than feedforward and feedback connections between nodes, are  
708 needed. This may be especially true when moving beyond models of sensory processing in the cortex.  
709 We would like to emphasize and encourage the adoption of a multigraph view of connectivity, in which  
710 two areas can be connected by multiple edges; each edge having an associated weight, type and  
711 subtype. We challenge the theoretical community to expand computational algorithms beyond those  
712 focused on classical graph structure. Additional data types that could predict directionality in cortical  
713 organization may also be added to these connections in the future. For example, ratios of specific  
714 interneuron types mapped across all cortical areas has also recently been related to hierarchical position  
715 in the mouse<sup>55</sup>.

716 The expansion of the Allen Mouse Connectivity Atlas to include mapping of projections from genetically-  
717 identified cell classes represents a big step toward a true *mesoscale* connectome. Here, we present the

718 addition of ~ 1000 new experiments to our online resource (<http://connectivity.brain-map.org/>), but focus  
719 only on the analysis of intracortical projection data. However, the complete brain-wide projection patterns  
720 are also already available for interested researchers to pursue a multitude of questions and analyses,  
721 Results incorporating subcortical inputs and outputs may alter our view of a hierarchy in interesting and  
722 important ways. Finally, one of the limitations inherent in the population-based mapping approach used  
723 here is that we are certainly still missing many details at a more fine-grained level of cell types. Cre lines  
724 used to label cell populations are rarely perfectly specific to a given cell class or type. Recent efforts and  
725 future work will undoubtedly further subdivide these broad classes of pyramidal neurons, at the level of  
726 areas as well as layers and projections, into specific cell types using morphology, physiology, and  
727 transcriptomics<sup>15,17</sup>. Here, we focused only on broad classes to derive general patterns of mesoscale  
728 cortical connectivity, which we believe will be instructive and informative for future connectome data from  
729 more refined cell types. However, future large-scale efforts aimed at mapping the projections of specific  
730 cell types rather than classes, and even single cells<sup>56</sup> will no doubt reveal additional principles of cell type-  
731 specific connectivity across the brain, moving us even closer to a full mesoscale connectome.

732

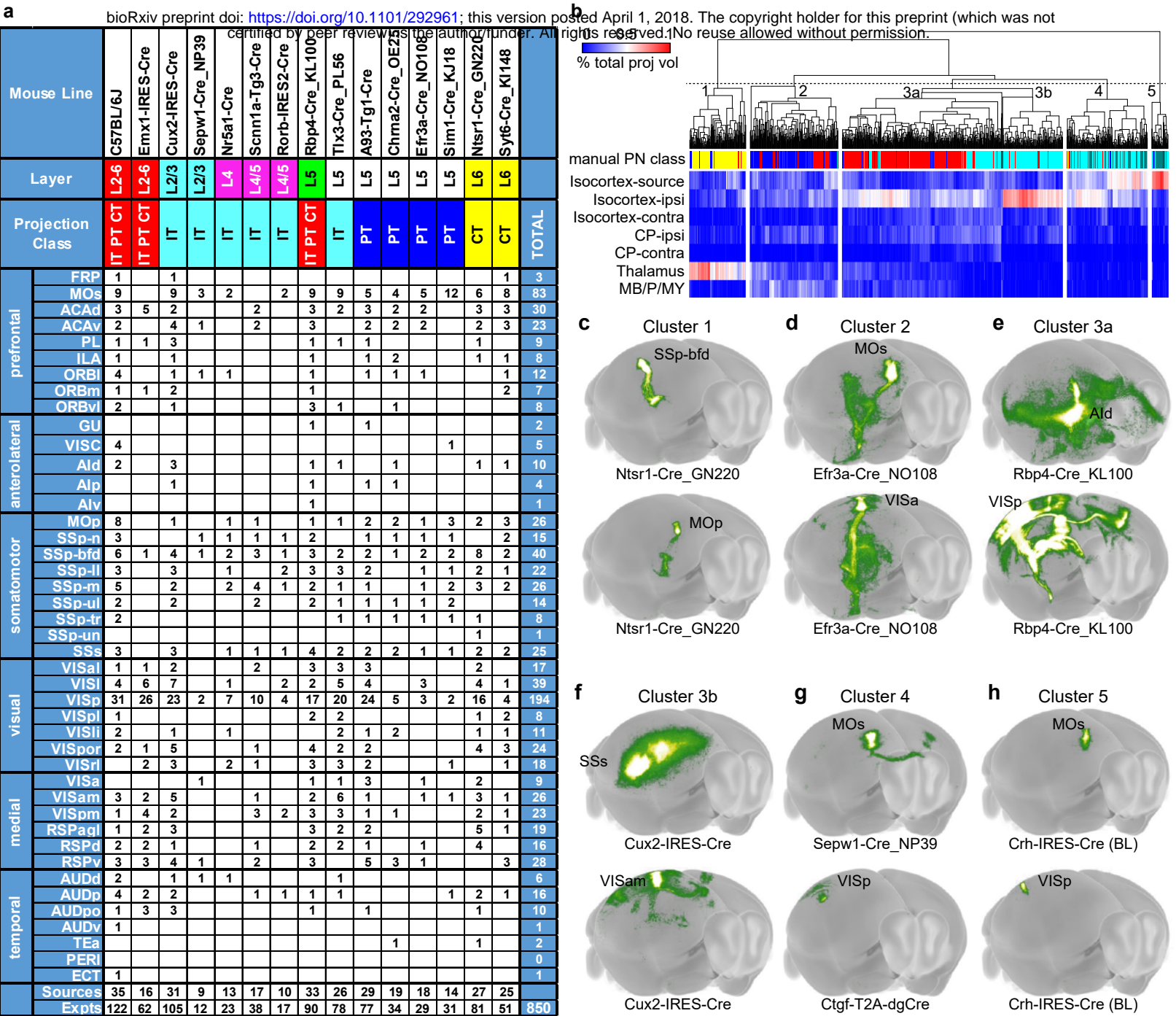


733 Figures

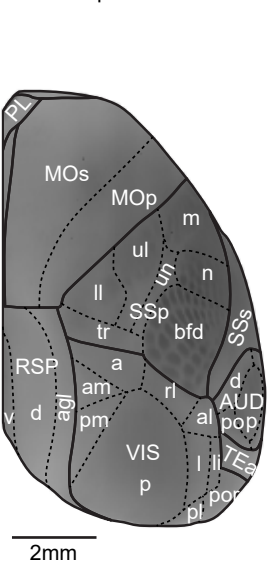
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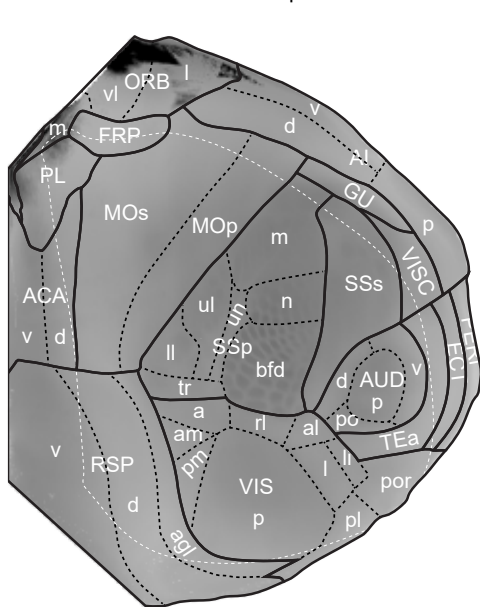
Figure 1



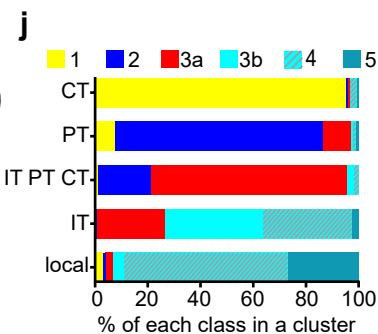
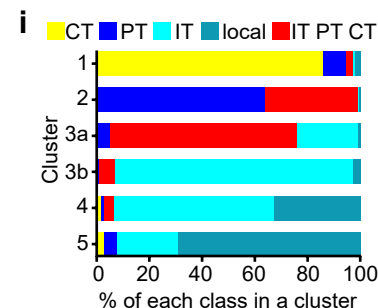
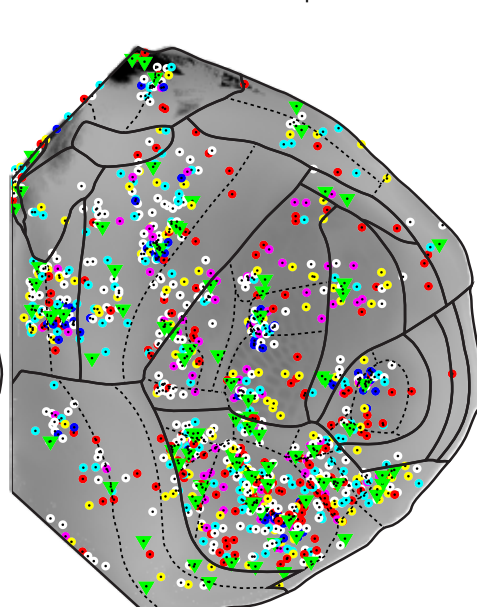
**k** Cortical surface map: top-down



**l** Cortical surface map: flat



**m** Centroid locations of all experiments

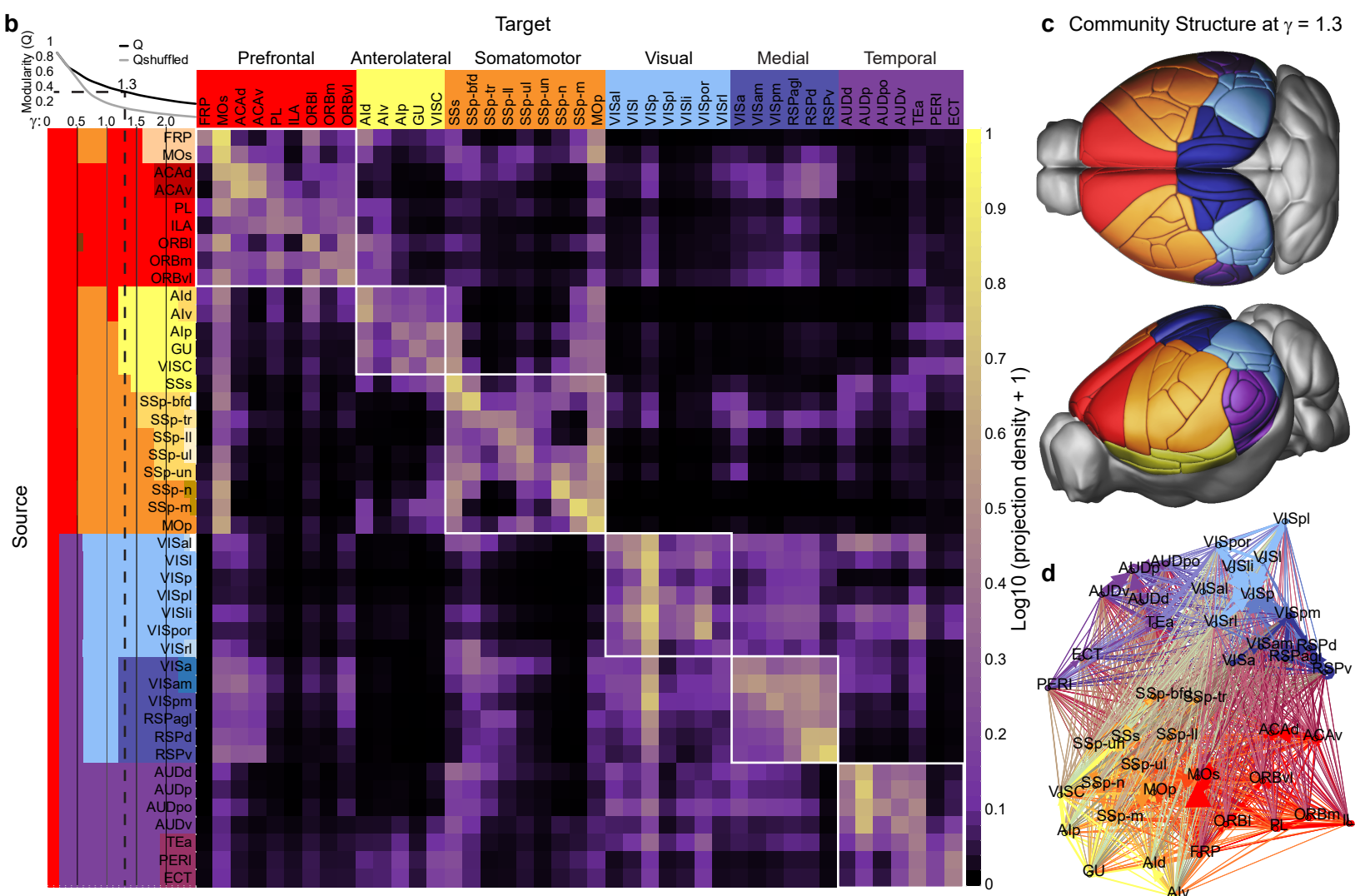
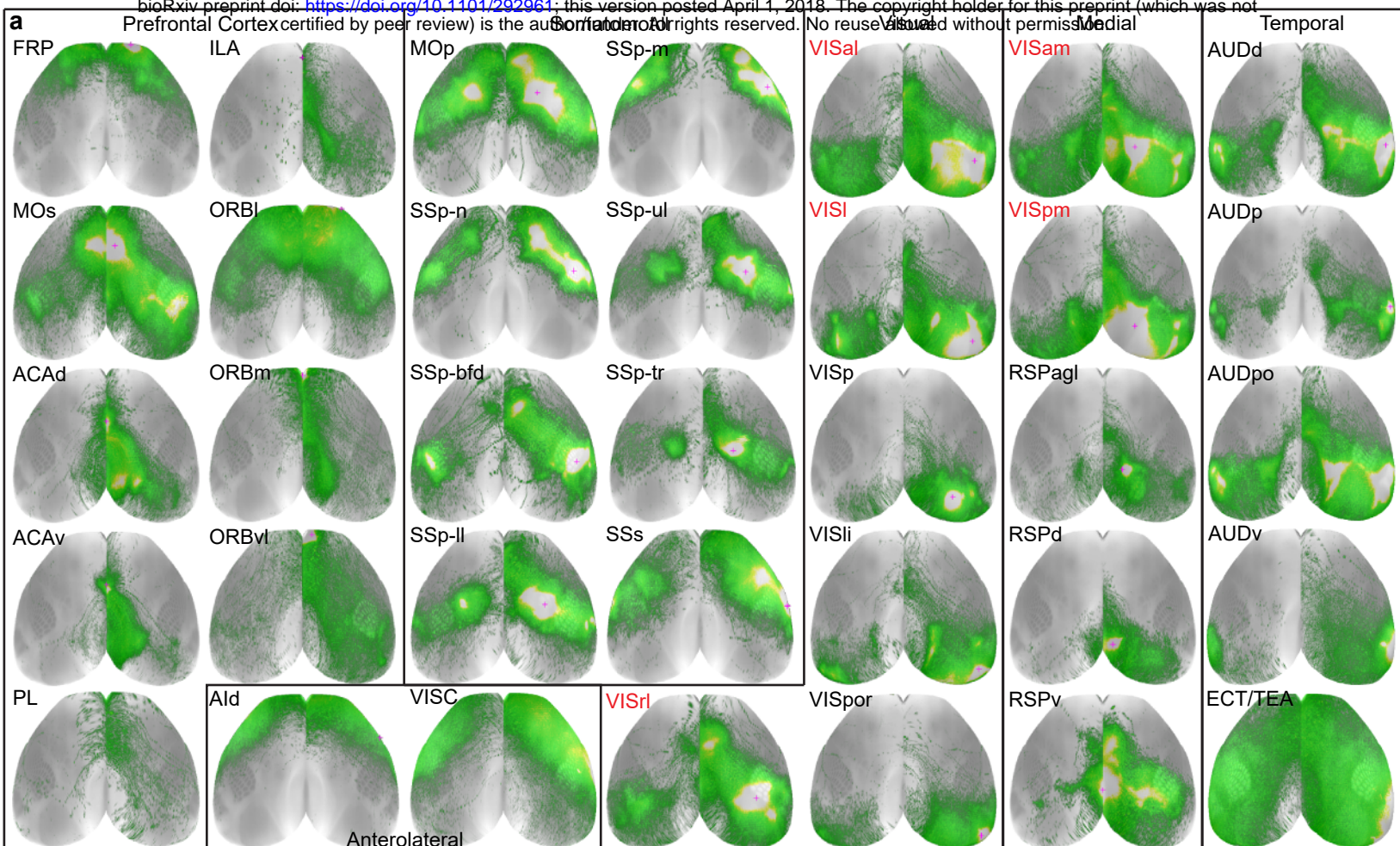


736 **Figure 1. Systematic generation of cortical projection mapping data by area and mouse line. (a)**  
737 Location and number of 850 tracer injection experiments across cortical areas and selected mouse lines.  
738 These 15 lines (C57Bl/6J through Syt6-Cre\_K1148) were used most extensively across cortical regions to  
739 map projections using Cre driver lines with expression preferentially in the layers and projection neuron  
740 classes indicated. **(b)** Hierarchical clustering of brain-wide projection patterns reveals classes of  
741 projection neurons. Each column of the heat map shows one of the 1082 injection experiments in the  
742 entire dataset (see Supplemental Table 1 for all experiments). Colors in the “manual PN class” are coded  
743 as in (a) for Projection Class, and show the manual assignment for each of the 1082 experiments. Rows  
744 show selected major brain regions to distinguish known classes of projection neurons (isocortex injection  
745 source, isocortex-ipsi and -contra, caudoputamen (CP)-ipsi and -contra, thalamus, and  
746 midbrain/pons/medulla combined (MB/P/MY)). Values in each cell of the matrix are the sum projection  
747 volume (segmented pixels) within the given brain region divided by the total brain projection volume per  
748 experiment. Unsupervised hierarchical clustering of the experiments (using Spearman rank correlations  
749 and average linkage method) identified five major clusters. Cluster 3 contains two additional subclusters  
750 (3a, 3b). **(c-h)** Maximum intensity projections of brain-wide axons from individual experiments are shown  
751 as representative examples from each cluster or subcluster. **(i)** The graph shows the percent of members  
752 in each cluster that were manually-assigned to one of the projection neuron classes. The majority class in  
753 each cluster was (in order from 1-5) 1=CT, 2=PT, 3a=IT PT CT, 3b=IT, 4=IT, and 5=local. **(j)** The graph  
754 shows the percent of experiments in each manually-assigned class that belonged to each cluster. The  
755 majority cluster for each class was CT=1, PT=2, IT PT CT=3a, IT=3b, and local=4. **(k)** Mouse isocortex is  
756 parcellated into 43 areas in the Allen 3D reference atlas. The positions of most areas are visible in our  
757 standard top-down view of the right hemisphere cortical surface. This view is obtained by projecting the  
758 maximum density voxels from the average template brain, used to construct the 3D reference atlas, along  
759 a curved coordinate system meant to match the columnar structure of the cortex (as opposed to a direct  
760 z-projection). **(l)** Areas that occupy ventral lateral, frontal and midline positions are better viewed in a  
761 flattened map of the mouse cortex. The flatmap is generated by constructing a 3D-to-2D mapping such  
762 that the 2D Euclidean distance of every point on the flatmap to a pair of anchor points are the same as  
763 their 3D geodesic distance (shortest path along surface), resulting in the coordinate along one axis  
764 formed by the anchor points. This process is repeated for a second pair of anchors point to form the  
765 second axis. The white dotted line indicates the boundaries of what is visible in the top-down view in **k**.  
766 **(m)** The position of each injection centroid is plotted on the flat cortical surface. Colors indicate the layer  
767 selectivity for excitatory projection neurons and the inhibitory Cre lines as shown in **a**. Green triangles are  
768 Rbp4-Cre\_KL100 experiments.

769

Figure 2

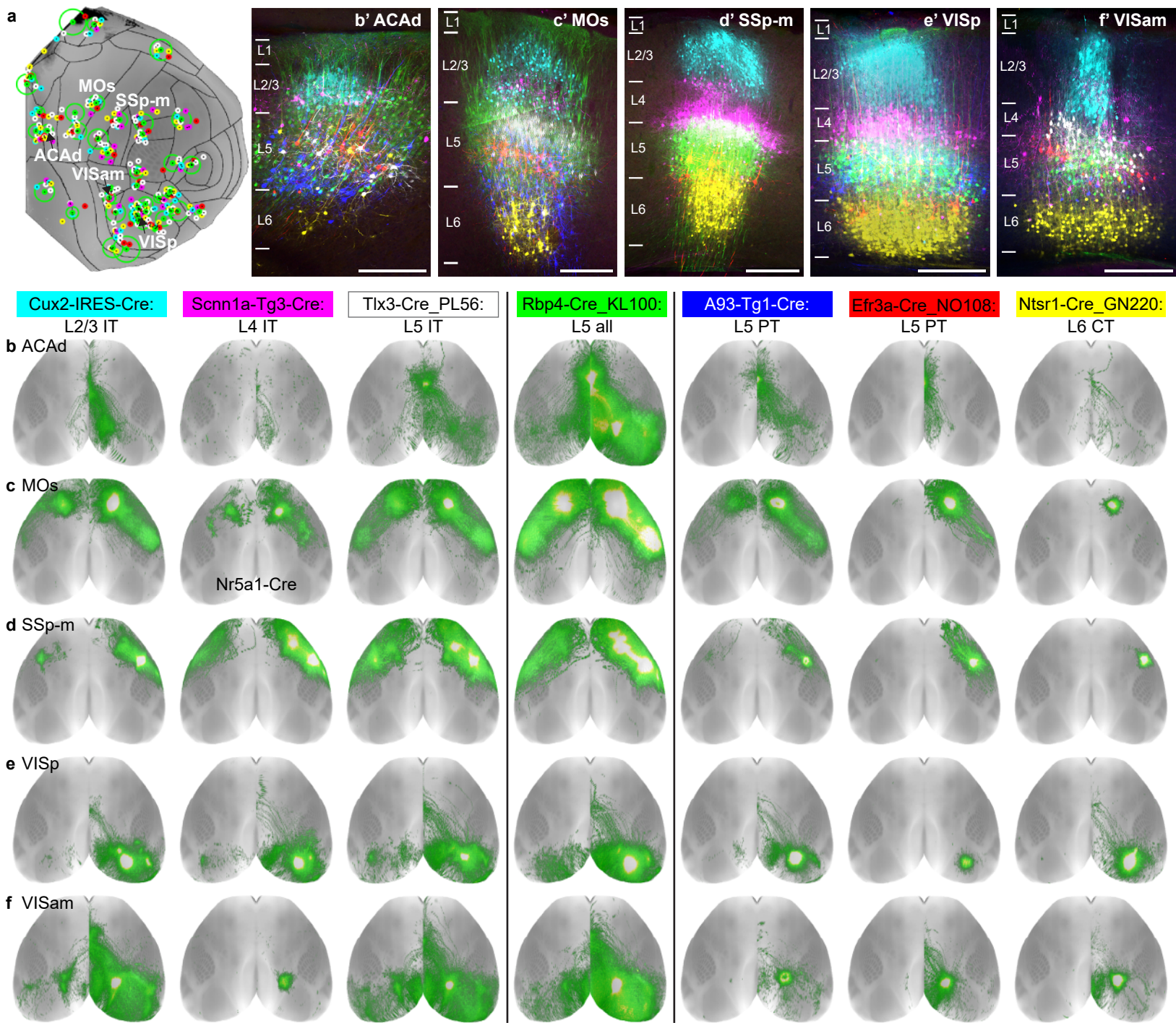
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770 **Figure 2. Modular organization of intracortical projection patterns based on the wild type**  
771 **connectivity matrix. (a)** Top down cortical surface views showing the relative projection densities of  
772 labeled axons (normalized within each experiment, white is saturation) originating from 35 cortical source  
773 areas in C57Bl6/J (black labels) or Emx1-IRES-Cre (red labels) mice. Red cross hairs indicate the  
774 location of the injection centroid. Some are not visible in the top down view. **(b)** Weighted connectivity  
775 matrix for 43 cortical areas. The data matrix was derived from the voxel-based model of Knox et al.,  
776 submitted, and shows the connection strength as log-transformed normalized projection density (the sum  
777 of predicted density per voxel in a target region normalized to that target's volume). Cortical areas are  
778 ordered first by module membership then by ontology order in the 3D Allen reference atlas. Colors along  
779 the matrix axes indicate community structure with varying levels of resolution ( $\gamma = 0-2.5$  on the y axis,  $\gamma =$   
780  $1.3$  only on the x-axis). The modularity metric (Q) is plotted for each level of  $\gamma$ , along with the Q value for a  
781 shuffled network containing the same weights. Community structure was determined independently for  
782 each value of  $\gamma$ , but colors were matched to show how communities split as the resolution parameter is  
783 increased. **(c)** Cortical regions color-coded by their community affiliation at  $\gamma = 1.3$  show spatial  
784 relationships. **(d)** Diagram shows the ipsilateral cortical network in 2D using a force-directed layout  
785 algorithm. Nodes are color coded by module. Edge thickness shows relative projection density and edges  
786 between modules are colored as a blend of the module colors.

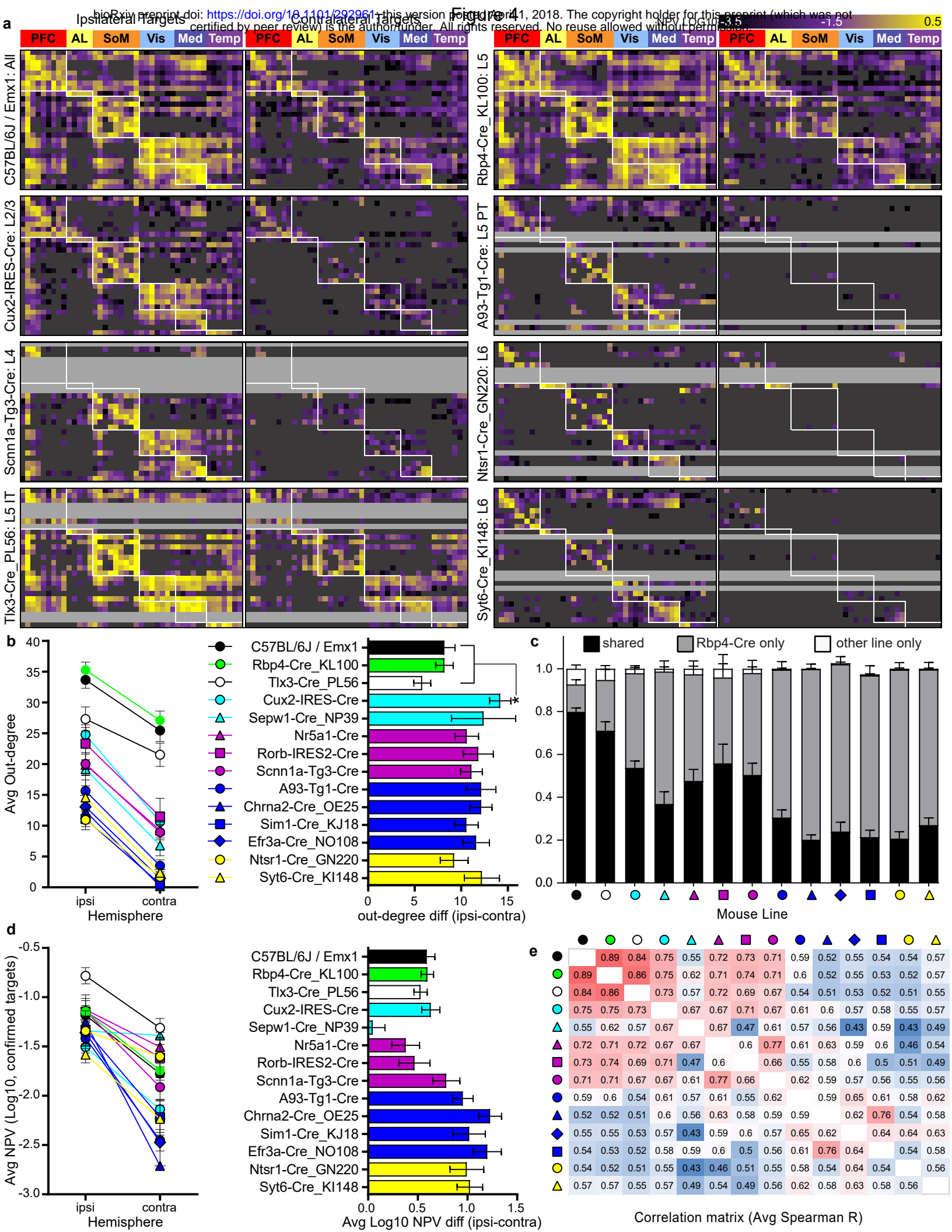
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Figure 3.



788 **Figure 3. Comparison of layer- and class-selective intracortical projection patterns. (a)** 43 groups of  
789 spatially-matched experiments to an Rbp4-Cre\_KL100 anchor were collated based on having a  
790 “complete” membership roster; representing L2/3 IT, L4 IT, L5 IT, L5 PT, L6 CT and the L5 IT PT CT data  
791 from Rbp4-Cre\_KL100. Each Rbp4-Cre experiment is shown as a green dot; all other experiments are  
792 color coded by layer and class as indicated. The green circle indicates the variance in distance to Rbp4  
793 for each group. The five labeled groups are the examples shown in **b-f** (ACAd, MOs, SSp-m, VISp, and  
794 VISam). **(b'-f')** 2-photon images acquired at the center of each injection site were manually overlaid by  
795 finding the best match between the top of L1 (pial surface) and bottom of L6 (white matter boundary)  
796 between each experiment, and then pseudocolored by Cre line to highlight the layer selectivity of Cre  
797 expression. Scale bar = 250  $\mu$ m. **(b-f)** Top down views of the labeled axonal projections across the cortex  
798 originating from the infected neurons shown in **b'-f'**. Three Cre lines that label IT projection classes in  
799 L2/3 (Cux2-IRES-Cre), L4 (Scnn1a-Tg3-Cre, or Nr5a1-Cre as indicated for MOs) and L5 (Tlx3-Cre\_PL56)  
800 are shown to the left of Rbp4-Cre\_KL100. Three lines that predominantly label PT or CT projection  
801 neurons in L5 (A93-Tg1-Cre, Efr3a-Cre\_NO108) and L6 (Ntsr1-Cre\_GN220) are shown to the right.  
802 These lines also have intracortical projections, but target a smaller number of areas.

803

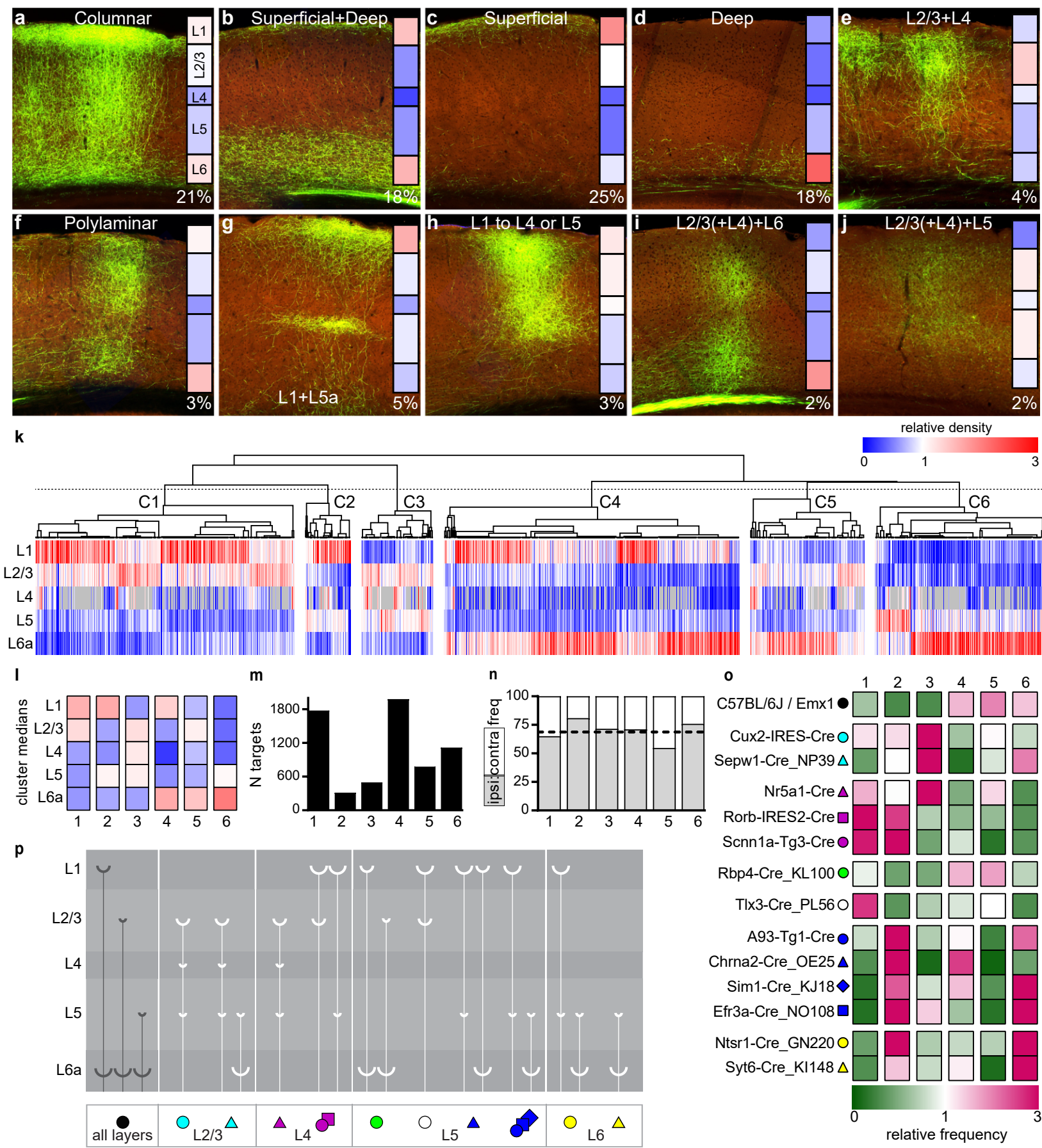




804 **Figure 4: Cre line and layer-specific cortical outputs. (a)** Eight directed, weighted, connectivity  
805 matrices (27 x 86) are shown for mouse lines representing projections labeled from different layers and  
806 cell classes. Each row of one matrix contains experimental data from one of 27 unique source areas.  
807 Columns show the 43 ipsilateral and 43 contralateral cortical target regions. Rows and columns follow the  
808 same module-based ordering in each matrix. Areas and connections belonging to the modules assigned  
809 using the ipsilateral voxel-based model data in Figure 2b are indicated by the white boxes. For every  
810 experiment, each of the 43 ipsilateral and 43 contralateral targets were inspected and assigned as  
811 containing either true positive or true negative axon terminal labeling. All true negatives (including passing  
812 fibers) were masked and colored dark grey. Rows for which an experiment was not completed are light  
813 grey. This was often because of low levels of Cre expression in those areas. The color map corresponds  
814 to log<sub>10</sub>-transformed normalized projection volumes in each target (range 10<sup>-3.5</sup> to 10<sup>0.5</sup>, truncated at both  
815 ends). **(b)** On the left, the average out-degree across all sources represented in each matrix for each Cre  
816 line is plotted for the ipsilateral and contralateral cortex. The difference in number of connections between  
817 the ipsi- and contra- hemispheres is shown for each line in the bar graph (right,b). **(c)** Binary present or  
818 absent calls for the targets of each experiment were compared to the presence/absence calls from the  
819 matched Rbp4-Cre\_KL100 anchor experiment. The average percentage of true positive targets shared by  
820 each line with its Rbp4 anchor experiment is plotted in the bar graph (black) as well as the average  
821 percentage of positive targets that are unique to Rbp4 (gray) or unique to the line indicated (white). **(d)**  
822 On the left, the average strength of all the connections (normalized projection volume) across source  
823 areas in each matrix are plotted for ipsilateral and contralateral hemisphere by Cre line. The difference in  
824 connection strengths between the ipsi- and contra-lateral hemispheres is shown for each line in the bar  
825 graph (right,d). **(e)** Average spearman correlation coefficients based on normalized projection volumes  
826 were calculated between all pairs of lines within each anchor group. Symbols or bars in **b-d** show mean  
827 +/- SEM.

828

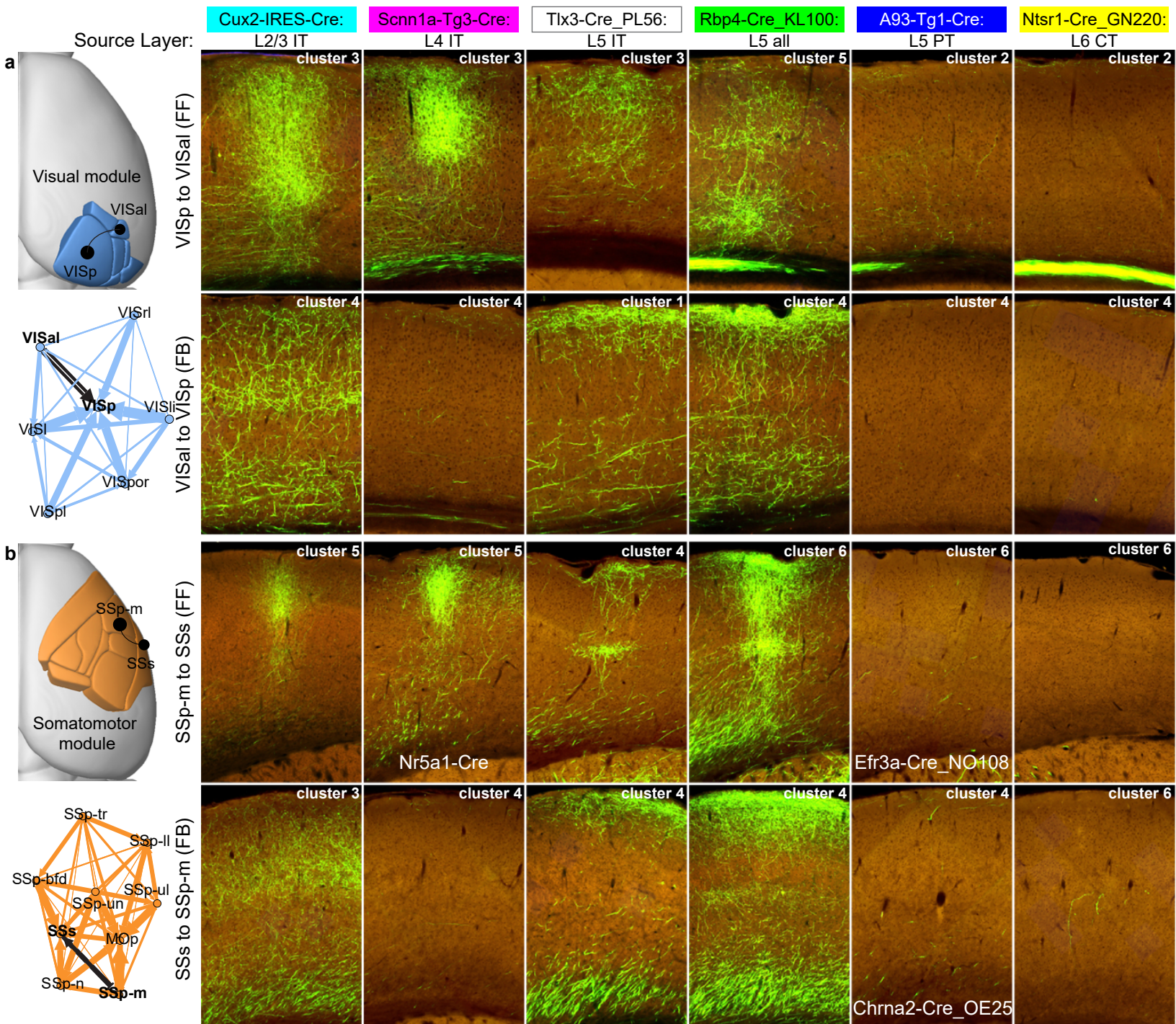
Figure 5



829 **Figure 5: Diverse target lamination patterns in mouse cortex. (a-j)** Relative densities of axon terminal  
830 labeling across layers for every cortical target were visually inspected for a subset of experiments, and  
831 then classified into one of ten categories based on overall observations. Four patterns occurred most  
832 frequently; **(a)** columnar, with relatively equal densities across all layers (21%), **(b)** superficial and deep  
833 layers in equal densities (18%), **(c)** superficial layers only (25%), or **(d)** deep layers only (18%). Additional  
834 patterns of note, although rare (<10%), included those in which L1 received relatively few axons **(e, i, j)**.  
835 Insets show the results of averaging informatively-derived quantification of relative layer density (the  
836 fraction of the total projection signal in each layer, scaled by the relative layer volumes) for all targets  
837 manually classified to that category. A relative density value of “1” (color = white) indicates that the  
838 fraction of axon labeling within a specific layer is equal to the relative size of that layer in that target, *i.e.*, it  
839 is neither more nor less dense than expected if axons were distributed evenly across layers, given  
840 differences in layer volumes. Values <1 indicate lower than expected density and >1 higher than  
841 expected density. Relative densities were color coded from 0 (blue) to 1 (white) to 3 (red). The color map  
842 key applies for panels a-l. **(k)** Unsupervised hierarchical clustering using spearman correlation and  
843 average linkages on the relative density values per layer. Each column is a unique combination of cre  
844 line, source area and target, after thresholding as described in the results. The dotted line indicates where  
845 the dendrogram was cut into 6 clusters. **(l)** Median relative density values by layer for each of 6 clusters.  
846 **(m)** Total number of targets in each cluster. **(n)** The frequency of ipsilateral and contralateral targets  
847 assigned to each cluster. The dotted line indicates the overall frequency of ipsilateral targets (68.77%).  
848 **(o)** The relative frequency of each Cre line appearing in one of the 6 clusters. The fraction of experiments  
849 in a cluster belonging to each Cre line was divided by the overall frequency of experiments from that Cre  
850 line in the complete dataset. A relative frequency value of “1” (color = white) indicates that Cre line  
851 appeared in that cluster with the same frequency as in the entire dataset. Values <1 (green) indicate  
852 lower than expected frequency, and >1 (pink) indicate higher than expected frequency of that Cre line in a  
853 cluster. **(p)** Schematic diagram showing the relationships between the layer and class of origin in the  
854 source area (Cre line symbols at the bottom) with the most frequent axon lamination patterns observed in  
855 the target area.

856

Figure 6



857 **Figure 6. Intra-module projection patterns between reciprocally connected areas originating from**  
858 **different layers/classes. (a)** In the visual module, VISp and VISal are reciprocally connected (black and  
859 white arrows). VISp is the de facto bottom of visual cortex hierarchies. The output to VISal from VISp is  
860 feedforward (FF). The reciprocal connection (VISal to VISp) is feedback (FB). **(b)** In the somatomotor  
861 module, the primary somatosensory cortex (SSp), like VISp, is the de facto bottom of the hierarchy. SSp-  
862 m sends feedforward projections to the secondary somatosensory region (SSs). SSs sends feedback  
863 projections to SSp-m. Modules from Figure 2b are shown spatially mapped on the cortex and as a force-  
864 directed network layout with the thickness of the lines corresponding to relative connection weights. 2P  
865 images in the approximate center of the axon termination fields for each target region show the laminar  
866 distribution of axons arising from labeled neurons in the different Cre lines, as indicated. Images were  
867 rotated so that the pial surface is always at the top of each panel. The cluster assignment for that line-  
868 source-target combination (columns **in Figure 5k**) is also indicated in each panel. One very striking  
869 difference between FF and FB connections was the strength and pattern of projections originating from  
870 L4 IT cells (second column). L4 IT cells in both modules strongly projected to the target in the FF  
871 direction, with patterns showing sparser axons in L1. In the FB direction, the L4 projection was weaker  
872 and ended in L1.

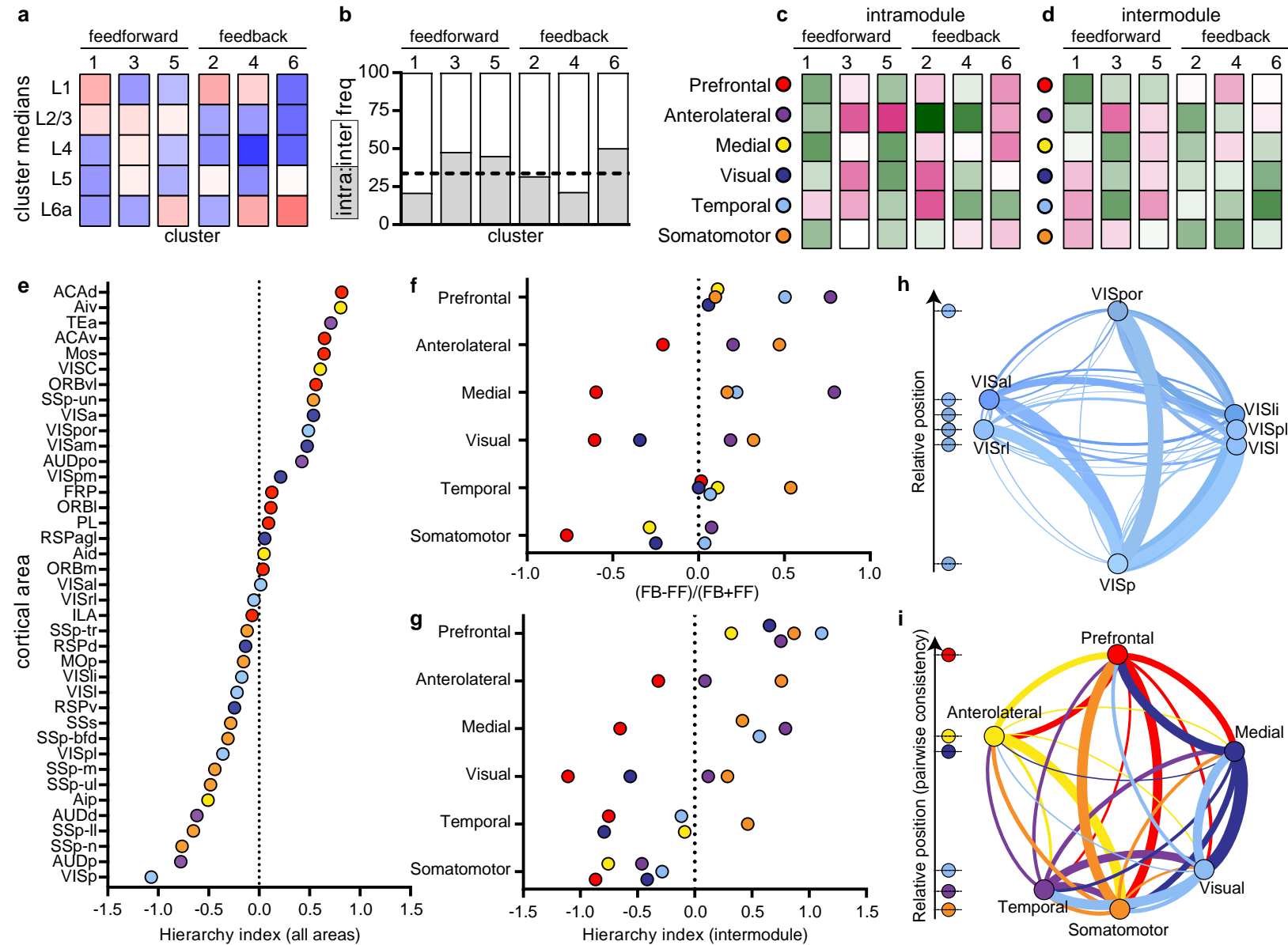
873



874 **Figure 7. Inter-module projection patterns between reciprocally connected areas originating from**  
875 **different layers/classes. (a)** Many reciprocal connections exist between areas in prefrontal and visual  
876 modules, e.g. VISp and ACAd. ACAd exerts top-down control of VISp activity, so we assume this  
877 connection is FB, and that the reverse is FF. **(b)** Many reciprocal, and stronger, connections also exist  
878 between nodes of the prefrontal and medial modules, e.g. ACAd and VISam. Modules from Figure 2b are  
879 shown spatially mapped on the cortex and as a force-directed network layout with the thickness of the  
880 lines corresponding to relative connection weights. Like in Figure 6, 2P images in the approximate center  
881 of the axon termination fields for each target region show the laminar distribution of axons arising from  
882 labeled neurons in the different Cre lines, as indicated. Images were rotated so that the pial surface is  
883 always at the top of each panel. The cluster assignment for that line-source-target combination (columns  
884 **in Figure 5k**) is also indicated in each panel.

885

Figure 8





886 **Figure 8. Organization of network modules into a hierarchy based on anatomical patterns of**  
887 **connections. (a)** The six layer patterns identified through clustering in **Figure 5k** were classified as either  
888 feedforward or feedback. Clusters 1,3, and 5 were considered as characteristic of different feedforward  
889 projections. Clusters 2,4, and 6 were considered characteristic of different feedback projections. **(b)** The  
890 relative frequency of inter-module and intra-module connections is plotted for each cluster. The dotted  
891 line indicates the overall frequency of intramodule connections (33.74%). The laminar patterns of clusters  
892 1 and 4 were relatively more frequent in intermodule connections, while clusters 3, 5, and 6 were  
893 associated more with intramodule connections. The relative frequencies within clusters are shown for  
894 each of the six modules as sources, for intramodule **(c)** and intermodule connections **(d)**. **(e)** 40 cortical  
895 areas are rank ordered by their hierarchical index scores and color coded by module assignment. **(f)** The  
896 relative differences in feedback to feedforward patterns between modules. Source module is indicated on  
897 the y-axis, and the relative differences in patterns between that module and every other target module is  
898 represented by the colored circles. Modules which had <10 connections were removed from analyses.  
899 Positive values indicate more FB than FF connections from the source (y-axis) to target (plotted circles)  
900 module. Negative values indicate more FF than FB connection types. **(g)** The difference between the  
901 values plotted in (f) for each pair of modules as source and target. Positive values indicate an overall  
902 feedback connection from the source module on the y-axis to the target module (circles). Negative values  
903 indicate a feedforward connection from source to target modules. All intermodule connections from the  
904 prefrontal cortex were feedback. Those originating from the somatomotor module were all feedforward.  
905 Thus, these two modules formed the top and bottom of an intermodule hierarchy. **(i)** Network diagram of  
906 visual module nodes. Edge width indicates relative connection strength (from **Figure 2b**). The direction of  
907 the curved line shows outputs (clockwise) and inputs (counter-clockwise) from each node. Nodes are  
908 positioned based on their hierarchical score, with VISp at the bottom and VISpor at the top. All of these  
909 areas have strong feedback to VISp **(j)** The intermodule network diagram shows each module as a node,  
910 with edge thicknesses based on the sum of connection weights from **Figure 2b**. Based on the data in **(g)**,  
911 we propose a hierarchical order of network modules that is consistent across levels. At the bottom is  
912 somatomotor, then temporal-lateral, visual, medial, ventral-lateral, and, at the top, prefrontal.

913

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923

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931 H.Z. All co-authors reviewed the manuscript.

## 932 Methods

### 933 *Mice*

934 Experiments involving mice were approved by the Institutional Animal Care and Use Committees of the  
935 Allen Institute for Brain Science in accordance with NIH guidelines. Sources of mouse lines are listed in  
936 Supplemental Table 1. Characterization of the expression patterns of Cre driver lines used in this study  
937 have previously been described<sup>23</sup>. Links to image series data are available through the Transgenic  
938 Characterization data portal (<http://connectivity.brain-map.org/transgenic>). Cre lines were derived on  
939 various backgrounds, but the majority were crossed to C57BL/6J mice and maintained as heterozygous  
940 lines upon arrival. Tracer injections were performed in male and female mice at an average age of P56 +  
941 10 days. Mice were group-housed in a 12-hour light/dark cycle. Food and water were provided ad libitum.

### 942 *Tracers and injection methods*

943 rAAV was used as an anterograde tracer. For most regions, stereotaxic coordinates were used to identify  
944 the appropriate location for a tracer injection<sup>57</sup>. For a subset of experiments in the left hemisphere, we first  
945 functionally mapped the visual cortex using intrinsic signal imaging (ISI) through the skull, described  
946 below. A pan-neuronal AAV expressing EGFP (rAAV2/1.hSynapsin.EGFP.WPRE.bGH, Penn Vector  
947 Core, AV-1-PV1696, Addgene ID 105539) was used for injections into wildtype C57BL/6J mice (stock no.  
948 00064, The Jackson Laboratory). To label genetically-defined populations of neurons, we used either a  
949 Cre-dependent AAV vector that robustly expresses EGFP within the cytoplasm of Cre-expressing infected  
950 neurons (AAV2/1.pCAG.FLEX.EGFP.WPRE.bGH, Penn Vector Core, AV-1-ALL854, Addgene ID 51502).  
951 or, a Cre-dependent AAV virus expressing a synaptophysin-EGFP fusion protein to more specifically label  
952 presynaptic terminals (AAV2/1.pCAG.FLEX.sypEGFP.WPRE.bGH, Penn Vector Core).

953 Functional mapping of visual field space by intrinsic signal optical imaging (ISI) was used in some cases  
954 to guide injection placement. Additional details of this procedure can be found online ([http://help.brain-  
955 map.org/display/mouseconnectivity/Documentation?preview=/2818171/10813533/Connectivity+Overview.  
956 pdf](http://help.brain-map.org/display/mouseconnectivity/Documentation?preview=/2818171/10813533/Connectivity+Overview.pdf)). Briefly, a custom 3D-printed headframe was attached to the skull, centered at 3.1 mm lateral and 1.3  
957 mm anterior to lambda on the left hemisphere. A transcranial window was made by securing a 7-mm  
958 glass coverslip onto the skull in the center of the headframe well. Mice were recovered for at least seven  
959 days before ISI mapping. ISI was then used to measure the hemodynamic response to visual stimulation  
960 across the entire field of view of a lightly anesthetized, head-fixed, mouse. The visual stimulus consisted  
961 of sweeping a bar containing a flickering black-and-white checkerboard pattern across a grey

962 background<sup>58</sup>. To generate a map, the bar was swept across the screen ten times in each of the four  
963 cardinal directions, moving at 9° per second. Processing of sign maps followed methods previously  
964 described<sup>59</sup>, with minor modifications. Phase maps were generated by calculating the phase angle of the  
965 pre-processed DFT at the stimulus frequency. The phase maps were used to translate the location of a  
966 visual stimulus displayed on the retina to a spatial location on the cortex. A sign map was produced from  
967 the phase maps by taking the sign of the angle between the altitude and azimuth map gradients.  
968 Averaged sign maps were produced from a minimum of three time series images, for a combined  
969 minimum average of 30 stimulus sweeps in each direction. Visual area segmentation and identification  
970 was obtained by converting the visual field map to a binary image using a manually-defined threshold and  
971 further processing the initial visual areas with split/merge routine<sup>59</sup>. Sign maps were curated and the  
972 experiment repeated if; (1) <6 visual areas were positively identified, (2) retinotopic metrics of V1 were out  
973 of bounds (azimuth coverage within 60-100 degrees and altitude coverage within 35-60 degrees) or, (3)  
974 auto-segmented maps needed to be annotated with more than 3 adjustments. Each animal had 3  
975 attempts to get a passing map.

976 All mice received one unilateral injection into a single target region. For injections using stereotaxic  
977 coordinates from bregma as a registration point, an incision was made to expose the skull and bregma  
978 was visualized using a stereomicroscope. A hole overlying the targeted area was made by first thinning  
979 the skull using a fine drill burr, then using a microprobe and fine forceps to remove the bone, revealing  
980 the brain surface. For ISI-guided injections, the glass coverslip of the transcranial window was removed  
981 by drilling around the edges and a small burr hole drilled, first through the Metabond and then through the  
982 skull using surface vasculature fiducials obtained from the ISI session as a guide. An overlay of the sign  
983 map over the vasculature fiducials was used to identify the target injection site. rAAV was delivered by  
984 iontophoresis with current settings of 3  $\mu$ A at 7 s 'on' and 7 s 'off' cycles for 5 min total, using glass  
985 pipettes (inner tip diameters of 10–20  $\mu$ m). Mice were perfused transcardially and brains collected 3  
986 weeks post-injection for Cre mice.

#### 987 *Serial two-photon tomography*

988 Imaging by serial two-photon (STP) tomography has been described<sup>4,60</sup>, and here we used the exact  
989 same procedures as our earlier published studies<sup>4,25</sup>.

#### 990 *Image data processing*

991 STP images were processed using the informatics data pipeline (IDP), which manages the processing  
992 and organization of the image and quantified data for analysis and display in the web application as  
993 previously described<sup>4,27</sup>. The two key algorithms are signal detection and image registration.

994 The signal detection algorithm was applied to each image to segment positive fluorescent signals from  
995 background. Image intensity was first rescaled by square root transform to remove second-order effects  
996 followed by histogram matching at the midpoint to a template profile. Median filtering and large kernel low  
997 pass filter was then applied to remove noise. Signal detection on the processed image was based on a  
998 combination of adaptive edge/line detection and morphological processing. Two variations of the  
999 algorithm were employed, depending on the virus used for that experiment; one was tuned for EGFP, and  
1000 one for SypEGFP detection. High-threshold edge information was combined with spatial distance-  
1001 conditioned low-threshold edge results to form candidate signal object sets. The candidate objects were  
1002 then filtered based on their morphological attributes such as length and area using connected component  
1003 labelling. For the SypEGFP data, filters were tuned to detect smaller objects (punctate terminal boutons  
1004 vs long fibers). In addition, high intensity pixels near the detected objects were included into the signal  
1005 pixel set. Detected objects near hyper-intense artifacts occurring in multiple channels were removed. We  
1006 developed an additional filtering step using a supervised decision tree classifier to filter out surface  
1007 segmentation artifacts (**Supplemental Figure 7**), based on morphological measurements, location  
1008 context and the normalized intensities of all three channels.

1009 The output is a full resolution mask that classifies each  $0.35 \mu\text{m} \times 0.35 \mu\text{m}$  pixel as either signal or  
1010 background. An isotropic 3-D summary of each brain is constructed by dividing each image into  
1011  $10 \mu\text{m} \times 10 \mu\text{m}$  grid voxels. Total signal is computed for each voxel by summing the number of signal-  
1012 positive pixels in that voxel. Each image stack is registered in a multi-step process using both global  
1013 affine and local deformable registration to the 3-D Allen mouse brain reference atlas as previously  
1014 described<sup>27</sup>. Segmentation and registration results are combined to quantify signal for each voxel in the  
1015 reference space and for each structure in the reference atlas ontology by combining voxels from the  
1016 same structure.

#### 1017 *Creation of the cortical top-down and flattened views for data visualization.*

1018 A standard z-projection of signal in a top-down view of the cortex mixes signal from multiple areas.  
1019 Visualizations of fluorescence in **Figures 1-3** instead project signal along a curved cortical coordinate  
1020 system that more closely matches the columnar structure of the cortex. This coordinate system was  
1021 created by first solving Laplace's equation between pia and white matter surfaces, resulting in  
1022 intermediate equi-potential surfaces. Streamlines were computed by finding orthogonal (steepest  
1023 descent) paths through the equi-potential field. Cortical signal can then be projected along these  
1024 streamlines for visualization.

1025 A cortical flatmap was also constructed to enable visualization of anatomical and projection information  
1026 while preserving spatial context for the entire cortex. The flatmap was created by computing the geodesic  
1027 distance (the shortest path between two points on a curve surface) between every point on the cortical  
1028 surface and two pairs of selected anchor points. Each pair of anchor points form one axis of the 2D  
1029 embedding of the cortex into a flatmap. The 2D coordinate for each point on the cortical surface is  
1030 obtained by finding the location such that the radial (circular) distance from the anchor points (in 2D)  
1031 equals to the geodesic distance that was computed in 3D. This procedure produces smooth mapping of  
1032 the cortical surface onto a 2D plane for visualization. This embedding does not preserve area and the  
1033 frontal pole and medial-posterior region is highly distorted. As such, all numerical computation is done in  
1034 3D space. Similar techniques are used for texture mapping on geometric models in the field of computer  
1035 graphics<sup>61</sup>.

#### 1036 *Network modularity analysis*

1037 The matrix of connection weights between cortical areas (**Figure 2b**) was obtained from a novel model  
1038 (Knox et al., submitted). Briefly, this model allows us to predict the structural connectivity strengths  
1039 between any given brain region in the mouse at the scale of voxels. This model combines the information  
1040 from the 'wild type' viral tracing experiments performed as a part of the Allen Mouse Brain Connectivity  
1041 Atlas. It uses the spatial information given by distances to injection sites to infer a connectivity strength  
1042 from a given voxel to every other voxel in the Allen 3D Reference Atlas.

1043 We analyzed the network structure of this graph using the Louvain Community Detection algorithm from  
1044 the Brain Connectivity Toolbox (<https://sites.google.com/site/bctnet/>)<sup>30,62</sup>. The Louvain algorithm uses a  
1045 greedy algorithm to define groups of nodes (modules) that are more connected to each other than they  
1046 are to other nodes outside their module. We determined the modularity at various levels of granularity by  
1047 varying the resolution parameter,  $\gamma$ , from 0-2.5 in steps of 0.1. For each value of  $\gamma$ , the modularity was  
1048 computed 1000x and each pair of regions received an affinity score between 0 and 1. The affinity score is  
1049 the probability of two regions being assigned to the same module weighted by the modularity score (Q)  
1050 for that iteration, thereby assigning higher weights to partitions with a higher modularity score. Each  
1051 region was assigned to the module with which it had the highest affinity, with the caveat that all structures  
1052 within a module had an affinity score  $\geq 0.5$  with all other members of the module. For each value of  $\gamma$ , we  
1053 also generated a shuffled matrix containing the same weights but with the source and target regions  
1054 randomized. The modularity for the cortical and hippocampal matrix (Q) and the shuffled matrix (Q<sub>shuffled</sub>)  
1055 were evaluated at each value of  $\gamma$ .

1056 *Clustering Analyses and Statistics*

1057 Unsupervised hierarchical clustering (Figure 1 and 5) was conducted with the online software, Morpheus,  
1058 (<https://software.broadinstitute.org/morpheus/>) for algorithms and for visualization of the dendrogram and  
1059 heat maps. Log-transforms were calculated on all values after adding a small value (0.5 minimum of the  
1060 true positive array elements) to avoid Log (0). Proximity between clusters was computed using average  
1061 linkages with spearman rank correlations as the distance metric. The clustering algorithm works  
1062 agglomeratively: initially assigning each sample to its own cluster and iteratively merging the most  
1063 proximal pair of clusters until finally all the clusters have been merged. To compare distances between  
1064 granular and agranular samples (those that lack a L4), the computation of the distance metric was  
1065 restricted to the set of shared layer projection fractions. In other words, we used the set of projection  
1066 fractions in all layers when evaluating granular-granular distances, whereas when evaluating agranular-  
1067 agranular or agranular-granular cortex, we used the set of all layers except L4. The software program  
1068 GraphPad Prism was used for statistical tests and generation of all graphs, and the software program  
1069 Gephi was used for visualization and layout of network diagrams.

1070 *Unsupervised discovery of hierarchy position*

1071 Following the classification of the laminar patterns in clusters, we use an unsupervised method to  
1072 simultaneously assign a direction to a cluster type and to construct a hierarchy.

1073 First consider a mapping function

1074 
$$M: [1, c] \cap N \rightarrow \{-1, 1\}$$

1075 which maps a type of connection cluster to either feedforward ( $M=1$ ) or feedback ( $M=-1$ ) type. We search  
1076 over the space of possible maps to see which map produces the most self-consistent hierarchy. Since  
1077 some transgenic line have different numbers of connections in different clusters, some maps will lead to  
1078 particular transgenic lines having very biased feedforward or feedback calls. Thus, we add a confidence  
1079 measure, which decreases the importance of the information provided by a transgenic line to the global  
1080 hierarchy if the calls from that transgenic line are biased.

1081 
$$conf(T) = 1 - \left| \langle M(C_{T_{i,j}}) \rangle_{i,j} \right| \quad (1)$$

1082 with a global confidence as an average over all the inter-areal connections above the threshold ( $10^{-1.5}$ )

1083 
$$conf_g = \langle conf(T) \rangle_{i,j} \quad (2)$$

1084 We define the hierarchical position of a source area based on the difference between the feedback and  
1085 feedforward connections originating from this area, normalized by the number of connections, which is  
1086 normalized by the confidence we have from different Cre lines providing information about the  
1087 directionality of the connection. The hierarchical position as a target is the difference between the  
1088 feedforward and feedback connections terminating in this area, normalized by the number of connections  
1089 and confidence. The hierarchical position of an area is defined as the sum of these measures:

1090 
$$H_i = \frac{1}{conf_g} (\langle M(C_{T_{i,j}}) \cdot conf(T) \rangle_j - \langle M(C_{T_{j,i}}) \cdot conf(T) \rangle_j) \quad (3)$$

1091 To test how self-consistent a hierarchy is we define the global hierarchy score:

1092 
$$H = \frac{1}{2 \cdot conf_g} \langle M(C_{T_{i,j}}) \cdot conf(T) \cdot (H_i - H_j) \rangle_{i,j} \quad (4)$$

1093 We performed an exhaustive search over all the maps M for the entire set of cortico-cortical connections,  
1094 and the most self-consistent hierarchy is obtained when connections of type 1,3 and 5 are of one type  
1095 and 2,4 and 6 are of the opposite type. Based on the position of the sensory areas, we conclude that type  
1096 1,3 and 5 are feedforward and 2,4 and 6 are feedback. It should be noted that a similar search inside of

1097 the visual module results in mapping connections 3 and 5 to feedforward and 1,2,4 and 6 to feedback.  
1098 Thus, it is likely that connection type 1 represent feedforward connections within a module and feedback  
1099 between.

## 1100 References

- 1101 1. Sporns, O., Tononi, G. & Kötter, R. The Human Connectome: A Structural Description of the  
1102 Human Brain. *PLoS Comput. Biol.* **1**, e42 (2005).
- 1103 2. Bohland, J. W. *et al.* A Proposal for a Coordinated Effort for the Determination of Brainwide  
1104 Neuroanatomical Connectivity in Model Organisms at a Mesoscopic Scale. *PLoS Comput. Biol.* **5**,  
1105 e1000334 (2009).
- 1106 3. Zingg, B. *et al.* Neural Networks of the Mouse Neocortex. *Cell* **156**, 1096–1111 (2014).
- 1107 4. Oh, S. W. *et al.* A mesoscale connectome of the mouse brain. *Nature* **508**, 207–214 (2014).
- 1108 5. Markov, N. T. *et al.* A weighted and directed interareal connectivity matrix for macaque cerebral  
1109 cortex. *Cereb. Cortex* **24**, 17–36 (2014).
- 1110 6. Bota, M., Sporns, O. & Swanson, L. W. Architecture of the cerebral cortical association  
1111 connectome underlying cognition. *Proc. Natl. Acad. Sci.* **112**, E2093–E2101 (2015).
- 1112 7. Scannell, J. W., Blakemore, C. & Young, M. P. Analysis of connectivity in the cat cerebral cortex.  
1113 *J. Neurosci.* **15**, 1463–83 (1995).
- 1114 8. Swanson, L. W., Hahn, J. D. & Sporns, O. Organizing principles for the cerebral cortex network of  
1115 commissural and association connections. *Proc. Natl. Acad. Sci.* **114**, E9692–E9701 (2017).
- 1116 9. Bullmore, E. & Sporns, O. Complex brain networks: graph theoretical analysis of structural and  
1117 functional systems. *Nat. Rev. Neurosci.* **10**, 186–198 (2009).
- 1118 10. Sporns, O. *Connectome Networks: From Cells to Systems. Micro-, Meso- and Macro-*  
1119 *Connectomics of the Brain* (Springer, 2016). doi:10.1007/978-3-319-27777-6\_8
- 1120 11. Wang, Q., Sporns, O. & Burkhalter, A. Network analysis of corticocortical connections reveals  
1121 ventral and dorsal processing streams in mouse visual cortex. *J. Neurosci.* **32**, 4386–99 (2012).
- 1122 12. Felleman, D. J. & Van Essen, D. C. Distributed Hierarchical Processing in the Primate Cerebral  
1123 Cortex. *Cereb. Cortex* **1**, 1–47 (1991).
- 1124 13. Rockland, K. S. & Pandya, D. N. Laminar origins and terminations of cortical connections of the  
1125 occipital lobe in the rhesus monkey. *Brain Res.* **179**, 3–20 (1979).
- 1126 14. Riesenhuber, M. & Poggio, T. Hierarchical models of object recognition in cortex. *Nat. Neurosci.* **2**,  
1127 1019–1025 (1999).
- 1128 15. Tasic, B. *et al.* Adult mouse cortical cell taxonomy revealed by single cell transcriptomics. *Nat.*  
1129 *Neurosci.* **19**, 335–346 (2016).
- 1130 16. Tasic, B. *et al.* Shared and distinct transcriptomic cell types across neocortical areas. *bioRxiv*  
1131 229542 (2017). doi:10.1101/229542
- 1132 17. Zeng, H. & Sanes, J. R. Neuronal cell-type classification: challenges, opportunities and the path  
1133 forward. *Nat. Rev. Neurosci.* **18**, 530–546 (2017).
- 1134 18. Sorensen, S. A. *et al.* Correlated gene expression and target specificity demonstrate excitatory  
1135 projection neuron diversity. *Cereb. Cortex* **25**, 433–449 (2015).
- 1136 19. Shepherd, G. M. G. Corticostriatal connectivity and its role in disease. *Nat. Rev. Neurosci.* **14**,  
1137 278–291 (2013).

- 1138 20. Harris, K. D. & Shepherd, G. M. G. The neocortical circuit: themes and variations. *Nat. Neurosci.*  
1139 **18**, 170–181 (2015).
- 1140 21. Gong, S. *et al.* Targeting Cre Recombinase to Specific Neuron Populations with Bacterial Artificial  
1141 Chromosome Constructs. *J. Neurosci.* **27**, 9817–9823 (2007).
- 1142 22. Gerfen, C. R., Paletzki, R. & Heintz, N. GENSAT BAC Cre-Recombinase Driver Lines to Study the  
1143 Functional Organization of Cerebral Cortical and Basal Ganglia Circuits. *Neuron* **80**, 1368–1383  
1144 (2013).
- 1145 23. Harris, J. A. *et al.* Anatomical characterization of Cre driver mice for neural circuit mapping and  
1146 manipulation. *Front. Neural Circuits* **8**, 1–16 (2014).
- 1147 24. Daigle, T. L. *et al.* A suite of transgenic driver and reporter mouse lines with enhanced brain cell  
1148 type targeting and functionality. *bioRxiv* 224881 (2017). doi:10.1101/224881
- 1149 25. Martersteck, E. M. *et al.* Diverse Central Projection Patterns of Retinal Ganglion Cells. *Cell Rep.*  
1150 **18**, 2058–2072 (2017).
- 1151 26. Coogan, T. A., Burkhalter, A. & Martin, K. Hierarchical organization of areas in rat visual cortex. *J.*  
1152 *Neurosci.* **13**, 3749–72 (1993).
- 1153 27. Kuan, L. *et al.* Neuroinformatics of the allen mouse brain connectivity atlas. *Methods* **73**, 4–17  
1154 (2015).
- 1155 28. Kim, E. J., Juavinett, A. L., Kyubwa, E. M., Jacobs, M. W. & Callaway, E. M. Three Types of  
1156 Cortical Layer 5 Neurons That Differ in Brain-wide Connectivity and Function. *Neuron* **88**, 1253–  
1157 1267 (2015).
- 1158 29. Olsen, S. R., Bortone, D. S., Adesnik, H. & Scanziani, M. Gain control by layer six in cortical  
1159 circuits of vision. *Nature* **483**, 47–52 (2012).
- 1160 30. Rubinov, M. & Sporns, O. Complex network measures of brain connectivity: Uses and  
1161 interpretations. *Neuroimage* **52**, 1059–1069 (2010).
- 1162 31. Sporns, O. & Betzel, R. F. Modular Brain Networks. *Annu. Rev. Psychol.* **67**, 613–640 (2016).
- 1163 32. Ria Ercsey-Ravasz, M. *et al.* A Predictive Network Model of Cerebral Cortical Connectivity Based  
1164 on a Distance Rule. *Neuron* **80**, 184–197 (2013).
- 1165 33. Jacomy, M., Venturini, T., Heymann, S. & Bastian, M. ForceAtlas2, a Continuous Graph Layout  
1166 Algorithm for Handy Network Visualization Designed for the Gephi Software. *PLoS One* **9**, e98679  
1167 (2014).
- 1168 34. Gămănuț, R. *et al.* The Mouse Cortical Connectome, Characterized by an Ultra-Dense Cortical  
1169 Graph, Maintains Specificity by Distinct Connectivity Profiles. *Neuron* **97**, 698–715.e10 (2018).
- 1170 35. Markov, N. T. *et al.* Weight Consistency Specifies Regularities of Macaque Cortical Networks.  
1171 *Cereb. Cortex* **21**, 1254–1272 (2011).
- 1172 36. Maunsell, J. H. & van Essen, D. C. The connections of the middle temporal visual area (MT) and  
1173 their relationship to a cortical hierarchy in the macaque monkey. *J. Neurosci.* **3**, 2563–86 (1983).
- 1174 37. Markov, N. T. *et al.* Anatomy of hierarchy: Feedforward and feedback pathways in macaque visual  
1175 cortex. *J. Comp. Neurol.* **522**, 225–259 (2014).
- 1176 38. D’Souza, R. D., Meier, A. M., Bista, P., Wang, Q. & Burkhalter, A. Recruitment of inhibition and  
1177 excitation across mouse visual cortex depends on the hierarchy of interconnecting areas. *Elife* **5**,  
1178 e19332 (2016).
- 1179 39. D’Souza, R. D. & Burkhalter, A. A Laminar Organization for Selective Cortico-Cortical  
1180 Communication. *Front. Neuroanat.* **11**, 1–13 (2017).

- 1181 40. Marshel, J. H., Garrett, M. E., Nauhaus, I. & Callaway, E. M. Functional Specialization of Seven  
1182 Mouse Visual Cortical Areas. *Neuron* **72**, 1040–1054 (2011).
- 1183 41. Huh, C. Y. L., Peach, J. P., Bennett, C., Vega, R. M. & Hestrin, S. Feature-Specific Organization of  
1184 Feedback Pathways in Mouse Visual Cortex. *Curr. Biol.* **28**, 114–120.e5 (2018).
- 1185 42. Zhang, S. *et al.* Selective attention. Long-range and local circuits for top-down modulation of visual  
1186 cortex processing. *Science* **345**, 660–5 (2014).
- 1187 43. Leinweber, M., Ward, D. R., Sobczak, J. M., Attinger, A. & Keller, G. B. A Sensorimotor Circuit in  
1188 Mouse Cortex for Visual Flow Predictions. *Neuron* **95**, 1420–1432.e5 (2017).
- 1189 44. Carlén, M. What constitutes the prefrontal cortex? *Science* **358**, 478–482 (2017).
- 1190 45. Avery, J. A. *et al.* Convergent gustatory and viscerosensory processing in the human dorsal mid-  
1191 insula. *Hum. Brain Mapp.* **38**, 2150–2164 (2017).
- 1192 46. Hanamori, T., Kunitake, T., Kato, K. & Kannan, H. Responses of Neurons in the Insular Cortex to  
1193 Gustatory, Visceral, and Nociceptive Stimuli in Rats. *J. Neurophysiol.* **79**, 2535–2545 (1998).
- 1194 47. Veinante, P. & Deschênes, M. Single-cell study of motor cortex projections to the barrel field in  
1195 rats. *J. Comp. Neurol.* **464**, 98–103 (2003).
- 1196 48. Douglas, R. J. & Martin, K. A. C. NEURONAL CIRCUITS OF THE NEOCORTEX. *Annu. Rev.*  
1197 *Neurosci.* **27**, 419–451 (2004).
- 1198 49. Fame, R. M., MacDonald, J. L. & Macklis, J. D. Development, specification, and diversity of  
1199 callosal projection neurons. *Trends Neurosci.* **34**, 41–50 (2011).
- 1200 50. Yamins, D. L. K. & DiCarlo, J. J. Using goal-driven deep learning models to understand sensory  
1201 cortex. *Nat. Neurosci.* **19**, 356–365 (2016).
- 1202 51. Rao, R. P. N. & Ballard, D. H. Predictive coding in the visual cortex: a functional interpretation of  
1203 some extra-classical receptive-field effects. *Nat. Neurosci.* **2**, 79–87 (1999).
- 1204 52. Cain, N., Iyer, R., Koch, C. & Mihalas, S. The Computational Properties of a Simplified Cortical  
1205 Column Model. *PLOS Comput. Biol.* **12**, e1005045 (2016).
- 1206 53. Coogan, T. A. & Burkhalter, A. Conserved patterns of cortico-cortical connections define areal  
1207 hierarchy in rat visual cortex. *Exp. brain Res.* **80**, 49–53 (1990).
- 1208 54. Shipp, S. The importance of being agranular: a comparative account of visual and motor cortex.  
1209 *Philos. Trans. R. Soc. B Biol. Sci.* **360**, 797–814 (2005).
- 1210 55. Kim, Y. *et al.* Brain-wide Maps Reveal Stereotyped Cell-Type-Based Cortical Architecture and  
1211 Subcortical Sexual Dimorphism. *Cell* **171**, 456–469.e22 (2017).
- 1212 56. Han, Y. *et al.* The logic of single-cell projections from visual cortex. *Nature* (2018).  
1213 doi:10.1038/nature26159
- 1214 57. Franklin, K. B. J. & Paxinos, G. *Paxinos and Franklin's The mouse brain in stereotaxic*  
1215 *coordinates.* (2012).
- 1216 58. Kalatsky, V. A. & Stryker, M. P. New paradigm for optical imaging: temporally encoded maps of  
1217 intrinsic signal. *Neuron* **38**, 529–45 (2003).
- 1218 59. Garrett, M. E., Nauhaus, I., Marshel, J. H. & Callaway, E. M. Topography and Areal Organization  
1219 of Mouse Visual Cortex. *J. Neurosci.* **34**, 12587–12600 (2014).
- 1220 60. Ragan, T. *et al.* Serial two-photon tomography for automated ex vivo mouse brain imaging. *Nat.*  
1221 *Methods* **9**, 255–8 (2012).



- 1222 61. Oliveira, G. N., Torchelsen, R. P., Comba, J. L. D., Walter, M. & Bastos, R. Geotextures: A Multi-  
1223 source Geodesic Distance Field Approach for Procedural Texturing of Complex Meshes. in *2010*  
1224 *23rd SIBGRAPI Conference on Graphics, Patterns and Images* 126–133 (IEEE, 2010).  
1225 doi:10.1109/SIBGRAPI.2010.25
- 1226 62. Blondel, V. D., Guillaume, J.-L., Lambiotte, R. & Lefebvre, E. Fast unfolding of communities in  
1227 large networks. *J. Stat. Mech. Theory Exp.* **2008**, P10008 (2008).
- 1228