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Connectivity of mouse somatosensory and prefrontal cortex examined with trans-synaptic tracing

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Information processing in neocortical circuits requires integrating inputs over a wide range of spatial scales, from local microcircuits to long-range cortical and subcortical connections. We used rabies virus–based trans-synaptic tracing to analyze the laminar distribution of local and long-range inputs to pyramidal neurons in the mouse barrel cortex and medial prefrontal cortex (mPFC). In barrel cortex, we found substantial inputs from layer 3 (L3) to L6, prevalent translaminar inhibitory inputs, and long-range inputs to L2/3 or L5/6 preferentially from L2/3 or L5/6 of input cortical areas, respectively. These layer-specific input patterns were largely independent of NMDA receptor function in the recipient neurons. mPFC L5 received proportionally more long-range inputs and more local inhibitory inputs than barrel cortex L5. Our results provide new insight into the organization and development of neocortical networks and identify important differences in the circuit organization in sensory and association cortices.

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The mammalian neocortex consists of discrete, but interconnected, functional areas that collectively encode features of the environment, form associations between stimuli and drive behavior by transforming sensory input into motor output. Individual areas contain microcircuits that are organized vertically in columns and horizontally in layers of distinct cell types. In the rodent somatosensory cortex, for example, barrel-related columns of cells tend to respond to stimulation of the same whisker[1,2] and are involved in sensory perception[3]. In contrast, cortical association areas such as the prefrontal cortex regulate cognitive functions and are not known to contain clear maps of sensory space[4].

Canonical circuits have been proposed as organizational features that are shared across sensory cortices[5–7]. In a canonical circuit model based largely on cat V1, information flows from thalamus→layer 4 (L4)→L2/3→L5/6. Mapping connectivity of the mouse barrel cortex largely supports the canonical circuit[8,9]. However, the canonical circuit represents a simplified overview of prominent cortical connections and many aspects of cortical circuit organization remain unknown. What is the spatial organization of transcolumnar connectivity in cortex? What are the cellular sources of long-range input to sensory cortex? What are the developmental mechanisms that give rise to layer-specific connectivity? To what extent does the canonical circuit in sensory cortices apply to association areas?

Previous pioneering studies generated maps of local and long-range inputs to specific cortical layers, but technical limitations leave the maps incomplete. Although *in vivo* dye fills allow for correlation of morphology with receptive field properties[10], synaptic connections cannot always be inferred from where dendrites and axons overlap[11]. Electrophysiological methods including paired recordings[8], glutamate uncaging[9,12,13] and channelrhodopsin-assisted circuit mapping (CRACM)[14,15] have enabled quantification of connectivity rates and synapse strengths between defined cell types. However, paired recordings and glutamate uncaging are limited to mapping local connections in a brain slice and CRACM can only be used to map one class of inputs at a time.

Modified rabies virus (RV) can be used to trace brain-wide monosynaptic inputs to genetically defined cell types[16,17]. Here we used RV trans-synaptic tracing to generate layer-specific input maps to L2/3, L5 and L6 pyramidal neurons in barrel cortex. By comprehensively mapping local, inhibitory, and long-range inputs, we constructed whole-brain input maps that would not be possible with alternative methodologies. We performed laminar analyses of inputs from barrel cortex and from other cortical regions in the sensorimotor network. To explore the developmental programs regulating layer specific cortical inputs, we coupled Cre-dependent tracing to cell type–specific knockout of the obligatory NMDA receptor subunit GluN1. Finally, we generated the first whole-brain input map to mPFC L5. Comparisons of mPFC and barrel cortex revealed differences in layer-specific microcircuit organization and in the relative contributions of inhibitory and long-range inputs to L5 neurons in each area.

RESULTS

Experimental strategy

We used RV tracing to probe the organization of inputs to excitatory neurons in L2/3, L5 and L6 of mouse barrel cortex. We injected two helper adeno-associated viruses (AAVs) that express target proteins in a Cre-dependent manner into mice in which the Cre recombinase is expressed in pyramidal neurons in specific layers (**Supplementary Fig. 1a**)[18]. The first AAV (CAG-FLEx-TVA^{66T}-mCherry) expressed a mutant receptor (TVA^{66T}) for EnvA, a coat protein for an avian virus, fused with mCherry. The second AAV (CAG-FLEx-G) expressed RV glycoprotein (G) necessary for the trans-synaptic transport of RV. We injected EnvA-pseudotyped RV 2 weeks later in which G was replaced with coding sequence of GFP (RV*dG* hereafter). Because there is no cognate receptor for EnvA in the mouse brain, RV*dG* only infects TVA-expressing cells. G expression from the second AAV complements the

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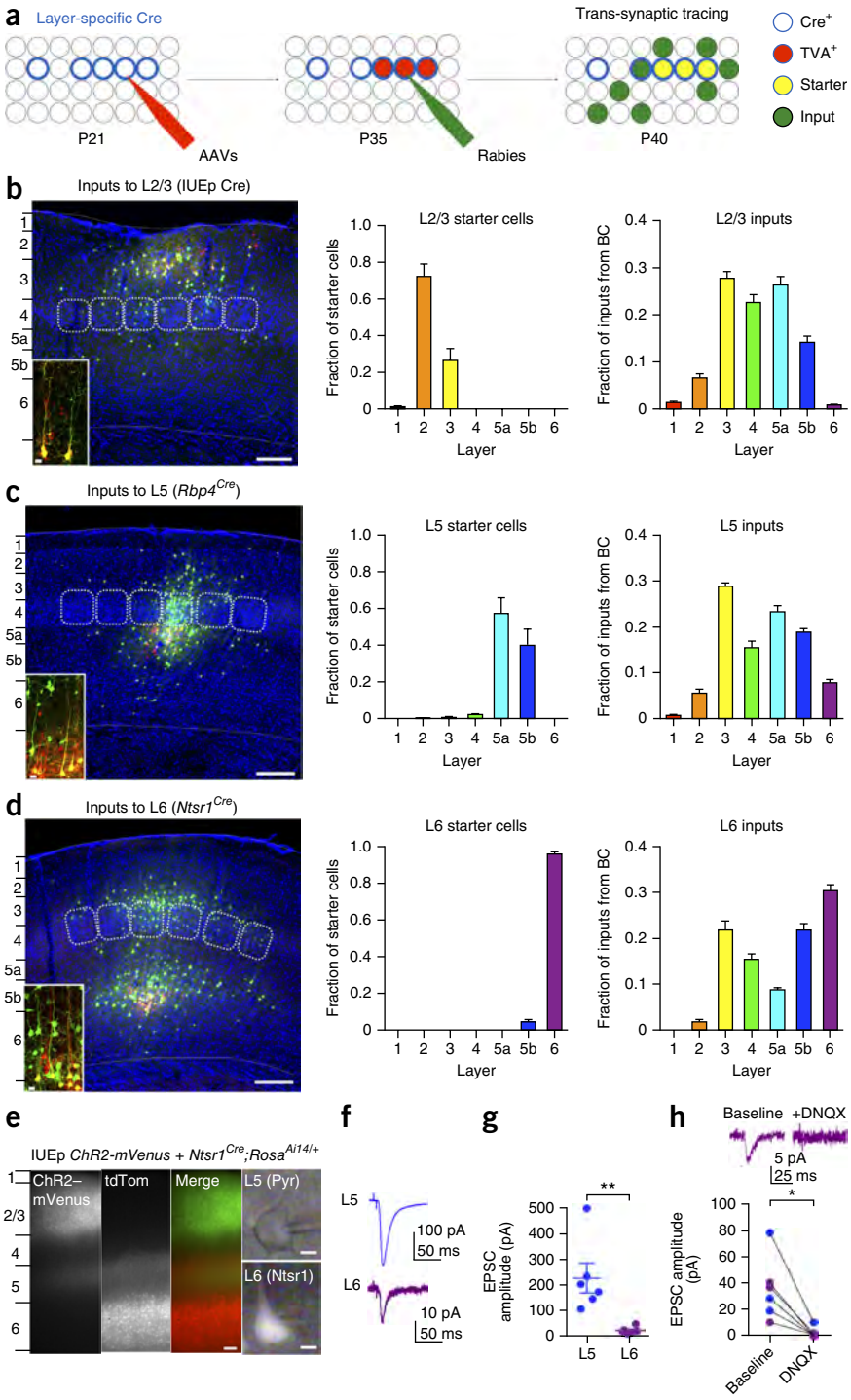

Figure 1 Layer-specific input tracing in mouse barrel cortex. (**a**) Experimental design and timeline of layer-specific RV tracing. (**b**–**d**) Layer-specific tracing in barrel cortex. Left, representative coronal sections showing local tracing (insets, confocal images of starter cells). Starter cells (SCs, middle) and local input (right) distributions are quantified according to cortical layers for SCs in L2/3 (**b**, SCs, 76 ± 27; total inputs, 1,148 ± 340; $n = 6$ mice), L5 (**c**, SCs, 84 ± 27; total inputs, 1,677 ± 359; $n = 9$ mice) and L6 (**d**, SCs, 117 ± 42; total inputs, 1,751 ± 542; $n = 9$ mice). Scale bars represent 200 μm (main panels) or 20 μm (insets). See **Supplementary Table 1** for numerical values and **Supplementary Figure 1** for controls for RV tracing. (**e**–**h**) L3→L6 CRACM. (**e**) Example coronal sections from an *Ntsr1*^{Cre};*Rosa*^{Ai14} mouse expressing ChR2-mVenus in L2/3. Right, recorded L5 pyramidal (pyr) and tdTomato+ L6 cell (Ntsr1). Scale bars represent 100 μm (left) and 10 μm (zoomed panels, right). (**f**) Traces from a L5 and L6 cell following laser stimulation of ChR2-expressing L3 axons in the presence of TTX, 4-AP and PTX. (**g**) Evoked EPSC amplitudes in L5 and L6 (L5, 205.2 ± 57.48 pA, $n = 6$ cells; L6, 23.05 ± 5.50 pA, $n = 6$ cells from 3 mice; $^{**}P = 0.0052$, Student's t test). (**h**) Representative trace (top) and quantification (bottom) of blockade of light-evoked EPSC following bath DNQX application (baseline, 35.89 ± 2.64 pA; DNQX, 2.64 ± 1.61 pA; $n = 6$ cells from 3 mice, $^{*}P = 0.01$, paired t test). All summary statistics are presented as mean ± s.e.m. See **Supplementary Table 6** for test results and P values.

RV*dG*, allowing retrograde monosynaptic tracing from Cre-expressing cells (**Fig. 1a**).

Starter cells expressed both mCherry and GFP, whereas input neurons expressed only GFP. Notably, the mutant TVA^{66T} eliminated background labeling[18], as there was no mCherry or GFP expression in animals lacking Cre (**Supplementary Fig. 1b**). When the AAV carrying CAG-FLEx-G was omitted, GFP expression was predominantly restricted to cells expressing TVA^{66T}-mCherry (**Supplementary Fig. 1c**), indicating that green-only cells in experimental animals represent presynaptic partners of starter cells (inputs hereafter) as a result of monosynaptic spread of RV*dG*. By injecting helper AAVs into mice that received *in utero* electroporation (IUEp) of a Cre-expressing plasmid at embryonic day 16 (E16), or into layer-specific Cre driver mice, we confined starter cells to L2/3 (IUEp-*Cre* or *SepW1*^{Cre}), L5 (*Rbp4*^{Cre}) and L6 (*Ntsr1*^{Cre}), respectively (**Fig. 1** and **Supplementary Fig. 1a**). We determined layer boundaries based on DAPI nuclear labeling and assigned starter cells and RV-labeled inputs to cortical layers using a custom MatLab program. Layer boundaries were consistent across Cre drivers (**Supplementary Fig. 1d**) and with immunostaining based on layer markers Cux1 (L2–4) and Ctip2 (L5b–6) (**Supplementary Fig. 1e**).

To characterize the starter cells defined by each Cre-driver, we crossed *Rbp4*^{Cre} and *Ntsr1*^{Cre} mice to tdTomato Cre reporter mice (*Rosa*^{Ai14}); as a result of technical limitations (Online Methods), we used Cre-dependent mCherry from AAV to characterize the *SepW1*^{Cre} expression pattern (**Supplementary Fig. 2**). Co-staining for the general neuronal marker NeuN in barrel cortex revealed that *SepW1*^{Cre} labeled ~31% of NeuN^{+} L2/3 cells, which represents a low estimate of labeling, as the viral reporter probably did not infect all of the L2/3 cells (**Supplementary Fig. 2a**,**g**). *Rbp4*^{Cre};*Rosa*^{Ai14} labeled ~42% of NeuN^{+} L5 cells (**Supplementary Fig. 2b**,**g**). *Ntsr1*^{Cre};*Rosa*^{Ai14} labeled ~68% of NeuN^{+} cells in L6 (**Supplementary Fig. 2f**,**g**), consistent with previous reports[19,20].

Layers 5b and 6 contain subcerebral and corticothalamic projection neurons, respectively[21]. We stained barrel cortex sections from

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Rbp4^{Cre};*Rosa*^{Ai14} mice for Ctip2, a marker for subcerebral projection neurons (SCPNs). ~50% of tdTomato^{+} cells in L5b expressed Ctip2 (**Supplementary Fig. 2c**,**g**), consistent with a previous report that *Rbp4*^{Cre} labels both SCPNs and inter-telencephalic projection neurons[22]. Previous work reported that corticothalamic projection neurons in somatosensory and visual cortex L6 are almost exclusively labeled in the *Ntsr1*^{Cre} line[20,23]. For simplicity, we used their respective layers to refer to the Cre driver names hereafter, keeping in mind that each Cre driver labeled only a subset of cells in that layer.

A prominent L3→L6 connection in barrel cortex

We mapped mouse barrel cortex so that we could compare RV tracing with other methods that have been used to describe this circuitry. In addition to describing layer-specific circuits in quantitative detail, trans-synaptic tracing might uncover new or understudied connections that were disrupted in brain slices used in previous mapping studies.

We traced from layer-restricted starter cells and mapped the layer identity of labeled inputs throughout all of barrel cortex. We define these inputs (located above, below or in a barrel visualized in L4 with DAPI staining) as local inputs hereafter. In all cases, starter cells had pyramidal morphology with apical dendrites extending toward the pia and were restricted to the layer of interest (**Fig. 1b**–**d** and **Supplementary Table 1**). For each brain, we calculated the number of inputs in each layer as a fraction of the total inputs in barrel cortex. Starter cells in each layer received characteristic sets of inputs (**Supplementary Table 2**). L2/3 starter cells produced by IUEp-*Cre* received the largest fractions of input from L3, L4 and L5a, L5 starter cells from L3, L5a and L5b, and L6 starter cells from L6, L5b, L3 and L4 (**Fig. 1b**–**d** and **Supplementary Table 1**). Consistent with previous work, starter cells received dense inputs from other cells in the starter cell layer[8]. Our data support the canonical pathways, including dense L4→L2/3 and L2/3→L5 connections, similar to previous reports[8,9]. We also observed a robust input from L3→L6 in barrel cortex that was not reported in a previous paired recording study and was observed as a weak connection using glutamate uncaging[8,9].

To validate that the L3→L6 synaptic connections are functional, we performed a CRACM experiment in which we used IUEp at E16 to express channelrhodopsin-2 fused with the fluorescent protein mVenus (ChR2-mVenus) in L2/3 of *Ntsr1*^{Cre};*Rosa*^{Ai14} mice. We prepared acute slices 1 month later and performed whole-cell patch recordings from labeled L6 cells while stimulating axons from L2/3 with a blue laser. To isolate monosynaptic excitatory postsynaptic currents (EPSCs), we voltage clamped the cells at −60 mV in the presence of tetrodotoxin (TTX), 4-aminopyridine (4-AP) and picrotoxin. In the same slices, we also recorded from L5 pyramidal cells to compare the strength of L2/3→L6 synapses to that of L2/3→L5 synapses, which are part of the canonical pathway.

In slices with robust ChR2-mVenus expression (**Fig. 1e**), 6 of 13 recorded L6 cells responded to laser stimulation with a short-latency EPSC, whereas, in the same slices, 6 of 6 recorded L5 cells responded. The amplitudes of the L2/3→L6 ESPCs were ten-fold smaller than L2/3→L5 EPSCs (**Fig. 1f**,**g**). L2/3→L5 and L2/3→L6 EPSCs were blocked by the AMPA-type glutamate receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX), indicating they were mediated by glutamate receptors (**Fig. 1h**). Given that we observed few L2→L6 inputs with RV tracing (**Fig. 1d**), most of the functional connections that we observed were likely L3→L6. Together, the RV tracing and CRACM results indicate that L3→L6 synapses are numerous, but weak. Axons from L2/3 neurons were visible in L5 (**Fig. 1e**), suggesting that they form synapses onto distal dendrites of L6 neurons and might be subject to substantial dendritic filtering. These results highlight the utility of RV tracing in identifying connections that are not easily detectable by electrophysiological methods.

Spatial distribution of inputs to barrel cortex

To understand how laminar sources of input change with distance from the starter cells, we analyzed the distribution of inputs in barrel cortex along the anterior-posterior (A-P) axis (across coronal sections; **Fig. 2a**) and medial-lateral (M-L) axis (within coronal sections; **Fig. 2b**). We generated heat maps showing the average distribution across space for inputs from different layers (**Fig. 2c**–**h**). Although inputs were widely distributed, they peaked in sections containing the most starter cells. Although most of the starter cells were contained in 360-μm-diameter regions, inputs extended across ~2 mm of barrel cortex. Individual barrels were ~200 μm in diameter (192.2 ± 2.3 μm, n = 3 mice). Thus, although the starter cells likely occupied 2–3 barrels along one axis, the inputs occupied up to 10 barrels. Because our starter cells spanned multiple barrels and septa, we did not attempt to draw conclusions about individual barrel or septa-related circuits from our data.

We observed layer-specific differences in the distributions of inputs with distance from the starter cells. L6 inputs exhibited target-dependent spread, providing clustered input to L5 and distributed input to L6. L4 inputs clustered above or below the starter cells, whereas L3 and L5 inputs spread out in space (**Fig. 2c**–**h**). To quantify differences along the A-P axis, we identified sections that contained 95% of the starter cells (central sections; **Fig. 2a**) and compared the layer distribution of those inputs to the distribution of inputs in peripheral sections (**Fig. 2c**–**e**). For the M-L axis, we focused on the central sections and determined the distance from each input or starter cell to a center line projected through the middle of the labeled cells (**Fig. 2b**). We defined a middle region that contained 95% of the starter cells in these sections and compared the layer distribution of inputs located in the middle and flanking side regions (**Fig. 2f**–**h**). L4 inputs to L2/3 (P = 0.003) and L5 (P = 0.006) significantly decreased along the A-P and M-L axes, respectively (**Fig. 2c**,**g**). L6 received significantly smaller fractions (A-P: P = 0.003; M-L: P = 0.0001) of L4 inputs with distance along both axes (**Fig. 2e**,**h**).

Overall, the laminar distribution of input varied predictably with distance from the starter cell population, with L4 neurons providing less relative input from distant locations to starter cells in all layers. Consistent with previous reports of axon arborization patterns[24], these data suggest that L4 primarily conveys intracolumnar information, whereas supra- and infragranular layers also convey information from adjacent barrels. Given that different classes of L6 neurons have unique intracortical axon patterns[25], the target-dependent spread of L6 inputs might reflect input from different cell types in L6.

Patterns of GABAergic inputs to barrel cortex

Inhibition is important for shaping receptive fields and determining the timing of neuronal responses[26]. To identify whether RV-labeled inputs were excitatory or inhibitory, we performed *in situ* hybridization using a mixture of probes that detect mRNAs for the GABA synthetic enzymes glutamic acid decarboxylase 1 and 2 (*Gad1/2*) in *SepW1*^{Cre}, *Rbp4*^{Cre}, and *Ntsr1*^{Cre} brains with layer-specific RV tracing in L2/3, L5 and L6, respectively. The *SepW1*^{Cre} line became available during the course of our study. Given that this line specifically labeled L2/3 neurons and yielded input patterns indistinguishable from the inputs to cells labeled with IUEp (no significant differences by t tests with Holm-Sidak corrections for multiple comparisons), we used *SepW1*^{Cre} driver for these experiments.

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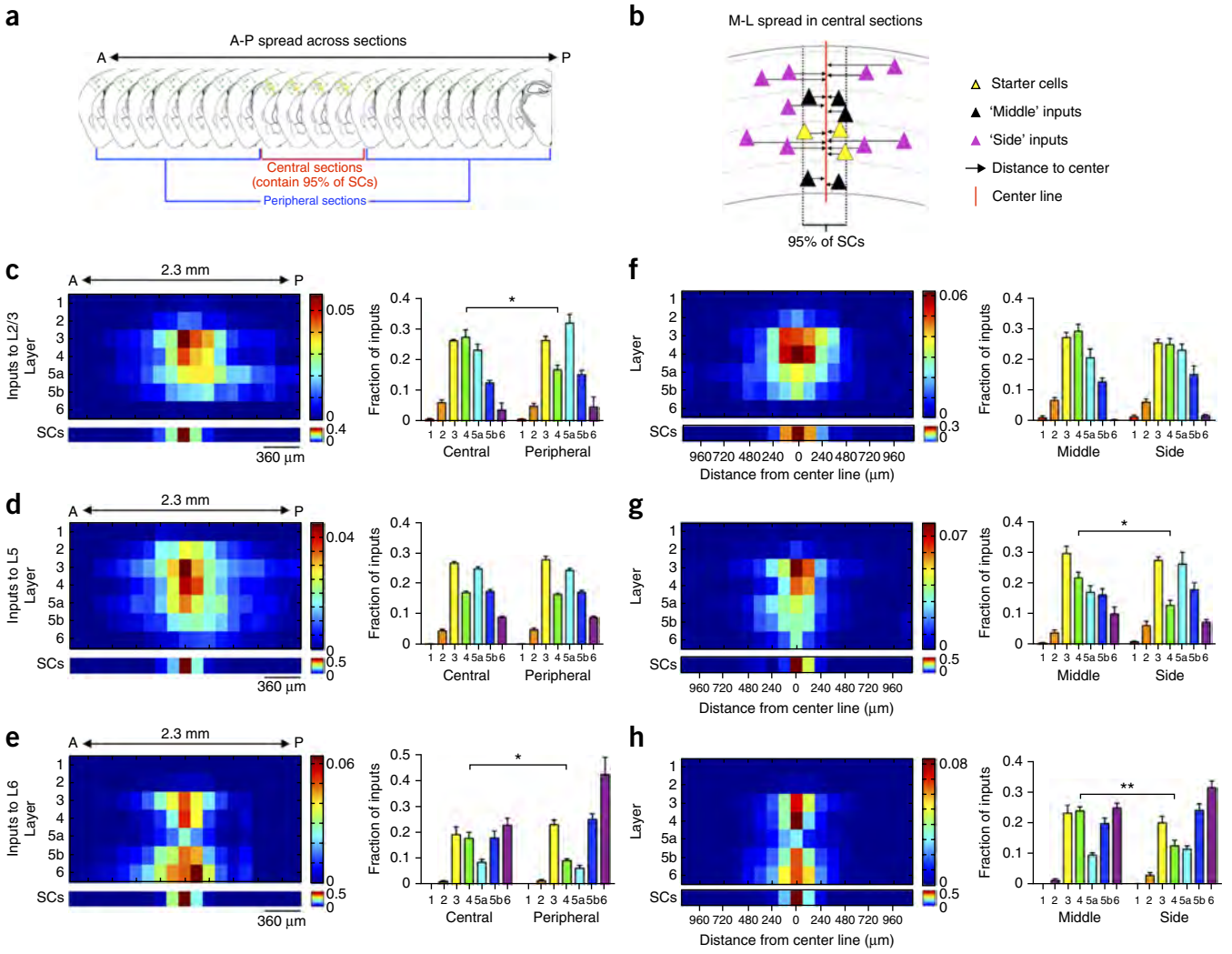

Figure 2 Spatial analyses of synaptic inputs from barrel cortex to starter cells in L2/3, L5 and L6. (**a**) Schematic of fractional analysis of inputs along the A-P axis. (**b**) Schematic of fractional analysis of inputs along M-L axis in the central sections. (**c**–**e**) Left, heat maps of distribution of starter cells (SCs, lower panels) and inputs (upper panels) along the A-P axis for inputs to L2/3 (n = 6 mice), L5 (n = 9 mice) and L6 (n = 9 mice). Colors represent the fraction of total barrel cortex inputs according to the index at right. Bin widths are 120 µm. Right, fraction of inputs in central versus peripheral sections (L4-L4, $*P = 0.003$, multiple t tests with Holm-Sidak correction for multiple comparisons ($\alpha = 0.05$)). (**f**–**h**) Left, heat maps of distribution of starter cells (SCs, lower panels) and inputs (upper panels) along the M-L axis for inputs to L2/3 (n = 5 mice), L5 (n = 6 mice) and L6 (n = 9 mice). Colors represent fraction of barrel cortex inputs in the sections analyzed. Bin widths are 120 µm. Right, quantification of fraction of inputs in middle versus side regions (L4-L4, $*P = 0.006$, $**P = 0.0001$, multiple t tests with Holm-Sidak correction for multiple comparisons ($\alpha = 0.05$)). Summary statistics presented as mean ± s.e.m. See **Supplementary Table 6** for test results and P values.

We quantified the number of *Gad*$^+$ inputs in starter cell-containing sections (**Fig. 3a**). Starter cells were largely restricted to the layer of interest (**Fig. 3b–d** and **Supplementary Table 3**) and the distribution of inhibitory inputs reflected the overall patterns of input, with a bias toward the same layer as the starter cells (**Fig. 3e–j** and **Supplementary Table 3**). GABAergic cells constituted 15, 9.8 and 9.5% of total inputs in counted sections of L2/3, 5 and 6 starter cells, respectively; the fraction of inputs that were GABAergic in each layer further differed for different starter cell populations (**Fig. 3k–m**). We observed a prevalence of inhibitory neurons in L1 and prominent intralaminar inhibition, consistent with previous reports[14,27]. We also observed large fractions of translaminar inhibition, especially from L2/3→L5 and L5→L2/3.

We examined the spatial spread of both *Gad*$^-$ and *Gad*$^+$ inputs to each layer along the M-L axis of barrel cortex in central sections. Although starter cells occupied 300–400 µm, *Gad*$^-$ (presumed excitatory) inputs spread throughout ~2 mm of cortex and maintained an hourglass shape with constriction in L4 (**Supplementary Fig. 3a–f**). *Gad*$^+$ inputs were more clustered than *Gad*$^-$ inputs, spanning ~1 mm of cortical space around the starter cells (**Supplementary Fig. 3a–f**), suggesting that excitatory and inhibitory inputs to the same starter cells span approximately ten and five barrel columns, respectively. *Gad*$^+$ inputs exhibited the most spread in the starter layer, indicating that the most lateral inhibition derives from the starter layer.

Long-range layer-specific input to barrel cortex

Barrel cortex is reciprocally connected with motor cortices, non-barrel–related S1 ($S1_{body}$), and S2 in sensorimotor processing circuits[13,28–30], but little is known about the laminar identity of long-range inputs to specific layers of barrel cortex. We identified numerous long-range inputs from $S1_{body}$, S2, M1 and M2 (**Fig. 4a–d**), from which we derived the first maps of projections from individual layers of M1,

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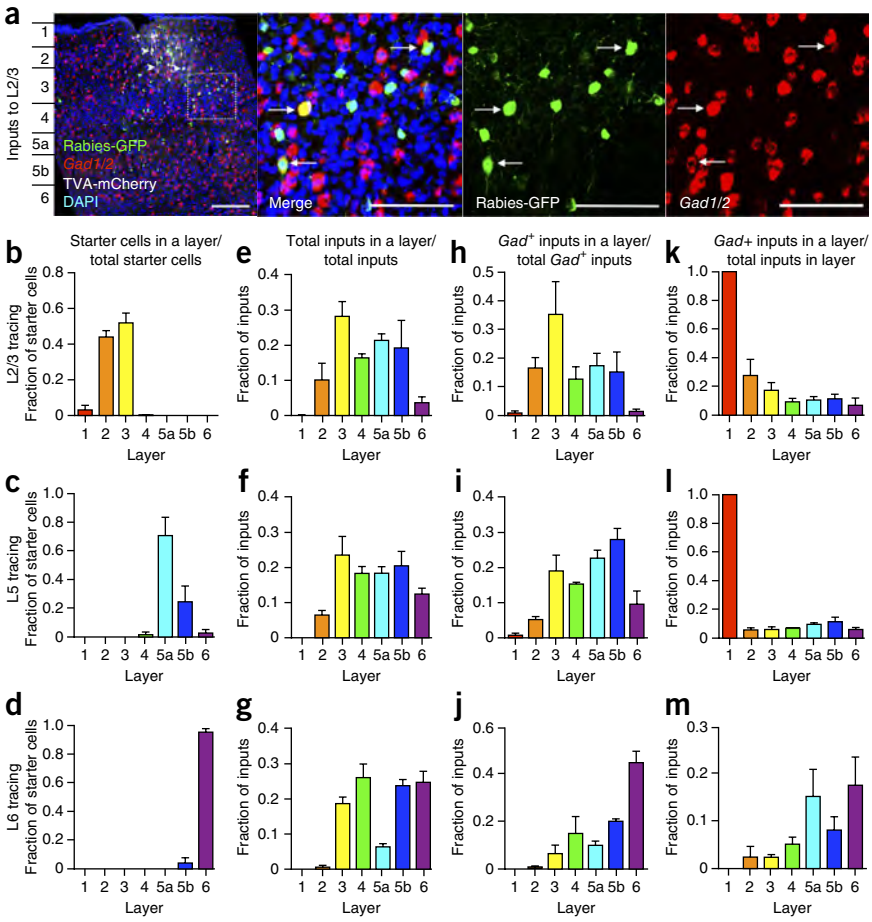

Figure 3 Analysis of local inhibitory inputs to starter cells in L2/3, L5 and L6. (**a**) Representative coronal sections of RV tracing to L2/3 in a *SepW1*^{Cre} mouse combined with *Gad1* and *Gad2 in situ* hybridization. Inhibitory inputs expressed both GFP (green) and *Gad1/2* (red) and are labeled with arrows. Starter cells expressed both GFP (green) and mCherry (white) and a subset are indicated with arrowheads. Excitatory inputs are green only. Scale bars represent 200 µm (first panel) or 100 µm (second to fourth panels, magnified from the dashed box in the first panel). (**b**–**d**) Starter cells are mostly restricted to the defined layer (L2/3 SCs, 48 ± 19, n = 3 mice; L5 SCs, 16 ± 2, n = 3 mice; L6 SCs, 95 ± 6, n = 3 mice). (**e**–**g**) Fraction of input (*Gad*$^+$ and *Gad*$^-$) in sections in which *Gad1/2 in situ* hybridization was performed (L2/3 total inputs, 342 ± 154, n = 3 mice; L5 total inputs, 983 ± 173, n = 3 mice; L6 total inputs, 605 ± 184, n = 3 mice). (**h**–**j**) Inhibitory inputs are distributed throughout all layers in a pattern that was specific to the layer origin of starter cells (L2/3 *Gad*$^+$ inputs, 91 ± 16, n = 3 mice; L5 *Gad*$^+$ inputs, 34 ± 7, n = 3 mice; L6 *Gad*$^+$ inputs, 52 ± 16, n = 3 mice). (**k**–**m**) Inhibitory inputs occupy specific fractions of inputs in each layer. Summary statistics presented as mean ± s.e.m. See **Supplementary Table 3** for numerical values.

M2, $S1_{body}$ and S2 to individual layers of barrel cortex. We also observed prominent input from ventral posteromedial (VPM) and posteromedial (POm) thalamic nuclei to L2/3, L5 and L6 (**Fig. 4c**,**e**–**g**), consistent with previous findings that L4 is not the only layer that receives thalamic input[31–34].

Compared with barrel cortex inputs, $S1_{body}$ and S2 provided fewer inputs from L5 to L2/3, whereas $S1_{body}$ and S2 inputs to L5 and L6 more closely resembled the local input patterns (**Figs. 1b**–**d** and **4h**–**m**). Long-range inputs to L2/3, L5 and L6 of barrel cortex came from progressively deeper layers, respectively. This pattern was most notable between barrel cortex and M1; whereas barrel cortex L2/3 received M1 inputs from L2–5b (**Fig. 4n**) and L5 received M1 inputs from L2–6 (**Fig. 4o**), L6 received M1 inputs almost exclusively from L5b–6 (**Fig. 4p**). M2 inputs to L5 and L6 originated mostly from L5 (**Fig. 4q**,**r**). Generally, superficial layers of barrel cortex received more long-range cortical input from superficial layers, whereas deep layers received more long-range cortical input from deep layers, suggesting distinct routes of information flow in feedback pathways.

Testing the role of NMDA receptors in shaping connectivity

Activity has a key role in circuit development[35–40], but little is known about its role in layer-specific synapse formation. By coupling coincident pre- and postsynaptic activity to calcium signaling, the NMDA receptor implements activity-dependent changes in wiring during development[36]. To examine whether activity sculpted input distributions, we traced inputs to neurons lacking GluN1, the obligatory subunit of NMDA receptors. We crossed layer-specific Cre lines to a conditional *GluN1* allele (*GluN1*^{fl}) and a *GluN1* null allele (*GluN1*$^{\Delta}$) to remove GluN1 from Cre-active cells (*Cre; GluN1*$^{fl/\Delta}$), a subset of which were starter cells for RV tracing (**Fig. 5a**). For L2/3 input tracing, we electroporated Cre *in utero* at E16 (**Supplementary Fig. 1a**). In both *Rbp4*^{Cre} and *Ntsr1*^{Cre} driver mice, Cre reporter expression was detected by postnatal day 1 (P1; **Supplementary Fig. 1a**), suggesting that *GluN1* was deleted well before the peak of cortical synaptogenesis around P14 (refs. 41,42).

To confirm that GluN1 was removed from Cre-expressing *GluN1*$^{fl/\Delta}$ cells, we recorded synaptic currents from TVA^{66T}-mCherry–labeled cells in *Ntsr1*^{Cre};*GluN1*$^{fl/+}$ and *Ntsr1*^{Cre};*GluN1*$^{fl/\Delta}$ mice. *GluN1*$^{fl/+}$ cells had robust GluA- and GluN-mediated EPSCs (**Fig. 5b**). In contrast, *GluN1*$^{fl/\Delta}$ cells had robust GluA-mediated EPSCs at −60 mV, but significantly reduced (P = 0.002) GluN-mediated EPSCs at +40 mV (**Fig. 5b**–**d**). In *GluN1*$^{fl/\Delta}$ cells, DNQX suppressed the EPSCs observed at +40 mV, indicating that they were GluA-mediated, whereas control cells maintained 20% of the EPSC at +40 mV after DNQX wash-in, indicating that much of that EPSC was mediated by GluN (**Supplementary Fig. 4**).

We compared the pattern of input to GluN1-lacking and control starter cells (**Figs. 1** and **5**). Cortical layer thicknesses were similar between conditions (**Supplementary Fig. 5a**–**d**), as were the patterns of input to GluN1-lacking L2/3, L5 and L6 starter cells (**Fig. 5e**–**m** and **Supplementary Table 4**), except for a significant increase (P = 0.006) in the fraction of L6→L5 inputs (**Fig. 5j**) and a significant decrease (P = 0.002) in the fraction of L6→L6 inputs for GluN1-lacking starter cells (**Fig. 5m**). We found no changes in the fractional distribution of long-range input to L5 and L6 from cortical areas and thalamus (**Supplementary Fig. 5e**,**f**). The laminar distribution of inputs was also similar for control and GluN1-lacking starter cells (**Supplementary Fig. 5e**,**f**), except that GluN1-lacking L5 starter cells received significantly more (P = 0.004) input from S2 L6 (**Supplementary Fig. 5e**).

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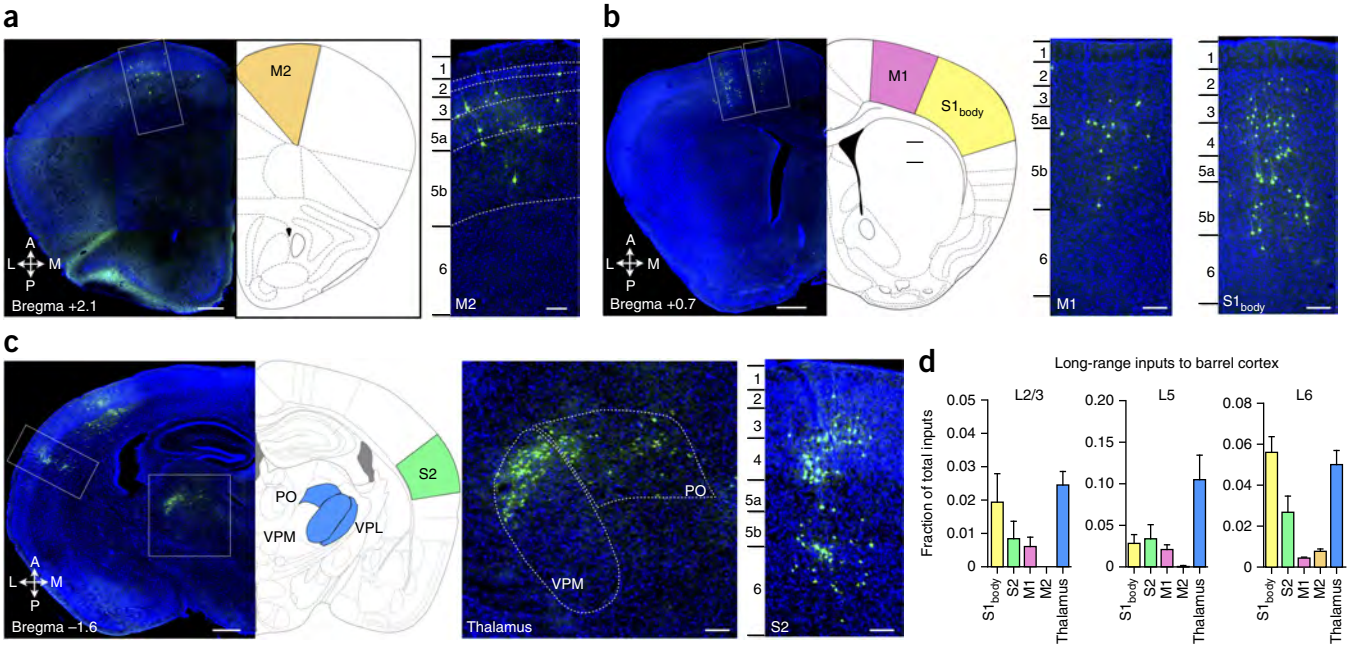

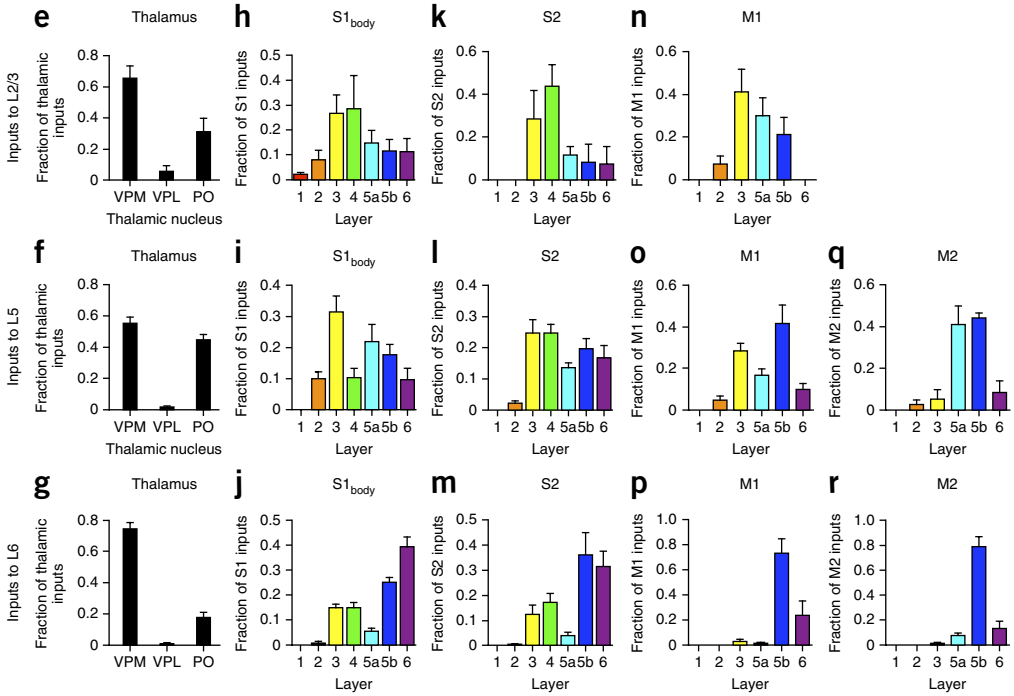

Figure 4 Laminar analyses of long-range inputs to starter cells in L2/3, L5 and L6. (**a**–**c**) Atlas locations and example images showing long-range inputs to barrel cortex L5 from secondary motor cortex (M2, **a**), primary motor cortex (M1, **b**), primary somatosensory cortex ($S1_{body}$, **b**), somatosensory thalamus (VPM/POm, **c**) and secondary somatosensory cortex (S2, **c**). Scale bars represent 500 µm (hemisection images) or 100 µm (zoomed images). (**d**) Quantification of fraction of total inputs contributed by each area. (**e**–**r**) Regional analysis of thalamic inputs (**e**–**g**) and laminar analysis of long-range inputs to L2/3 (n = 6 mice), L5 (n = 9 mice) and L6 (n = 9 mice) from $S1_{body}$ (**h**–**j**), S2 (**k**–**m**), M1 (**n**–**p**) and M2 (**q**,**r**). Summary statistics presented as mean ± s.e.m. Schematics are modified after ref. 50.

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Thus, eliminating GluN1 signaling had minimal effects on patterns of input, with the exception of particular connections involving deep-layer neurons. These loss-of-function data suggest that NMDA receptors in L6 neurons normally promote the formation or maintenance of recurrent connections from other L6 neurons, whereas NMDA receptors in L5 normally inhibit formation or maintenance of L6 inputs from barrel cortex and S2.

Comparing local input to mPFC and barrel cortex L5

We next tested whether layer-specific connectivity patterns in sensory cortex also apply to association cortex. Of the Cre lines we used for layer-specific input mapping in barrel cortex, only *Rpb4*^{Cre} maintained layer specificity in mPFC. We traced the inputs to L5 cells in mPFC and compared the results to barrel cortex. To characterize the potential starter cells in mPFC L5, we immunostained mPFC sections from *Rbp4*^{Cre};*Rosa*^{Ai14} mice with antibodies to NeuN or Ctip2 (**Supplementary Fig. 2d**,**e**). *Rbp4*^{Cre};*Rosa*^{Ai14} labeled ~46% of NeuN^{+} L5 cells and 51% of tdTomato^{+} cells in L5b expressed Ctip2, similar to what we observed in barrel cortex (**Supplementary Fig. 2g**).

mPFC starter cells were located in infralimbic (IL) and prelimbic (PL) cortex (**Fig. 6a**) in L5a and L5b with a small fraction in L3 (**Fig. 6b** and **Supplementary Table 5**). Layers were assigned on the basis of DAPI staining for each section and were consistent with layer boundaries determined by expression of Cux1 (L3) and Ctip2 (L5b/6) (**Supplementary Fig. 6a**,**b** and Online Methods). Control experiments revealed minimal local background (**Supplementary Fig. 6c**,**d**).

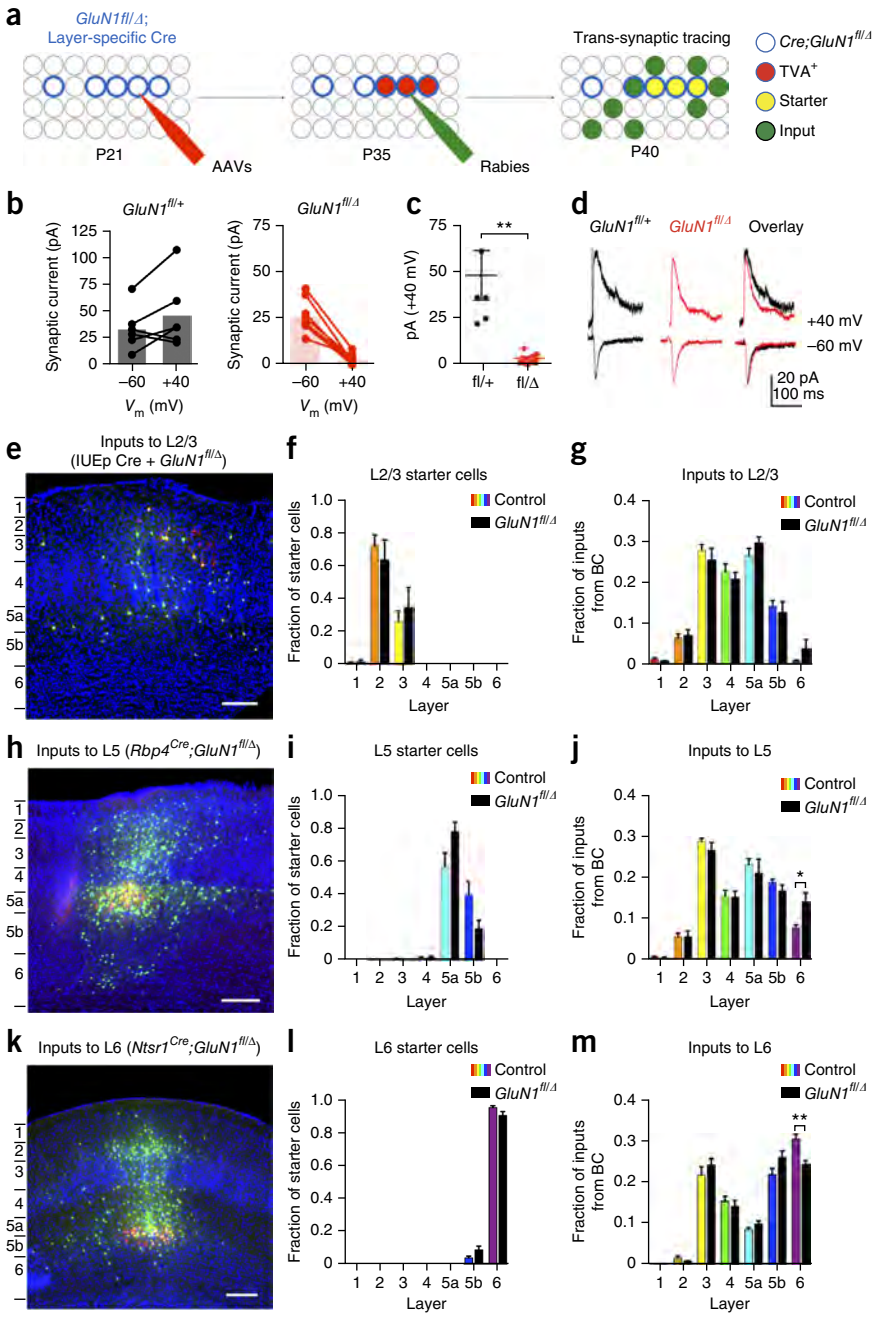

Figure 5 Layer-specific tracing from starter cells lacking GluN1. (**a**) Experimental design. RV tracing was performed as described in **Figure 1**, except on mice in which starter cells (as well as other Cre-expressing cells in the starter cell layer, blue circles) lacked GluN1. (**b**–**d**) Electrophysiological characterization of *GluN1* mutant cells. (**b**) Synaptic currents measured in *Ntsr1*^{Cre};*GluN1*$^{fl/+}$ (heterozygous control, left, −60 mV, 34.6 ± 8.4 pA; +40 mV, 47.7 ± 13.3 pA; $n = 6$ cells from 2 mice) and *Ntsr1*^{Cre};*GluN1*$^{fl/\Delta}$ (mutant, right, −60 mV, 26.2 ± 3.1 pA; +40 mV, 3.1 ± 1.0 pA; $n = 8$ cells from 2 mice). (**c**) Quantification of NMDA synaptic current in heterozygous control (black) and mutant (red) (fl/+, 47.7 ± 13.3 pA, $n = 6$ cells from 2 mice; fl/Δ, 3.1 ± 1 pA, $n = 8$ cells from 2 mice; $^{**}P = 0.002$, Student's *t* test). (**d**) Example recordings from *Ntsr1*^{Cre};*GluN1*$^{fl/+}$ (black) and *Ntsr1*^{Cre};*GluN1*$^{fl/\Delta}$ (red) cells. (**e**,**h**,**k**) Representative coronal sections of layer-specific RV tracing in which starter cells lack GluN1. Scale bars represent 200 µm. (**f**,**i**,**l**) Starter cells are mostly restricted to the defined layer. (**g**,**j**,**m**) Comparisons of the patterns of input to control (same as data in **Fig. 1**) versus GluN1-lacking starter cells (L2/3 SCs, 18 ± 10; inputs, 564 ± 117, $n = 4$ mice; L5 SCs, 152 ± 64; inputs, 4,447 ± 1559, $n = 6$ mice L6→L5, $^{*}P = 0.006$; L6 SCs, 146 ± 26; inputs, 1,583 ± 348, $n = 7$ mice, L6→L6, $^{**}P = 0.002$; multiple *t* tests with Holm-Sidak correction for multiple comparisons ($\alpha = 0.05$)). Summary statistics presented as mean ± s.e.m. See **Supplementary Table 4** for numerical values. See **Supplementary Table 6** for test results and *P* values.

Inputs were distributed throughout all layers, with the largest fraction from L5a and L5b (**Fig. 6c** and **Supplementary Table 5**). Given that mPFC lacks a detectable L4, we removed L4 inputs from our barrel cortex analysis to simplify the comparison. Compared with barrel cortex, mPFC L5 cells received a greater fraction of input from L1, L5b and L6 at the expense of L3 (**Fig. 6c**). Given that L3 is thinner and L5 is thicker in mPFC than in barrel cortex (**Supplementary Fig. 6b** and **Supplementary Fig. 1d**), layer thickness could contribute to these differences. After normalizing input distribution by layer thickness, L5 cells in mPFC still received more L1 input at the expense of L3, but the fractional input from L5 was comparable (**Supplementary Fig. 6e**). Thus, although layer thickness may explain the fractional differences in L5 recurrent connectivity, it alone cannot account for the distinct patterns of translaminar input between barrel cortex and mPFC.

We also analyzed the distribution of local inputs along the A-P and dorsal-ventral (D-V) axes. As in barrel cortex (**Fig. 2**), starter cells were contained within 360 µm diameter regions of mPFC; inputs peaked within sections that contained the most starter cells but also extended across ~2 mm of mPFC (**Fig. 6d**,**e**). We observed trends suggesting that L5 inputs spread more with distance along the A-P (**Fig. 6d**) and D-V (**Fig. 6e**) axes compared with inputs from other layers of mPFC.

To characterize the local GABAergic inputs to mPFC L5 cells, we performed *Gad1* and *Gad2 in situ* hybridization on sections containing starter cells (**Fig. 6f**,**g**). Similar to barrel cortex, we observed that the pattern of inhibition in mPFC reflected the overall pattern of input with a slight bias toward inputs from L5 (**Fig. 6h**). The percent of inhibitory input was relatively consistent across layers, ranging from 18–27% (**Fig. 6i**). Notably, there was a 2.5-fold increase in the GABAergic fraction of local inputs to mPFC L5 cells compared with barrel cortex L5 (**Fig. 6j**), indicating that ratio of local excitatory to inhibitory inputs to L5 pyramidal cells varies between cortical areas.

mPFC L5 receives proportionally more long-range input

mPFC and barrel cortex L5 cells received markedly different fractional inputs from local versus long-range areas. Whereas barrel

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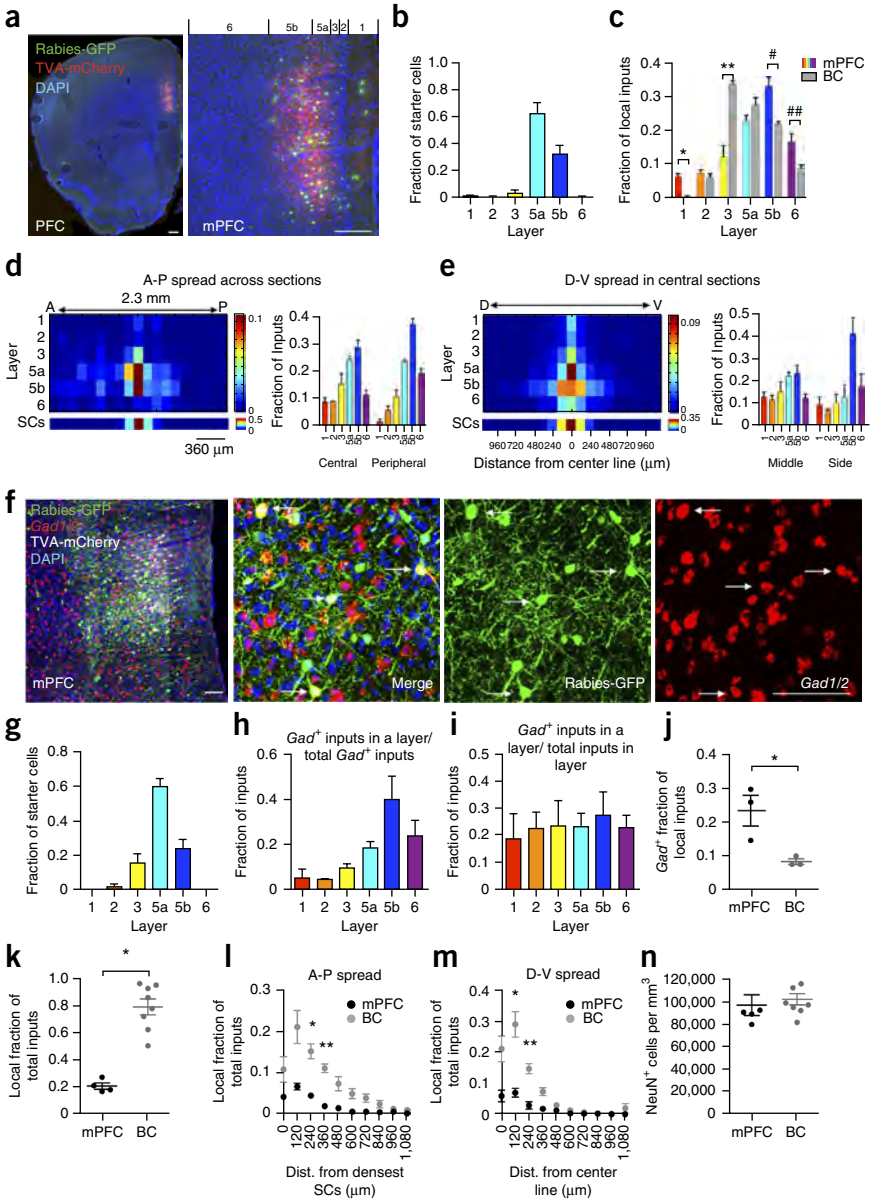

Figure 6 Local input to mPFC L5. (**a**) Low (left) and high (right) magnification example images of tracing from L5 mPFC neurons. Scale bar represents 200 µm. (**b**) Fraction of starter cells by layer. (**c**) Fraction of local inputs by layer (colored; SCs, 114 ± 33; total inputs, 2,641 ± 664; $n = 4$ mice; L1-L1, $^{*}P = 3.03 \times 10^{-7}$; L3-L3, $^{**}P = 2.53 \times 10^{-6}$; L5b-L5b, $^{\#}P = 0.0002$; L6-L6, $^{\#\#}P = 0.002$; multiple t tests with Holm-Sidak correction for multiple comparisons ($\alpha = 0.05$)) in comparison with barrel cortex (BC, gray, $n = 8$ mice). (**d**,**e**) Left, heat maps of distribution of starter cells (SCs, lower panel) and inputs (upper panel) in each layer along the (**d**) A-P or (**e**) D-V axis. Colors represent fraction of total mPFC cells according to the index at right. Bin widths are 120 µm. Right, fraction of mPFC inputs in central versus peripheral sections (**d**; $n = 4$ mice) or in middle versus side regions (**e**; $n = 4$ mice). (**f**) Tracing from L5 starter cells with *Gad1* and *Gad2 in situ* hybridization. Arrows indicate *Gad*+ inputs. Scale bar represents 100 µm. (**g**) Fraction of starter cells by layer ($n = 3$ mice). (**h**) Fraction of total *Gad*+ cells by layer ($n = 3$ mice). (**i**) *Gad*+ fraction of total inputs in each layer ($n = 3$ mice). (**j**) Comparison of *Gad*+ fraction of local inputs to L5 mPFC versus L5 BC (mPFC, 0.24 ± 0.04, $n = 3$ mice; BC, 0.09 ± 0.008, $n = 3$ mice; $^{*}P = 0.03$, Student's t test). (**k**) Comparison of the local fraction of total inputs to mPFC and BC L5 (mPFC, 0.21 ± 0.02, $n = 4$ mice; BC, 0.79 ± 0.6, $n = 8$ mice; $^{*}P = 5.14 \times 10^{-5}$, Student's t test). (**l**) Spread of local inputs along the A-P axis in mPFC ($n = 4$ mice) or BC ($n = 8$ mice), presented as fraction of total inputs (240 µm, $^{*}P = 0.0009$; 360 µm, $^{**}P = 0.0005$; multiple t tests with Holm-Sidak correction for multiple comparisons ($\alpha = 0.05$)). (**m**) Spread of local inputs along the D-V axis in mPFC ($n = 4$ mice) or BC ($n = 8$ mice), presented as fraction of total inputs (120 µm, $^{*}P = 0.004$; 240 µm, $^{**}P = 0.0007$; multiple t tests with Holm-Sidak correction for multiple comparisons ($\alpha = 0.05$)). (**n**) Quantification of cell density based on NeuN staining (mPFC, 97,633 ± 9,010 cells per mm^3, $n = 5$ sections from 2 mice; BC, 102,760 ± 4,876 cells per mm^3, $n = 8$ sections from 3 mice; $P = 0.59$, Student's t test). Summary statistics presented as mean ± s.e.m. See **Supplementary Table 5** for numerical values. See **Supplementary Table 6** for test results and P values.

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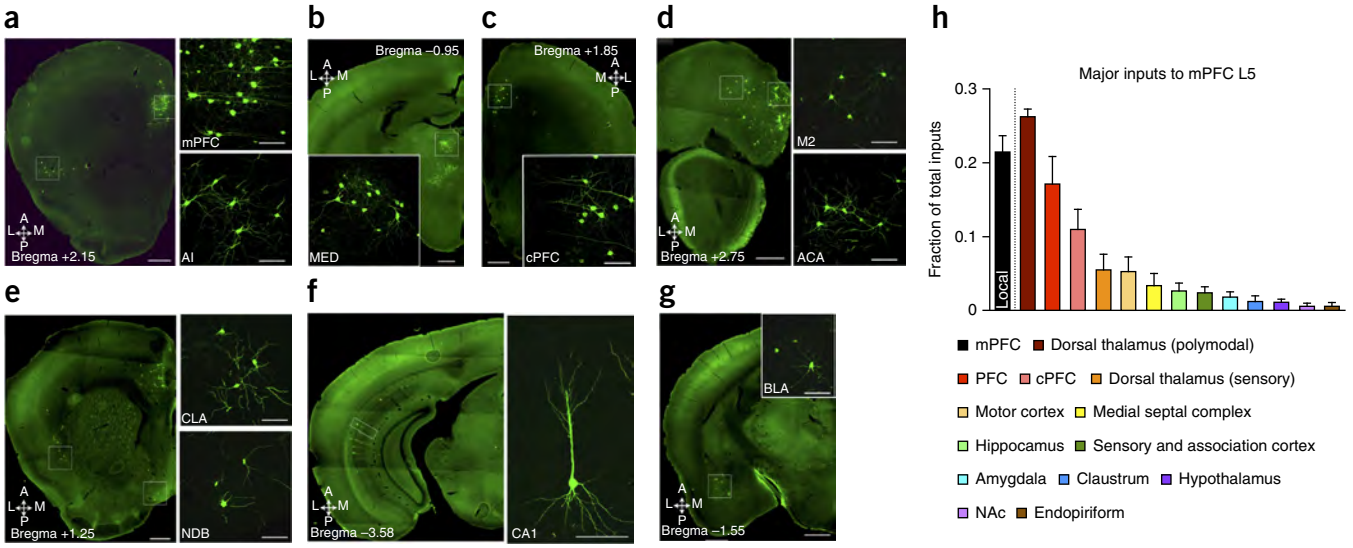
cortex L5 cells received, on average, 79% of their total inputs from other cells in ipsilateral barrel cortex, mPFC L5 cells only received 21% of their inputs from other ipsilateral mPFC cells (**Fig. 6k**). This could be because barrel cortex is approximately twice the volume of mPFC (barrel cortex per hemisphere = 4.1 ± 0.4 mm^3, $n = 5$ mice; mPFC per hemisphere = 1.9 ± 0.02 mm^3, $n = 4$ mice). However, most of the inputs in barrel cortex and mPFC were located within 1 mm of the starter cells (**Figs. 2d**,**g** and **6d**,**e**), suggesting the volumes containing local inputs in each area were similar. Alternatively, the local connection probability could vary by region.

To determine whether the spread of inputs to L5 starter cells differs between barrel cortex and mPFC, we plotted the local fraction of total inputs along the A-P (**Fig. 6l**) and D-V (**Fig. 6m**; M-L for barrel cortex) axes for each area. We did not observe differences in the fraction of local inputs at sites distant (360–1,080 µm) from starter cells, suggesting that mPFC and barrel cortex starter cells receive local inputs from similar volumes of cortical space. However, we observed significantly smaller fractions (A-P 240 µm: $P = 0.0009$; A-P 360 µm: $P = 0.0005$; D-V 120 µm: $P = 0.004$; D-V 240 µm: $P = 0.0007$) of inputs to mPFC L5 at sites close to the starter cells along both axes (**Fig. 6l**,**m**). This was not a result of a reduced number of potential inputs, as the density of neurons in mPFC and barrel cortex was similar (**Fig. 6n**). Thus, the decreased fraction of local input to mPFC compared to barrel cortex is not a result of differences in cortical size or available inputs, but instead may reflect distinct local connection probabilities between the two areas.

Major long-range inputs to L5 mPFC neurons originated from other prefrontal areas, including agranular insula (AI; **Fig. 7a**), dorsal polymodal thalamic nuclei (~26%; **Fig. 7b**), contralateral mPFC (cPFC, ~11%; **Fig. 7c**) and motor areas (~5%; **Fig. 7d**), with more minor inputs from claustrum (CLA; **Fig. 7e**), CA1 cells throughout the D-V axis of the hippocampus (**Fig. 7f**), and the basolateral



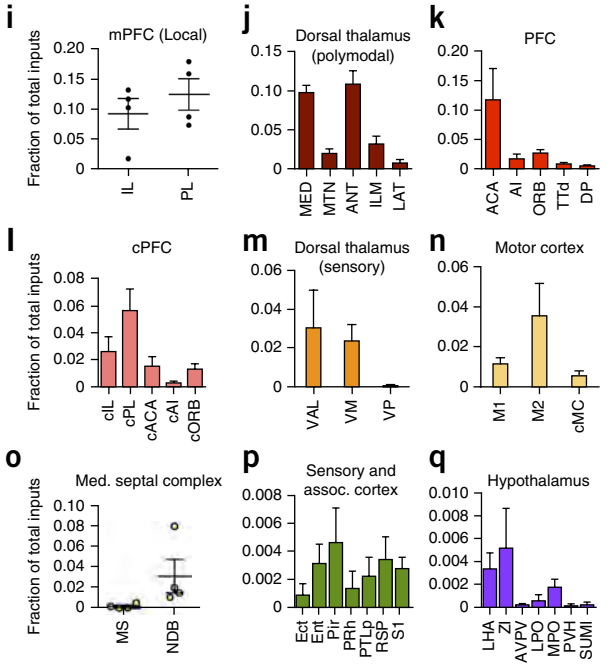


Figure 7 Long-range input to mPFC L5. (**a**–**g**) Example images of long-range inputs to L5 mPFC. Scale bars represent 500 µm (hemisection images) or 100 µm (zoomed images). (**h**) Quantification of major inputs to L5 mPFC ($n = 4$ mice). (**i**–**q**) Detailed quantification of subregional inputs to mPFC L5 ($n = 4$ mice). AVPV, anteroventral periventricular nucleus; Ect, ectorhinal cortex; Ent, entorhinal cortex; LHA, lateral hypothalamic area; LPO, lateral preoptic area; MPO, medial preoptic area; MS, medial septum; NDB, nucleus of the diagonal band; Pir, piriform cortex; PRh, perirhinal cortex; PTLp, posterior parietal association area; PVH, paraventricular hypothalamic nucleus; RSP, retrosplenial cortex; SUMI, supramammillary nucleus, lateral part; ZI, zona inserta. Summary statistics are presented as mean ± s.e.m.

amygdala (BLA; **Fig. 7g**,**h**). Local mPFC inputs came about equally from IL and PL (**Fig. 7i**). In the dorsal thalamus, we observed inputs from the medial (MED), midline (MTN), anterior (ANT), intralaminar (ILM) and lateral (LAT) groups, with MED and ANT each contributing approximately 10% of the overall inputs to L5 (**Fig. 7j**). The anterior cingulate area (ACA) was the major source of PFC input, contributing about 10% of the total inputs to mPFC L5, with smaller contributions from agranular insula, orbital area (ORB), tenia tecta (TTd) and the dorsal peduncular area (DP) (**Fig. 7k**). cPFC contributed ~11% of the overall input (**Fig. 7l**). Dorsal sensory thalamus (**Fig. 7m**) and motor cortex (MC; **Fig. 7n**) each contributed 5% of the overall input. Finally, mPFC L5 cells received smaller fractions of input from the medial septal complex (MSC; **Fig. 7o**), sensory and association cortices (**Fig. 7p**), and various hypothalamic nuclei (**Fig. 7q**). These data are largely consistent with classical retrograde tracer studies that mapped inputs to mPFC[43]. We found that neurons from these regions provided monosynaptic input directly onto L5 pyramidal neurons. In contrast with barrel cortex, which is a dedicated circuit for processing sensorimotor information, mPFC circuits integrated information from diverse brain regions involved in a wide variety of cognitive functions and behaviors. Furthermore, the large fraction of local inhibitory inputs in mPFC (**Fig. 6j**) likely participates in feedforward inhibitory circuits driven by long-range inputs such that the local inhibition reflects the overall excitatory drive to L5 cells in a given cortical area rather than scaling with the number of local excitatory inputs.

DISCUSSION

We used RV trans-synaptic tracing to generate local and long-range input maps to genetically defined pyramidal cells in L2/3, L5 and L6 of the mouse barrel cortex and to mPFC L5 (**Fig. 8** and see **Supplementary Fig. 7** for raw numbers). Although it is a powerful tool for visualizing the distribution of inputs to specific neuronal populations, RV tracing does not label all inputs and lacks information about the strength of labeled connections. If RV tracing is biased toward certain connections, the relative input maps may not accurately represent overall connectivity. We are not aware of such biases, but this potential caveat should be kept in mind when interpreting our results. On the other hand, RV tracing labels connections in intact tissue and has the potential to identify many connections that are not easily detected in slice recordings or glutamate uncaging, including long-range connections from cortical areas and subcortical inputs. The Cre mouse lines that we used allowed us to trace from a mixed population of L5 neurons (*Rbp4*^{Cre}) and a relatively pure population of L6 cortico-thalamic neurons (*Ntsr1*^{Cre}). Using additional Cre lines with unique cell-type specificity or tracing inputs to functionally defined cell types will further expand our understanding of cortical circuit organization. Ultimately, the combination of anatomy-based studies such as RV tracing with functional connectivity data will be

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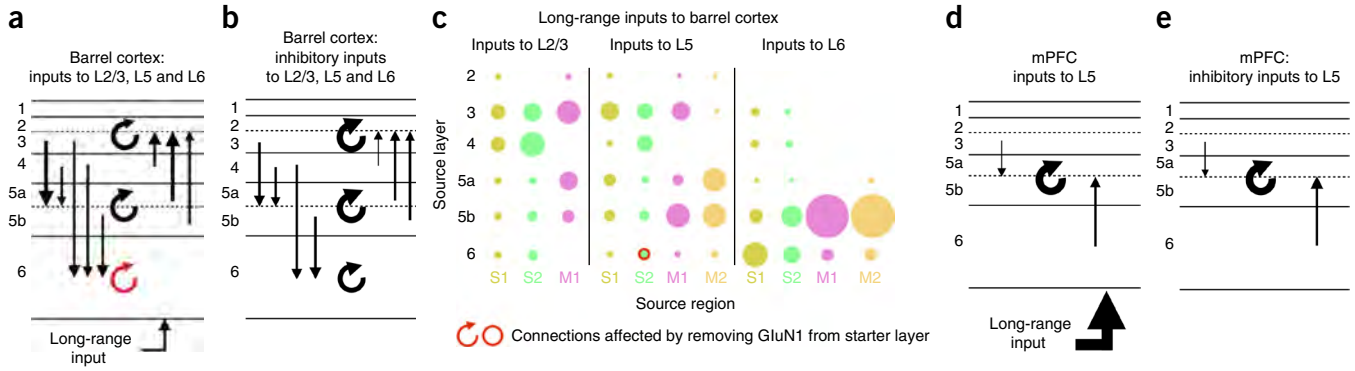

Figure 8 Summary of main findings. (**a**) Summary of major connections in barrel cortex. (**b**) Summary of major inhibitory connections in barrel cortex. (**c**) Summary of laminar analysis of long-range inputs to barrel cortex L2/3, L5 and L6. (**d**) Summary of major connections in mPFC. (**e**) Summary of major inhibitory connections in mPFC. Arrow thickness in **a**, **b**, **d** and **e**, and circle diameter in **c** correspond to strength of fractional inputs. Only inputs that consisted of more than 10% of total inputs to a given layer are represented by arrows. Dotted lines between L2 and L3, as well as between L5a and L5b, indicate that, although our experiments distinguished the input from these layers (arrows leaving from the middle of these layers), we treated them collectively as recipient of inputs (arrows pointing to the dotted lines).

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required to more completely understand the contributions of different connections to cortical information processing.

Our RV-based layer-specific input map identified cortical pathways that have been described with other methods, as well as prominent L3→L6 connections (**Figs. 1d** and **8a**) that were not apparent in electrophysiological studies in brain slices[8,9]. CRACM confirmed that L3→L6 connections are functional, but weak, compared with L3→L5 connections (**Fig. 1e–h**). Compared with somatic recordings, RV tracing may be more sensitive in detecting weak synaptic connections. Consistent with this notion, a recent RV trans-synaptic tracing study observed substantial L2/3→L6 connections in V1 (ref. 44), whereas an electrophysiological mapping study reported sparse and weak connections from L2/3→L6 in V1 (ref. 45). It will be important to assess how these weak connections interact at the level of the dendrite, as they could powerfully affect the output of the cell through supralinear interactions with simultaneously active synapses or back-propagating action potentials[46].

We observed a preferential superficial→superficial and deep→deep layer connectivity structure between motor and barrel cortices, with inputs to layer 6 being particularly biased toward deep layers (**Fig. 8c**). Motor cortex is strongly activated before and during whisking, and it sends information about self-generated movements back to barrel cortex. It will be interesting to test whether layer-specific pathways carry different kinds of information about exploratory whisking, and whether the preferential superficial→superficial and deep→deep layer connectivity applies to other long-range cortical-cortical projections.

The mutant TVA receptor that we used, despite reducing Cre-independent, non-specific labeling to zero (**Supplementary Fig. 1b**), may preferentially reduce the efficiency of long-range input tracing[18]. Thus, the fraction of long-range input that we reported is likely an underestimate. Nevertheless, the relative laminar distribution of long-range input should still be informative, as should be the comparison between barrel cortex and mPFC (**Fig. 8a,b,d,e**). We observed that mPFC L5 cells receive a much larger fraction of their total input from long-range sources. mPFC L5 cells also received a substantially larger fraction of local inhibitory inputs than barrel cortex, suggesting that many mPFC inhibitory cells participate in feedforward inhibitory circuits driven by long-range inputs[47]. Thus, the large local fraction of inhibitory inputs to mPFC L5 neurons may participate in diverse inhibitory microcircuits recruited by different behaviorally relevant long-range inputs.

Removing NMDA receptor activity from starter cells revealed that NMDA receptors may preferentially influence a subset of connections originating from L6 neurons (**Fig. 8a,c**), potentially related to the activity dependence of L6 axon targeting seen during visual cortex development[37]. Our data also indicate that the distribution of long-range inputs and the layer specificity of cortical inputs are largely independent of the presence of NMDA receptors in the starter cells. Our results contrast with the critical role of NMDA receptor activity in determining connectivity onto reelin-expressing interneurons[48] and suggest that NMDA receptors differentially regulate circuit development in distinct cortical cell types.

Although RV tracing can reveal information about labeled presynaptic partners, including morphology, transmitter phenotype and position in cortex, it may not detect changes in synaptic strength or functional specificity, such as preferential connectivity for neurons with similar tuning properties[49], which may require NMDA receptor activity. Our starter cells were not confined to individual barrels, so subtle effects influencing connectivity in and between barrels may also have been beyond our detection limit, but the overall pattern of layer-specific connections was largely independent of NMDA receptor and, by extension, correlated neuronal activity. Thus, molecular determinants likely have a large role in layer-specific cortical connectivity. Our strategy of RV tracing in combination with layer-specific knockout of genes provides a means to test candidate molecular determinants in the future.

METHODS

Methods and any associated references are available in the online version of the paper.

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

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AUTHOR CONTRIBUTIONS

L.A.D., D.S.B. and L.L. designed the experiments. L.A.D. and D.S.B. performed and analyzed layer-specific trans-synaptic tracing experiments. L.A.D. performed slice recording and CRACM experiments. K.D. performed and analyzed *in situ* hybridization experiments. L.A.D., D.S.B. and L.L wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. All animal procedures followed animal care guidelines approved by Stanford University's Administrative Panel on Laboratory Animal Care (APLAC). Both male and female mice from mixed CD1 and C57/Bl6 backgrounds were used. Mice were group housed. *SepW1*^{Cre} mice[22] were obtained from the US National Institutes of Health, *Rbp4*^{Cre} and *Ntsr1*^{Cre} mice[51] were obtained from the Mutant Mouse Regional Resource Center, *GluN1*$^{fl/fl}$ mice[52] and Cre-dependent tdTomato reporter *Rosa*^{Ai14} mice[53] were obtained from the Jackson Laboratories. To characterize Cre drivers, *Rbp4*^{Cre}*and Ntsr1*^{Cre} were crossed to *Rosa*^{Ai14} mice (**Supplementary Fig. 2**). While *SepW1*^{Cre} allowed us to generate L2/3-restricted starter cells at P21 (**Fig. 3**), crossing *SepW1*^{Cre} to *Rosa*^{Ai14} mice resulted in tdTomato labeling throughout the entire brain (data not shown), suggesting that *SepW1*^{Cre} is active in most cells early in development and becomes layer restricted with age. Thus, to characterize L2/3 cells defined by *SepW1*^{Cre}, we injected large volumes of AAV (CAG-FLEx-TVA^{66T}-mCherry) into barrel cortex at P25 and analyzed the expression pattern at P40. The *GluN1*$^{\Delta}$ allele was generated by crossing *GluN1*$^{fl/fl}$ to a germline Cre (*hprt*^{Cre}). To study the effect of GluN1 knockout in L2/3, we crossed *GluN1*$^{fl/fl}$ to *GluN1*$^{\Delta/+}$ mice and performed *in utero* electroporation of a Cre-expressing plasmid at E16. To study the effects of *GluN1* knockout in L5 and L6, we generated *Rbp4*^{Cre};*GluN1*$^{\Delta/+}$ and *Ntsr1*^{Cre};*GluN1*$^{\Delta/+}$ mice, respectively, and crossed them to *GluN1*$^{fl/fl}$ mice. *GluN1*$^{fl/+}$ mice were combined with *GluN1*$^{+/+}$ mice in the control group for all analyses.

DNA constructs. DNAs encoding Cre-2A-BFP-3x Myc were subcloned in frame into the pCAG vector. Briefly, pCAG was digested with BsrG1 and EcoR1 and Cre and 2A-BFP-3x myc fragments were subcloned in simultaneously using an InFusion kit (Clontech).

***In utero* electroporation.** Timed-pregnant mice (E16) were anaesthetized with 3% isoflurane (vol/vol) and given preoperative injection of Buprenex at 0.05–0.1 mg per kg. The abdomen was swabbed with iodine. A small vertical incision was made in the skin and abdominal wall and embryos were gently exposed. Each embryo was injected with 1–2 µl of DNA solution and 0.01% Fast Green (vol/vol) using a pressure-controlled glass pipette. After each injection, the embryos were moistened with phosphate-buffered saline (PBS) and voltage steps via tweezertrodes (BTX, 5 mm round, platinum, BTX electroporator) were applied at a 45° angle with respect to the interaural line to target barrel cortex. Voltage was 36 V for five 50-ms pulses at 1 Hz as described previously[54]. The embryos were returned to the abdomen, which was sutured, followed by suturing of the skin. The procedure typically lasted 20 min.

Viral procedures. AAV vectors containing CAG-FLEx-G (2×10^{11} genomic copies (GC) per ml) and CAG-FLEx-TVA^{66T}-mCherry (10^{12} GC per ml) were described previously[18] and produced by the University of North Carolina viral vector core. EnvA-pseudotyped RV*dG* (10^{10} IU per ml) was produced in-house based on established procedures[55]. For trans-synaptic labeling, 0.1 µl of 1:1 mixture of AAV2 or AAV8 CAG-FLEx-G and AAV2 CAG-FLEx-TVA^{66T}-mCherry was injected into the brain at P21 by using a stereotactic apparatus (KOPF). During surgery, animals were anesthetized with 65 mg per kg ketamine and 13 mg per kg xylazine (Lloid Laboratories). For barrel cortex injections, the pipette was placed 0.25 mm posterior and 3.1 mm lateral from the bregma and 0.25 or 0.4 mm from the brain surface to target L2/3 or L5, respectively, or 0.7 mm posterior and 3.5 mm lateral from bregma and 0.85 mm from the brain surface for L6. For mPFC injections, the needle was placed 1.8 mm anterior, 0.3 mm lateral, and 2.5 mm deep from bregma. After recovery, animals were housed in regular 12-h dark/light cycle with food and water *ad libitum*. 2 weeks later, 0.5 µl of EnvA-pseudotyped RV*dG* was injected into the same brain location under anesthesia. After recovery, animals were housed in a biosafety room for 5 d to allow RV*dG* infection, trans-synaptic spread, and expression of sufficient amount of GFP to label presynaptic cells.

Histology. Animals were perfused transcardially with PBS followed by 4% paraformaldehyde (PFA, vol/vol). Brains were dissected, post-fixed in 4% PFA for 12–24 h, and placed in 30% sucrose (vol/vol) for 24–48 h. They were then embedded in Optimum Cutting Temperature (OCT, Tissue Tek) and stored at −80 °C until sectioning. 60-µm floating sections were collected into PBS. For Cre line characterization and layer analysis, sections were incubated in 0.3% PBST (vol/vol) and 10% donkey serum (vol/vol) for 1 h and then stained with mouse anti-NeuN (Millipore MAB377, 1:1,000), rat anti-Ctip2 (Abcam ab18465, 1:200) or rabbit anti-Cux1 (Santa Cruz SC-13024, 1:500) for 1–4 nights at 4 °C in 0.3% PBST and 5% donkey serum. All sections washed 3 × 10 min in PBS and also stained with NeuroTrace Blue (1:1,000) in 0.3% PBST for 2 h, followed by DAPI (1:10,000 of 5 mg ml^{-1}, Sigma-Aldrich) in PBS for 10–15 min, and then washed once more with PBS before mounting onto Superfrost Plus slides and coverslipping with Fluorogel (Electron Microscopy Sciences). The sections were imaged at 5× using a Leica Ariol Slide Scanner microscope with an SL200 slide loader, and scanner images were processed with custom software[56].

***In situ* hybridization.** *Gad1* and *Gad2 in situ* hybrization probes were prepared and hybridized in *SepW1*^{Cre} (L2/3), *Rbp4*^{Cre} (L5) and *Ntsr1*^{Cre} (L6) trans-synaptic tracing brains as previously described[56]. Five to seven starter cell containing sections were analyzed per brain. Images were collected on a Zeiss 780 confocal microscope.

Electrophysiology. At P28, the brain was removed and placed in ice-cold carbogenated slicing artificial cerebrospinal fluid (ACSF) that contains (in mM) 83 NaCl, 2.5 KCl, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 22 glucose, 72 sucrose, 0.5 $CaCl_2$, and 3.3 $MgSO_4$. We cut 300-µm coronal sections on a Leica vibratome. Slices were allowed to recover at 31 °C for 40 min and then at 23–25 °C for 30 min to 6 h. Slices were then placed in carbogenated recording ACSF (119 NaCl, 2.5 KCl, 26 $NaHCO_3$, 1 NaH_2PO_4, 1.5 $MgSO_4$, 2.5 $CaCl_2$, and 11 glucose, all in mM) that contained 50 µM picrotoxin (Sigma). For CRACM experiments, TTX (Tocris, 1 µM) and 4-AP (Sigma, 100 µM) were also included in the recording solution. Signals were recorded with a 5×gain, low-pass filtered at 2 kHz, digitized at 10 kHz (Molecular Devices Multiclamp 700B), and analyzed with pClamp 10 (Molecular Devices). Whole-cell recordings were made using 3–5 MΩ pipettes filled with an internal solution that contained (in mM) 123 cesium gluconate, 8 NaCl, 1 $CaCl_2$, 10 EGTA, 10 HEPES, and 10 glucose, pH 7.3 with CsOH, 280–290 mOsm. Series resistance (R_s) and input resistance (R_{in}) were monitored throughout the experiment by measuring the capacitive transient and steady-state deñection, respectively, in response to a −5-mV test pulse. Responses were evoked by stimulating the white matter with a platinum two-contact cluster electrode (FHC) 100–200 µm lateral to the recording site, or for CRACM experiments, by photostimulating ChR2^{+} axon terminals with an optical fiber coupled to a diode-pumped solid-state 473 laser positioned ~300 µm from the recording site. Cells were clamped at −60 mV to measure GluA (AMPA-receptor)-mediated excitatory postsynaptic currents (EPSC) and then at +40 mV to measure a compound EPSC that was quantified at 50 ms after stimulus onset, at which point the GluA-mediated currents had largely decayed. Analysis was based on the average of ten sweeps. mCherry- and tdTomato-positive L6 cells were visually identified based on red fluorescence (Olympus BX51WI).

Data analysis. Regions were identified based on DAPI and NeuroTrace signal with reference to an atlas[50]. Barrel cortex was identified based on the presence of L4 barrels visible with nuclear stains. $S1_{body}$ was adjacent to barrel cortex and had a thick L4, but lacked barrel structures. Similarly, S2 was identified adjacent and ventral to $S1_{body}$ based on the reference atlas. M1 and M2 were identified based on their location as defined by the reference atlas, and characterized by the disappearance of L4 and expansion of L5. M2 was flanked by the cingulate area on the medial side, and M1 was flanked by $S1_{body}$ on the lateral side[57]. Finally, thalamic nuclei were defined based on the reference atlas with aid from nuclear stains. Dorsal polymodal thalamus groups were defined based on the Allen Reference Atlas (Allen Institute for Brain Science, http://mouse.brain-map.org/).

Data was analyzed using custom MatLab software (code available from the authors on request). All GFP-labeled cells were clicked by the user. Section order was maintained during tissue processing and imaging, so each cell had coordinates specifying the anterior-posterior position within the brain, and the precise location within that section. For each section, the pial surface of the region of interest was outlined, and the lower boundary of each layer was identified based on differences in the density of DAPI staining. The distance-to-pia for every cell was calculated based on its coordinates, and layer identity was assigned by comparing this distance to the defined layer boundaries. To boost confidence in our layer-assignments, we stained BC and mPFC sections for neuronal markers for L2/3/4 (Cux1) and 5b/6 (Ctip2) and calculated layer boundaries based on marker

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expression patterns. In both areas, DAPI-based layer assignments were consistent with neuronal marker-based assignments (**Supplementary Figs. 1b** and **6a**).

To generate A-P heat maps, inputs were combined into 120-μm bins, and divided by the total cells in barrel cortex. Distributions were aligned between animals on the bin with the most starter cells. The M-L analysis was limited to the sections that contained 95% of the starter cells. The median center of mass for inputs in each layer was calculated within each section, and the center line was projected through the centers of mass. Distance-to-center was measured for every cell as the minimum distance to the center line, and given a sign to indicate medial or lateral direction from center. Distances were combined across sections within each animal. Middle cells were defined as those with distances-to-center within the range containing 95% of the starter cells. Side cells were those with distances-to-center outside this range. To generate the M-L heat maps, cells were combined into 120 μm bins centered on the center line, and combined across sections within each animal. Cell counts for each bin were divided by the total number of cells in the sections analyzed, and fractions for each bin were averaged across animals. A-P and M-L heat maps both show the average fraction of cells per bin across all animals. D-V heat maps for mPFC were generated identically to the M-L heat maps. Statistics and graphing were performed using Prism software (GraphPad). Data was analyzed blind to genotype. Brains with inefficient tracing (<100 inputs), or major tissue damage were excluded from analysis.

Statistical analyses. A supplementary methods checklist is available summarizing statistical tests and results. Data randomization was not applicable to our study, and no statistical methods were used to predetermine sample sizes but our sample sizes are similar to those reported in previous publications[18]. Data distributions were assumed to be normal but were not formally tested. When performing multiple *t* tests, statistical significance was determined using the Holm-Sidak method, with alpha = 5%. Each row was analyzed individually, without assuming a consistent s.d. All *t* tests were two-sided. For a complete list of statistical tests and *P* values, see **Supplementary Table 6**.

A **Supplementary Methods Checklist** is available.

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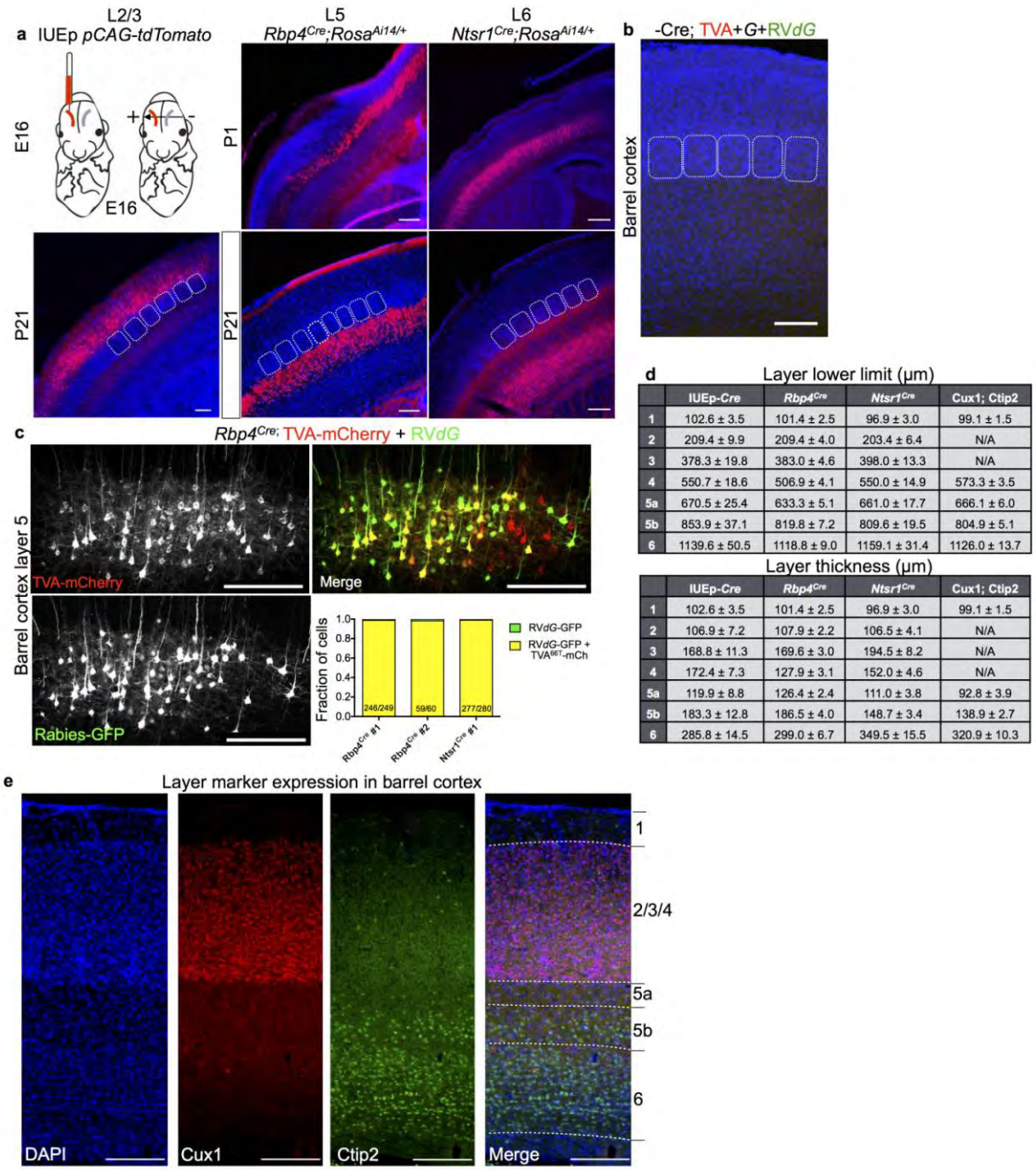


d

Layer lower limit (μm)

	IUEp-*Cre*	*Rbp4*^{Cre}	*Ntsr1*^{Cre}	Cux1; Ctip2
1	102.6 ± 3.5	101.4 ± 2.5	96.9 ± 3.0	99.1 ± 1.5
2	209.4 ± 9.9	209.4 ± 4.0	203.4 ± 6.4	N/A
3	378.3 ± 19.8	383.0 ± 4.6	398.0 ± 13.3	N/A
4	550.7 ± 18.6	506.9 ± 4.1	550.0 ± 14.9	573.3 ± 3.5
5a	670.5 ± 25.4	633.3 ± 5.1	661.0 ± 17.7	666.1 ± 6.0
5b	853.9 ± 37.1	819.8 ± 7.2	809.6 ± 19.5	804.9 ± 5.1
6	1139.6 ± 50.5	1118.8 ± 9.0	1159.1 ± 31.4	1126.0 ± 13.7

Layer thickness (μm)

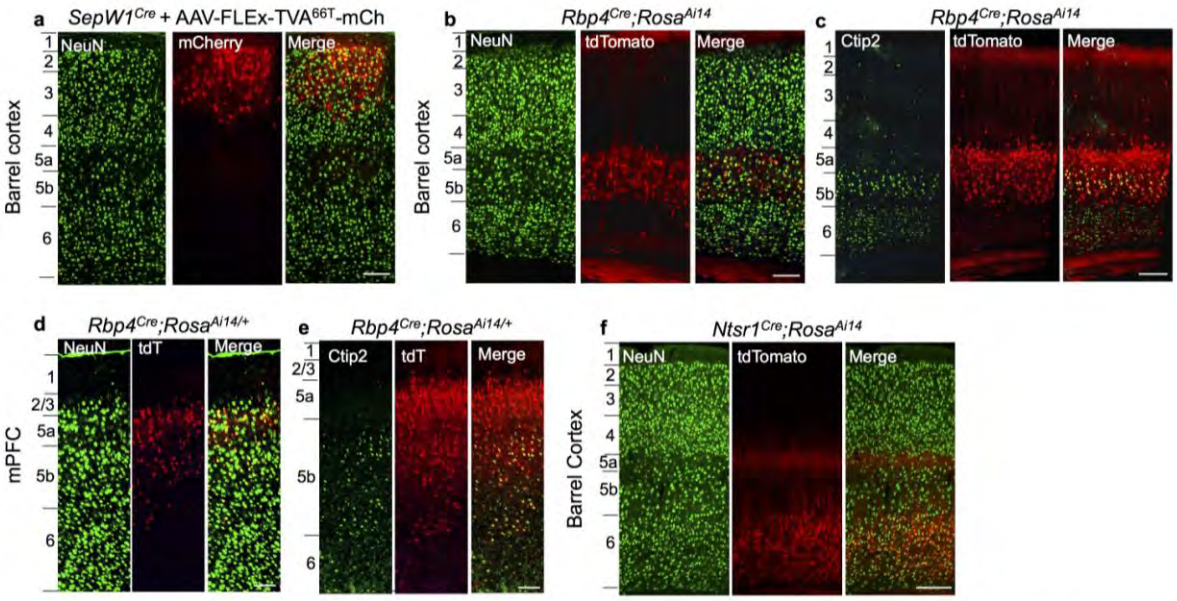
	IUEp-*Cre*	*Rbp4*^{Cre}	*Ntsr1*^{Cre}	Cux1; Ctip2
1	102.6 ± 3.5	101.4 ± 2.5	96.9 ± 3.0	99.1 ± 1.5
2	106.9 ± 7.2	107.9 ± 2.2	106.5 ± 4.1	N/A
3	168.8 ± 11.3	169.6 ± 3.0	194.5 ± 8.2	N/A
4	172.4 ± 7.3	127.9 ± 3.1	152.0 ± 4.6	N/A
5a	119.9 ± 8.8	126.4 ± 2.4	111.0 ± 3.8	92.8 ± 3.9
5b	183.3 ± 12.8	186.5 ± 4.0	148.7 ± 3.4	138.9 ± 2.7
6	285.8 ± 14.5	299.0 ± 6.7	349.5 ± 15.5	320.9 ± 10.3

Supplementary Figure 1

Controls for rabies tracing

(**a**) Characterization of reagents and procedures. L2/3 neurons were electroporated *in utero* with *pCAG-tdTomato* and Cre-driver lines were crossed to Ai14 mice. Images show that tdTomato expression in barrel cortex was restricted to the intended layers at P21, and both Cre lines were active at P1. Individual barrels in L4 at P21 are outlined with dotted lines. (**b**) We observed 0 mCherry-positive or GFP-positive cells when tracing from wild-type mice that did not express Cre (n = 3 mice) using the trans-synaptic tracing experimental conditions. (**c**) Example image from a control tracing experiment when AAV carrying CAG-FLEx-G was

omitted. Graphs show quantification of the number of red+green (yellow cells) divided by total green cells. **(d)** Tables list the lower limits (upper) and thicknesses (lower) of each layer for each set of experiments (IUEp-*Cre,* n = 6 mice; $Rbp4^{Cre}$, n = 9 mice; $Ntsr1^{Cre}$, n = 9 mice, layer markers, n = 6–8 sections from 2 mice). There were no significant differences between layer limits (Two-way ANOVA, F = 2.66, P = 0.07) or layer thicknesses (Two-way ANOVA, F = 1.39, P = 0.25). (**e**) Staining for layer markers Cux1 (L2-4) and Ctip2 (L5b/6) in barrel cortex. Scale bars represent 200 μm. Summary statistics presented as mean ± s.e.m. See **Supplementary Table 6** for exact test results and P values.



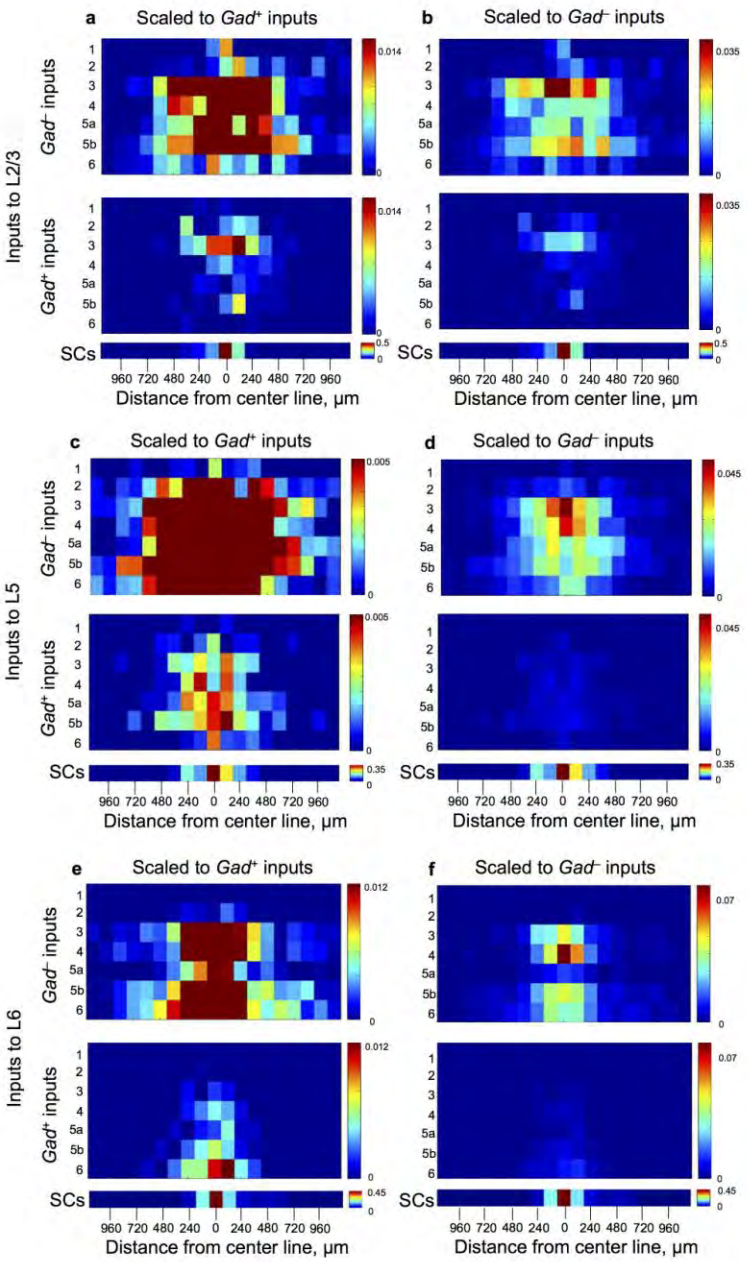
g

	Barrel cortex							mPFC		
	L2/3	L2	L3	L5	L5a	L5b	L6	L5	L5a	L5b
SepW1/NeuN	0.31±0.01	0.37±0.07	0.28±0.02							
Rbp4/NeuN				0.42±0.02	0.34±0.02	0.48±0.04		0.46±0.06	0.58±0.04	0.39±0.07
Rbp4/Ctip2				0.59±0.04		0.59±0.04		0.59±0.03		0.59±0.03
Ctip2/Rbp4				0.30±0.04		0.50±0.07		0.25±0.03		0.51±0.06
Ntsr1/NeuN							0.68±0.02			

Supplementary Figure 2

Characterization of Cre driver lines

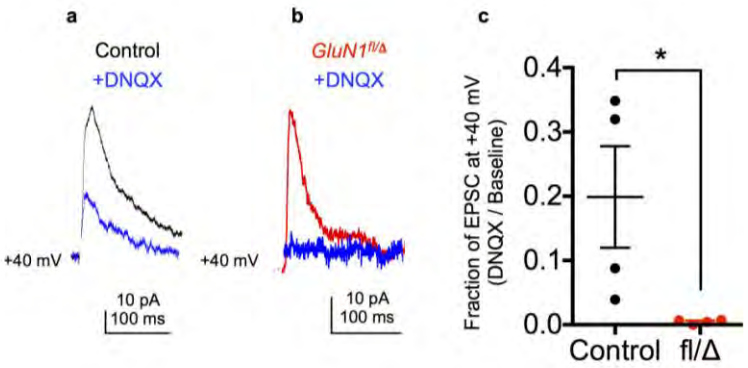
(**a**) Characterization of *SepW1*^{Cre}. *SepW1*^{Cre} mice were injected with AAV carrying CAG-FLEx-TC^{66T}-mCherry in barrel cortex at P25 and perfused at P40. Sections of barrel cortex were stained with antibodies against NeuN (*n* = 8 sections from 2 mice). (**b-e**) Characterization of *Rbp4*^{Cre}. Brain sections from P40 *Rbp4*^{Cre}*;Rosa*^{Ai14} mice were stained for NeuN or Ctip2. (**b**) NeuN staining in barrel cortex (*n* = 6 slices from 2 mice). (**c**) Ctip2 staining in barrel cortex (*n* = 12 slices from 2 mice). (**d**) NeuN staining in mPFC (*n* = 5 slices from 2 mice). (**e**) Ctip2 staining in mPFC (*n* = 5 slices from 2 mice). (**f**) Characterization of *Ntsr1*^{Cre}. Barrel cortex sections from P40 *Ntsr1*^{Cre}*;Rosa*^{Ai14} mice were stained for NeuN (*n* = 10 slices from 2 mice). (**g**) Quantification of marker colocalization. Summary statistics presented as mean ± s.e.m.



Supplementary Figure 3

Spatial analyses of *Gad*$^+$ and *Gad*$^-$ synaptic inputs from barrel cortex to starter cells in L2/3, L5, and L6 along the M-L Axis

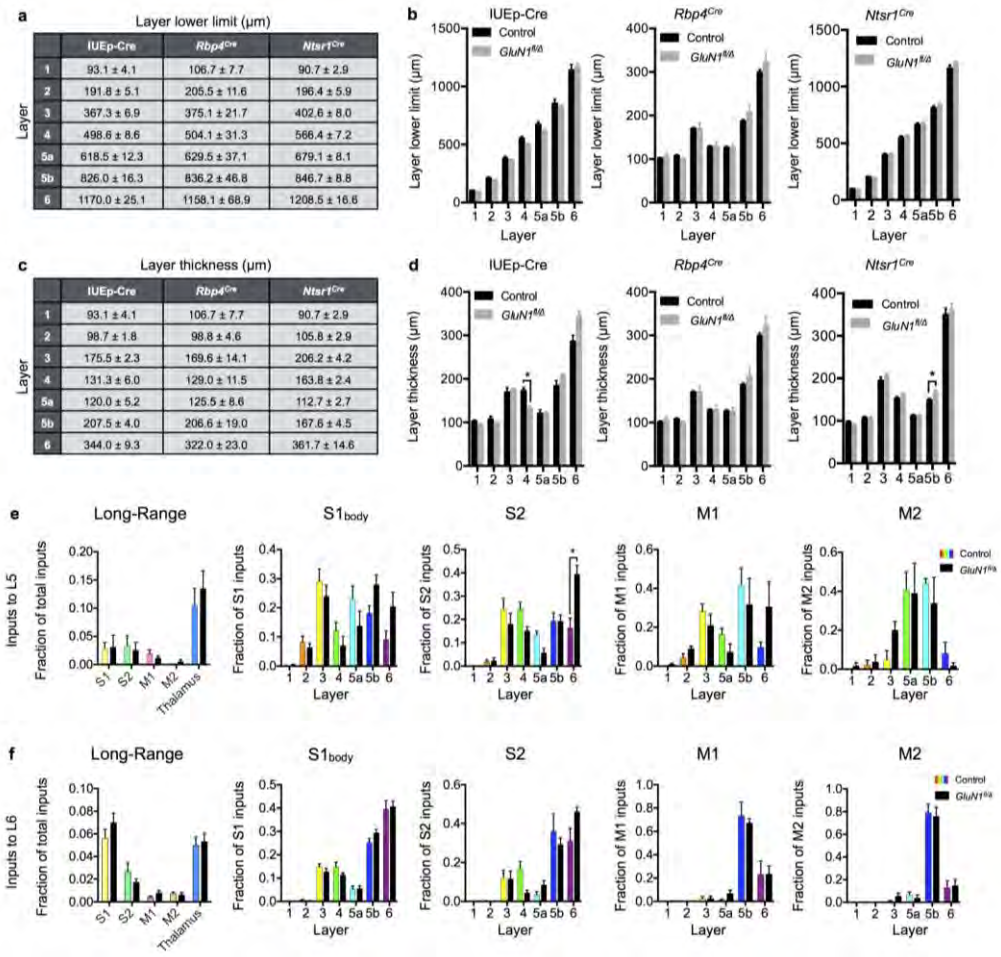
(**a-f**) Heat maps showing distribution of *Gad*$^-$ inputs (upper panels), *Gad*$^+$ inputs (middle panels) and starter cells (SCs, lower panels) in each layer along the M-L axis (n = 3 mice each). Inputs to L2/3 (**a**), L5 (**c**), and L6 (**e**) scaled to highest fraction of *Gad*$^+$ inputs. Inputs to L2/3 (**b**), L5 (**d**), and L6 (**f**) scaled to highest fraction of *Gad*$^-$ inputs. Colors represent fraction of barrel cortex inputs within the sections analyzed. Bin widths are 120 µm.



Supplementary Figure 4

Characterization of the EPSCs in control and GluN1-lacking neurons

(**a**) Example EPSCs recorded from a WT L6 neuron voltage-clamped at +40 mV while stimulating white matter. DNQX (blue trace) reduced the amplitude of the EPSC. (**b**) Example EPSCs recorded from a *GluN1*$^{fl/\Delta}$ L6 neuron voltage-clamped at +40 mV while stimulating white matter. DNQX (blue trace) completely abolished the EPSC. (**c**) Quantification of fraction of EPSC remaining after DNQX wash-in (Control: 0.20 ± 0.07, n = 4 cells, from 2 mice; *Grin1*$^{fl/\Delta}$: 0.005 ± 0.001, n = 4 cells from 2 mice; P = 0.04, Student's t-test). Summary statistics presented as mean s.e.m. See **Supplementary Table 6** for exact test results and P values.



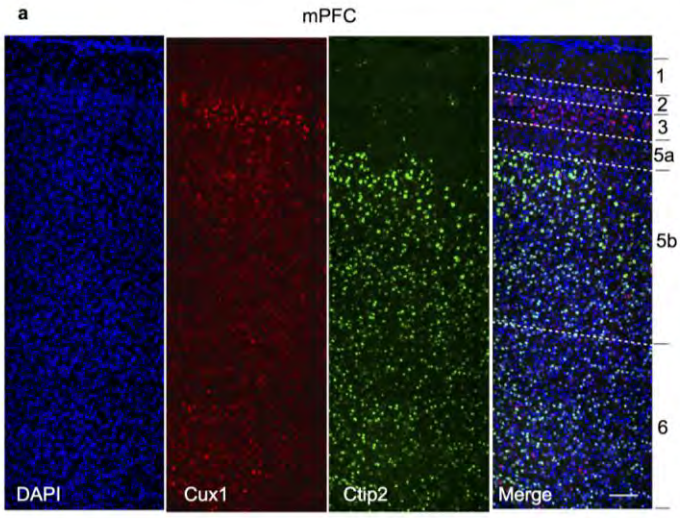
Layer	IUEp-Cre	*Rbp4*^{Cre}	*Ntsr1*^{Cre}
1	93.1 ± 4.1	106.7 ± 7.7	90.7 ± 2.9
2	191.8 ± 5.1	205.5 ± 11.6	196.4 ± 5.9
3	367.3 ± 6.9	375.1 ± 21.7	402.6 ± 8.0
4	498.6 ± 8.6	504.1 ± 31.3	566.4 ± 7.2
5a	618.5 ± 12.3	629.5 ± 37.1	679.1 ± 8.1
5b	826.0 ± 16.3	836.2 ± 46.8	846.7 ± 8.8
6	1170.0 ± 25.1	1158.1 ± 68.9	1208.5 ± 16.6

Layer	IUEp-Cre	*Rbp4*^{Cre}	*Ntsr1*^{Cre}
1	93.1 ± 4.1	106.7 ± 7.7	90.7 ± 2.9
2	98.7 ± 1.8	98.8 ± 4.6	105.8 ± 2.9
3	175.5 ± 2.3	169.6 ± 14.1	206.2 ± 4.2
4	131.3 ± 6.0	129.0 ± 11.5	163.8 ± 2.4
5a	120.0 ± 5.2	125.5 ± 8.6	112.7 ± 2.7
5b	207.5 ± 4.0	206.6 ± 19.0	167.6 ± 4.5
6	344.0 ± 9.3	322.0 ± 23.0	361.7 ± 14.6

Supplementary Figure 5

Layer assignments for tracing from starter cells lacking GluN1 and comparison of long-range inputs to control and GluN1-lacking starter cells

(**a**) Layer limits were assigned based on the DAPI signal for each section. Table lists the lower limits of each layer for each set of experiments (IUEp-*Cre*;*GluN1*$^{fl/\Delta}$, *n* = 4 mice; *Rbp4*^{Cre};*GluN1*$^{fl/\Delta}$, *n* = 6 mice; *Ntsr1*^{Cre};*GluN1*$^{fl/\Delta}$, *n* = 7 mice). (**b**) Comparison of layer limits between control and *GluN1*$^{fl/\Delta}$ animals. (**c**) Layer thicknesses derived from the limits based on DAPI signal within each section. Table lists the thickness of each layer for each set of experiments (IUEp-*Cre*;*GluN1*$^{fl/\Delta}$, *n* = 4 mice; *Rbp4*^{Cre};*GluN1*$^{fl/\Delta}$, *n* = 6 mice; *Ntsr1*^{Cre};*GluN1*$^{fl/\Delta}$, *n* = 7 mice). (**d**) Comparison of layer thickness between control and *GluN1*$^{fl/\Delta}$ animals. (**e**) Laminar analysis of long-range inputs from to control (colors) and GluN1-lacking (black) starter cells in L5 from S1 (control: *n* = 9 mice; *GluN1*$^{fl/\Delta}$: *n* = 4 mice), S2 (control: *n* = 5 mice; *GluN1*$^{fl/\Delta}$: *n* = 5 mice; L6 comparison, *P* = 0.004), M1 (control: *n* = 7 mice; *GluN1*$^{fl/\Delta}$: *n* = 4 mice), and M2 (control: *n* = 5 mice; *GluN1*$^{fl/\Delta}$: *n* = 3 mice). (**f**) Laminar analysis of long-range inputs from to control (colors) and GluN1-lacking (black) starter cells in L6 from S1 (control: *n* = 9 mice; *GluN1*$^{fl/\Delta}$: *n* = 7 mice), S2 (control: *n* = 9 mice; *GluN1*$^{fl/\Delta}$: *n* = 7 mice; L6 comparison, *P* = 0.003), M1 (control: *n* = 8 mice; *GluN1*$^{fl/\Delta}$: *n* = 7 mice), and M2 (control: *n* = 8 mice; *GluN1*$^{fl/\Delta}$: *n* = 6 mice). * denotes significant *P* values from multiple *t*-tests with Holm-Sidak correction for multiple comparisons (α=0.05). Summary statistics presented as mean ± s.e.m. See **Supplementary Table 6** for exact test results and *P* values.

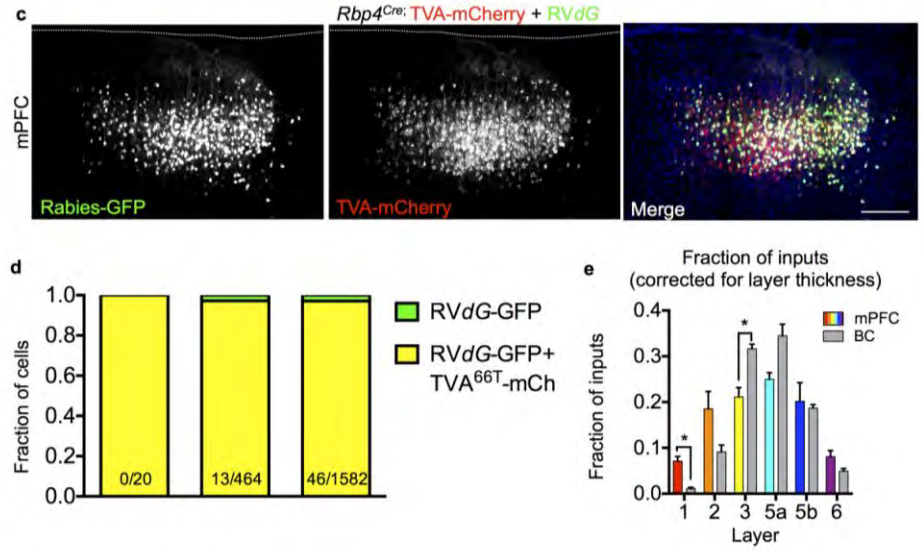


b Layer lower limit (μm)

	DAPI	Cux/Ctip2
1	124.1 ± 14.0	108.9 ± 5.5
2	187.6 ± 21.0	162.4 ± 6.2
3	271.3 ± 47.2	227.7 ± 3.9
5a	387.6 ± 53.9	349.8 ± 4.8
5b	625.0 ± 54.8	643.0 ± 10.9
6	908.2 ± 61.8	999.8 ± 21.9

Layer thickness (μm)

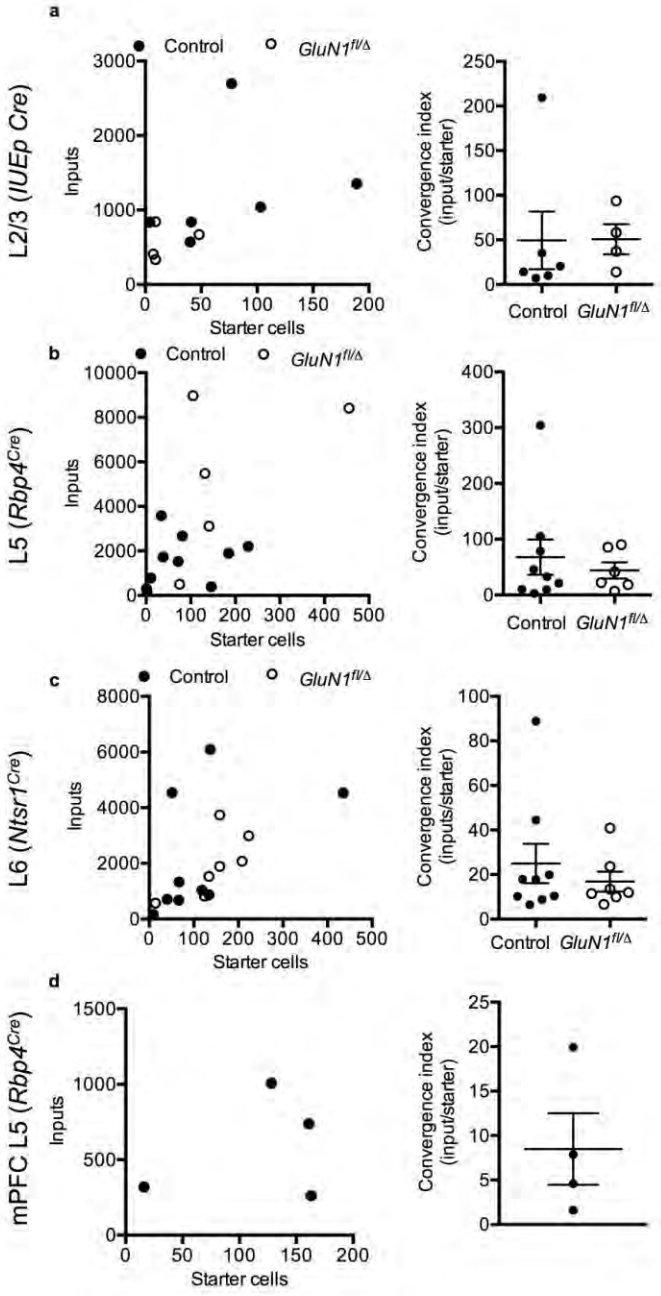
	DAPI	Cux/Ctip2
1	124.1 ± 14.0	108.9 ± 5.5
2	63.5 ± 16.8	53.6 ± 3.2
3	83.7 ± 31.4	65.3 ± 4.1
5a	116.3 ± 7.8	122.1 ± 3.7
5b	237.4 ± 42.8	293.3 ± 6.9
6	283.3 ± 32.3	356.8 ± 15.1



Supplementary Figure 6

Controls for layer-specific tracing to mPFC

(**a**) Example images showing staining for layer markers Cux1 (L3) and Ctip2 (L5b/6). Scale bar represents 100 μm. (**b**) Summary of layer lower limits (upper) assigned based on DAPI or layer markers and layer thickness (lower) assigned based on DAPI or layer markers. (**c**) Example images showing rabies tracing in mPFC without CAG-FLEx-G. Green rabies-infected neurons have almost 100% overlap with red *TVA^{66T}-mCherry*-positive neurons. (**d**) Results of control tracing experiments (n = 3 mice) without rabies glycoprotein, which mediates trans-synaptic spread. CAG-FLEx-TVA^{66T}-mCherry was injected into *Rbp4^{Cre}* animals in mPFC. Two weeks later, RV*dG*-EGFP was injected into the same location. Graphs show quantification of the sum of red+green (yellow) divided by the total green cells. (**e**) Comparison of layer distribution of total inputs to L5 of mPFC and barrel cortex, normalized by layer thickness for each respective area (mPFC: n = 4 mice; BC: n = 8 mice; L1: $P = 6.31\times10^{-6}$, L3: P = 0.0002). * denotes significant P values from multiple t-tests with Holm-Sidak correction for multiple comparisons (α=0.05). Summary statistics presented as mean ± s.e.m. See **Supplementary Table 6** for exact test results and P values.



Supplementary Figure 7

Raw numbers of inputs and starter cells for tracing in Barrel Cortex and mPFC

Plots of raw numbers of inputs vs. starter cells (left) and convergence index (# inputs cells / # starter cells) (right) for barrel cortex (**a**) L2/3, (**b**) L5, and (**c**) L6, and (**d**) mPFC L5 tracing experiments. Summary statistics presented as mean±SEM.

	L2/3 (n=6)		L5 (n=9)		L6 (n=9)	
Layer	Fraction of SCs (mean±SEM)	Fraction of Inputs (mean±SEM)	Fraction of SCs (mean±SEM)	Fraction of Inputs (mean±SEM)	Fraction of SCs (mean±SEM)	Fraction of Inputs (mean±SEM)
1	0.012±0.01	0.013±0.003	0.001±0.001	0.006±0.001	0.000±0.000	0.001±0.0005
2	0.723±0.06	0.066±0.009	0.004±0.002	0.055±0.009	0.000±0.000	0.017±0.005
3	0.264±0.07	0.278±0.014	0.001±0.005	0.288±0.007	0.000±0.000	0.218±0.020
4	0.000±0.00	0.226±0.019	0.018±0.006	0.154±0.015	0.000±0.000	0.154±0.012
5a	0.000±0.00	0.265±0.018	0.570±0.085	0.232±0.014	0.000±0.000	0.086±0.005
5b	0.000±0.00	0.143±0.012	0.398±0.086	0.187±0.008	0.043±0.011	0.219±0.014
6	0.000±0.00	0.009±0.002	0.000±0.00	0.077±0.008	0.957±0.011	0.305±0.012

Supplementary Table 1. Quantification of layer-specific input tracing in barrel cortex. Tabulated are fraction of starter cells (SCs) and fraction of inputs to starter cells in barrel cortex L2/3, L5 and L6.

		Starter cell populations		
		L2/3 vs L5	L2/3 vs L6	L5 vs L6
Layer	1	0.06	6.2x10^–4	0.004
	2	0.44	2.8x10^–4	0.003
	3	0.52	0.04	0.004
	4	0.01	0.004	0.997
	5a	0.17	3.8x10^–8	4.8x10^–8
	5b	0.01	2.2x10^–3	0.08
	6	1.7x10^–5	4.1x10^–11	3.5x10^–11

Supplementary Table 2. Uncorrected p-values for multiple t-tests.
Uncorrected p-values from multiple t-tests comparing input distributions for different starter cell populations. Red shading indicates p-values that were significant after Holm-Sidak correction for multiple comparisons (α=0.05).

a	L2/3 (n=3)			
Layer	Fraction of SCs (mean±SEM)	Fraction of Total Inputs (Gad^+ & Gad^-) (mean±SEM)	Gad^+ Fraction of *Gad*+ Inputs (mean±SEM)	Gad^+/Total Inputs in Layer (mean±SEM)
1	0.032±0.03	0.001±0.001	0.007±0.008	1.000±0.000
2	0.443±0.06	0.102±0.049	0.167±0.036	0.274±0.113
3	0.522±0.10	0.282±0.044	0.352±0.118	0.163±0.061
4	0.004±0.003	0.167±0.012	0.129±0.042	0.086±0.025
5a	0.000±0.00	0.215±0.020	0.176±0.041	0.097±0.022
5b	0.000±0.00	0.195±0.079	0.153±0.070	0.104±0.039
6	0.000±0.00	0.038±0.019	0.017±0.008	0.063±0.053

b	L5 (n=3)			
Layer	Fraction of SCs (mean±SEM)	Fraction of Total Inputs (Gad^+ & Gad^-) (mean±SEM)	Gad^+ Fraction of Gad^+ Inputs (mean±SEM)	Gad^+/Total Inputs in Layer (mean±SEM)
1	0.000±0.000	0.001±0.0003	0.006±0.003	1.000±0.000
2	0.000±0.000	0.065±0.014	0.052±0.009	0.072±0.006
3	0.000±0.000	0.237±0.051	0.188±0.044	0.072±0.014
4	0.019±0.019	0.184±0.020	0.153±0.002	0.075±0.002
5a	0.713±0.129	0.186±0.017	0.227±0.021	0.108±0.007
5b	0.243±0.228	0.205±0.043	0.277±0.031	0.128±0.024
6	0.026±0.026	0.123±0.020	0.097±0.034	0.067±0.018

c	L6 (n=3)			
Layer	Fraction of SCs (mean±SEM)	Fraction of Total Inputs (Gad^+ & Gad^-) (mean±SEM)	Gad^+ Fraction of Gad^+ Inputs (mean±SEM)	Gad^+/Total Inputs in Layer (mean±SEM)
1	0.000±0.00	0.000±0.000	0.000±0.000	
2	0.000±0.00	0.006±0.004	0.007±0.007	0.024±0.024
3	0.000±0.00	0.185±0.019	0.072±0.034	0.024±0.006
4	0.000±0.00	0.260±0.036	0.156±0.070	0.064±0.017
5a	0.007±0.007	0.065±0.007	0.106±0.017	0.153±0.058
5b	0.093±0.043	0.237±0.016	0.204±0.014	0.821±0.028
6	0.900±0.048	0.246±0.032	0.455±0.048	0.178±0.060

Supplementary Table 3. Quantification of local inhibitory inputs to starter cells in barrel cortex L2/3, L5 and L6. Tabulated are fraction of starter cells (SCs), fraction of total inputs, Gad^+ fraction of total Gad^+ inputs, and Gad^+/total inputs per layer for SCs in **(a)** L2/3, **(b)** L5, and **(c)** L6.

	L2/3 (n=4)		L5 (n=6)		L6 (n=7)	
Layer	Fraction of SCs (mean±SEM)	Fraction of Inputs (mean±SEM)	Fraction of SCs (mean±SEM)	Fraction of Inputs (mean±SEM)	Fraction of SCs (mean±SEM)	Fraction of Inputs (mean±SEM)
1	0.015±0.016	0.007±0.002	0.000±0.00	0.003±0.002	0.000±0.00	0.001±0.001
2	0.635±0.124	0.071±0.015	0.000±0.00	0.056±0.017	0.000±0.00	0.009±0.001
3	0.349±0.121	0.254±0.029	0.000±0.00	0.262±0.021	0.000±0.00	0.242±0.015
4	0.000±0.00	0.207±0.017	0.014±0.006	0.154±0.018	0.000±0.00	0.142±0.013
5a	0.000±0.00	0.296±0.015	0.821±0.052	0.230±0.034	0.000±0.00	0.100±0.007
5b	0.000±0.00	0.126±0.026	0.165±0.047	0.164±0.017	0.091±0.024	0.261±0.016
6	0.000±0.00	0.038±0.023	0.000±0.00	0.130±0.022	0.909±0.024	0.244±0.009

Supplementary Table 4. Quantification of layer-specific input tracing from starter cells lacking GluN1 in barrel cortex. Tabulated are fraction of starter cells (SCs) and fraction of inputs to starter cells lacking GluN1 in barrel cortex L2/3, L5 and L6.

	L5 (n=4)		L5 *Gad* (n=3)		
Layer	Fraction of SCs (mean±SEM)	Fraction of Inputs (mean±SEM)	Fraction of SCs (mean±SEM)	*Gad*$^+$ Fraction of *Gad*$^+$ Inputs (mean±SEM)	*Gad*$^+$/Total Inputs in Layer (mean±SEM)
1	0.006±0.003	0.070±0.009	0.000±0.000	0.050±0.041	0.184±0.098
2	0.004±0.004	0.073±0.012	0.017±0.017	0.046±0.004	0.228±0.059
3	0.020±0.017	0.128±0.011	0.150±0.063	0.097±0.016	0.233±0.095
5a	0.643±0.117	0.225±0.022	0.597±0.050	0.184±0.027	0.234±0.045
5b	0.321±0.100	0.334±0.036	0.236±0.061	0.399±0.102	0.275±0.086
6	0.004±0.004	0.170±0.032	0.000±0.000	0.238±0.072	0.230±0.045

Supplementary Table 5. Quantification of layer-specific input tracing from mPFC L5. Tabulated are fraction of starter cells (SCs) and fraction of total mPFC inputs (left), and fraction of SCs, *Gad*$^+$ fraction of total *Gad*$^+$ inputs, and *Gad*$^+$/total inputs per layer (right).

Figure Panel	Comparison	t/F value and dF	p-value
1g	L5 vs L6 amplitude	t(10) = 3.554	0.0052
1h	baseline vs DNQX	t(5) = 3.992	0.01
2c	L1 - L1	t(10) = 0.03	0.98
	L2 - L2	t(10)=0.91	0.39
	L3 - L3	t(10)=0.07	0.94
	L4 - L4	t(10)=3.82	0.003
	L5a - L5a	t(10)=2.56	0.028
	L5b - L5b	t(10)=1.68	0.12
	L6 - L6	t(10)=0.27	0.79
2d	L1 - L1	t(16) = 0.16	0.87
	L2 - L2	t(16)= 0.42	0.68
	L3 - L3	t(16)= 1.00	0.33
	L4 - L4	t(16)= 0.87	0.39
	L5a - L5a	t(16)= 0.52	0.61
	L5b - L5b	t(16)= 0.40	0.69
	L6 - L6	t(16)= 0.08	0.93
2e	L1 - L1	t(16) = 1.11	0.28
	L2 - L2	t(16)=0.43	0.67
	L3 - L3	t(16)=1.10	0.29
	L4 - L4	t(16)=3.56	0.003
	L5a - L5a	t(16)=1.46	0.16
	L5b - L5b	t(16)= 2.03	0.06
	L6 - L6	t(16)=2.73	0.01
2f	L1 - L1	t(8) = 0.31	0.76
	L2 - L2	t(8)=0.40	0.7
	L3 - L3	t(8)=0.87	0.41
	L4 - L4	t(8)=1.44	0.19
	L5a - L5a	t(8)=0.72	0.49
	L5b - L5b	t(8)=0.79	0.45
	L6 - L6	t(8)=2.92	0.02
2g	L1 - L1	t(10) = 1.06	0.31
	L2 - L2	t(10)=1.26	0.23
	L3 - L3	t(10)=0.84	0.42
	L4 - L4	t(10)=3.43	0.006
	L5a - L5a	t(10)=2.08	0.06
	L5b - L5b	t(10)=0.56	0.58
	L6 - L6	t(10)=1.09	0.3
2h	L1 - L1	t(16) = 1.45	0.17
	L2 - L2	t(16)=1.64	0.12
	L3 - L3	t(16)=0.95	0.35
	L4 - L4	t(16)=5.06	0.0001

	L5a - L5a	t(16)=1.46	0.16
	L5b - L5b	t(16)=1.53	0.14
	L6 - L6	t(16)=2.47	0.025
5c	fl/+ vs fl/D	t(12)=3.90	0.002
5g	L1 - L1	t(8) = 1.21	0.26
	L2 - L2	t(8)=0.31	0.76
	L3 - L3	t(8)=0.81	0.44
	L4 - L4	t(8)=0.69	0.51
	L5a - L5a	t(8)=1.21	0.26
	L5b - L5b	t(8)=0.62	0.55
	L6 - L6	t(8)=1.57	0.16
5j	L1 - L1	t(13) = 1.15	0.27
	L2 - L2	t(13)=0.04	0.97
	L3 - L3	t(13)=1.18	0.26
	L4 - L4	t(13)=0.09	0.93
	L5a - L5a	t(13)=0.64	0.53
	L5b - L5b	t(13)=1.20	0.25
	L6 - L6	t(13)=3.25	0.006
5m	L1 - L1	t(14)=0.16	0.88
	L2 - L2	t(14)=1.25	0.23
	L3 - L3	t(14)=0.95	0.36
	L4 - L4	t(14)=0.69	0.5
	L5a-L5a	t(14)=1.56	0.14
	L5b - L5b	t(14)=1.99	0.07
	L6 - L6	t(14)=3.87	0.002
6c	L1 - L1	t(11) = 10.9	3.03×10^{-7}
	L2 - L2	t(11)=0.76	0.46
	L3 - L3	t(11)=8.83	2.53×10^{-6}
	L5a - L5a	t(11)=1.40	0.19
	L5b - L5b	t(11)=5.64	0.0002
	L6 - L6	t(11)=3.96	0.002
6d	L1 - L1	t(6) = 3.79	0.009
	L2 - L2	t(6)= 2.00	0.09
	L3 - L3	t(6)= 1.04	0.34
	L5a - L5a	t(6)= 0.76	0.48
	L5b - L5b	t(6)= 2.47	0.048
	L6 - L6	t(6)= 3.46	0.01
6e	L1 - L1	t(6) = 0.83	0.44
	L2 - L2	t(6)= 2.14	0.08
	L3 - L3	t(6)= 0.88	0.41
	L5a - L5a	t(6)= 1.63	0.15
	L5b - L5b	t(6)= 2.18	0.07

	L6 - L6	t(6)= 0.86	0.42
6j	BC vs mPFC	t(4) = 3.26	0.03
6k	BC vs mPFC	t(10) = 6.73	5.14×10^{-5}
6l	BC vs mPFC: 0	t(10) = 1.70	0.12
	120	t(10)=2.81	0.02
	240	t(10)=4.66	0.0009
	360	t(10)=5.01	0.0005
	480	t(10)=2.62	0.03
	600	t(10)=2.18	0.05
	720	t(10)=1.94	0.08
	840	t(10)=2.62	0.22
	960	t(10)=2.18	0.55
	1,080	t(10)=1.94	0.25
6m	BC vs mPFC: 0	t(9) = 2.63	0.03
	120	t(9)=3.85	0.004
	240	t(9)=5.02	0.0007
	360	t(9)=3.05	0.01
	480	t(9)=2.51	0.03
	600	t(9)=2.31	0.05
	720	t(9)=1.76	0.11
	840	t(9)=1.06	0.32
	960	t(9)=1.16	0.28
	1,080	t(9)=0.98	0.35
6n	BC vs mPFC	t(11) = 0.55	0.59
Results pg.5 ¶ 1	L1 - L1	t(7) = 2.44	0.04
IUEp vs	L2 - L2	t(7)=1.04	0.33
SepWI-Cre	L3 - L3	t(7)=0.11	0.91
	L4 - L4	t(7)=2.04	0.08
	L5a - L5a	t(7)=1.74	0.12
	L5b - L5b	t(7)=0.95	0.37
	L6 - L6	t(7)=2.30	0.06
Supp. 1d top	Layer	F(6, 147) = 1339	<0.0001
	Cre line	F(2, 147) = 2.66	0.07
	interaction	F(12, 147) = 0.80	0.65
Supp. 1d	Layer	F(6, 147) = 321.6	<0.0001
bottom	Cre line	F(2, 147) = 1.39	0.25
	interaction	F(12, 147) = 8.16	<0.0001
Supp table 3.	L1 - L1	t(13) = 2.03	0.06
column 1	L2 - L2	t(13) = 0.80	0.44
L2/3 vs L5	L3 - L3	t(13) = 0.66	0.52
	L4 - L4	t(13) = 2.99	0.01

	L5a - L5a	t(13) = 1.47	0.17
	L5b - L5b	t(13) = 3.01	0.01
	L6 - L6	t(13) = 6.62	$1.7x10^{-5}$
Supp table 3.	L1 - L1	t(13) = 4.47	$6.2x10^{-4}$
column 2	L2 - L2	t(13) = 4.92	$2.8x10^{-4}$
L2/3 vs L6	L3 - L3	t(13) = 2.24	0.04
	L4 - L4	t(13) = 3.42	0.004
	L5a - L5a	t(13) = 11.42	$3.8x10^{-8}$
	L5b - L5b	t(13) = 3.80	$2.2x10^{-3}$
	L6 - L6	t(13) = 19.89	$4.1x10^{-11}$
Supp table 3.	L1 - L1	t(16) = 3.32	0.004
Column 3	L2 - L2	t(16) = 3.43	0.003
L5 vs L6	L3 - L3	t(16) = 3.34	0.004
	L4 - L4	t(16) = 0.003	0.997
	L5a - L5a	t(16) = 9.61	$4.8x10^{-8}$
	L5b - L5b	t(16) = 1.90	0.08
	L6 - L6	t(16) = 15.79	$3.5x10^{-11}$
Supp. 4c	control vs fl/D	t(6) = 2.46	0.04
Supp. 5b	L1 - L1	t(8) = 1.74	0.12
left	L2 - L2	t(8)=1.36	0.21
	L3 - L3	t(8)=0.43	0.68
	L4 - L4	t(8)=2.15	0.064
	L5a - L5a	t(8)=1.57	0.16
	L5b - L5b	t(8)=0.58	0.58
	L6 - L6	t(8)=0.46	0.66
Supp. 5b	L1 - L1	t(13) = 0.80	0.43
middle	L2 - L2	t(13) = 0.39	0.7
	L3 - L3	t(13) = 0.23	0.82
	L4 - L4	t(13) = 0.12	0.9
	L5a - L5a	t(13) = 0.14	0.89
	L5b - L5b	t(13) = 0.47	0.65
	L6 - L6	t(13) = 0.77	0.46
Supp. 5b	L1 - L1	t(14)=1.48	0.16
right	L2 - L2	t(14)=0.78	0.45
	L3 - L3	t(14)=0.28	0.78
	L4 - L4	t(14)=0.90	0.38
	L5a - L5a	t(14)=0.84	0.41
	L5b - L5b	t(14)=1.57	0.14
	L6 - L6	t(14)=1.27	0.22
Supp. 5d	L1 - L1	t(8) = 1.73	0.12
left	L2 - L2	t(8)=0.88	0.4
	L3 - L3	t(8)=0.47	0.65

	L4 - L4	t(8)=3.79	0.005
	L5a - L5a	t(8)=0.008	0.99
	L5b - L5b	t(8)=1.48	0.18
	L6 - L6	t(8)=2.97	0.017
Supp. 5d	L1 - L1	t(13) = 0.80	0.44
middle	L2 - L2	t(13) = 2.02	0.066
	L3 - L3	t(13) = 0.004	1
	L4 - L4	t(13) = 0.11	0.91
	L5a - L5a	t(13) = 0.13	0.9
	L5b - L5b	t(13) = 1.36	0.2
	L6 - L6	t(13) = 1.21	0.25
Supp. 5d	L1 - L1	t(14)=1.48	0.16
right	L2 - L2	t(14)=0.11	0.91
	L3 - L3	t(14)=1.16	0.26
	L4 - L4	t(14)=2.08	0.06
	L5a - L5a	t(14)=0.35	0.73
	L5b - L5b	t(14)=3.42	0.004
	L6 - L6	t(14)=0.56	0.58
Supp. 5e	S1-S1	t(12) = 0.13	0.89
long range	S2-S2	t(12)=0.31	0.76
	M1-M1	t(12)=0.97	0.34
	M2-M2	t(12)=1.68	0.12
	Thal.-Thal.	t(12)=0.64	0.54
Supp. 5e S1	L1 - L1	t(11) = 1.06	0.31
	L2 - L2	t(11) = 0.56	0.59
	L3 - L3	t(11) = 0.74	0.47
	L4 - L4	t(11) = 1.10	0.29
	L5a - L5a	t(11) = 1.19	0.26
	L5b - L5b	t(11) = 2.15	0.05
	L6 - L6	t(11) = 2.08	0.06
Supp. 5e S2	L1 - L1	t(8) = 0.57	0.58
	L2 - L2	t(8)=0.27	0.79
	L3 - L3	t(8)=1.00	0.34
	L4 - L4	t(8)=2.81	0.02
	L5a - L5a	t(8)=3.16	0.01
	L5b - L5b	t(8)=0.003	0.99
	L6 - L6	t(8)=4.05	0.004
Supp. 5e M1	L1 - L1	t(9) = 2.02	0.07
	L2 - L2	t(9)=1.57	0.15
	L3 - L3	t(9)=1.07	0.31
	L5a - L5a	t(9)=1.72	0.12
	L5b - L5b	t(9)=0.65	0.53

	L6 - L6	t(9)=2.02	0.07
Supp. 5e M2	L1 - L1	t(6) = 1.37	0.22
	L2 - L2	t(6)=0.35	0.74
	L3 - L3	t(6)=2.14	0.08
	L5a - L5a	t(6)=0.11	0.92
	L5b - L5b	t(6)=0.98	0.37
	L6 - L6	t(6)=0.85	0.43
Supp. 5fe	S1-S1	t(14)=1.17	0.26
long rang	S2-S2	t(14)=1.02	0.32
	M1-M1	t(14)=1.76	0.1
	M2-M2	t(14)=0.50	0.62
	Thal.-Thal.	t(14)=0.31	0.76
Supp. 5f S1	L2 - L2	t(14)=0.79	0.44
	L3 - L3	t(14)=1.02	0.33
	L4 - L4	t(14)=1.32	0.21
	L5a - L5a	t(14)=0.11	0.91
	L5b - L5b	t(14)=1.87	0.08
	L6 - L6	t(14)=0.25	0.8
Supp. 5f S2	L1 - L1	t(14)=0.875	0.39
	L2 - L2	t(14)=0.875	0.39
	L3 - L3	t(14)=0.11	0.91
	L4 - L4	t(14)=2.69	0.02
	L5a - L5a	t(14)=1.88	0.08
	L5b - L5b	t(14)=0.64	0.53
	L6 - L6	t(14)=1.96	0.07
Supp. 5f M1	L3 - L3	t(13)=0.02	0.99
	L5a - L5a	t(13)=2.01	0.07
	L5b - L5b	t(13)=0.46	0.65
	L6 - L6	t(13)=0.02	0.98
Supp. 5f M2	L2 - L2	t(12)= 0.86	0.41
	L3 - L3	t(12) = 1.86	0.09
	L5a - L5a	t(12) = 0.78	0.45
	L5b - L5b	t(12) = 0.27	0.79
	L6 - L6	t(12)=0.21	0.84
Supp. 6e	L1 - L1	t(11) = 8.03	6.31×10^{-6}
	L2 - L2	t(11) = 2.82	0.02
	L3 - L3	t(11) = 5.26	0.0003
	L5a - L5a	t(11) = 2.41	0.034
	L5b - L5b	t(11) = 0.54	0.6
	L6 - L6	t(11) = 2.62	0.02

Supplementary Table 6. List of test statistics and p values