**Fig. 1. Patch-seq mapping reveals transcriptomic and morpho-electric diversity of excitatory neurons in monkey DLPFC layers 4–6.**

**(a)** NeuN staining reveals the cortical layering in the monkey dorsolateral prefrontal cortex (DLPFC), with layers labeled from L1 to L6.

**(b)** Patch-seq mapping demonstrates comprehensive coverage of all excitatory neuron populations across cortical layers, including L2/3 IT, L4 IT, L5 IT1, L5 IT2, L5 ET, L6 IT1, L6 IT2, L6 CT, and L6b.

**(c** Distribution of neurons mapped to their corresponding cortical layers, showing the fraction of observed versus expected sampling across transcriptomic types.

**(d)** Normalized cortical depth of patch-seq-mapped neurons across different transcriptomic types across cortical layers.

**(e)** Representative morphologies and electrophysiological firing patterns of transcriptomically identified cell clusters in L4-6. Layer 4 includes stellate cells, Layer 5 consists of three subgroups, and Layer 6 comprises four subgroups.

**(f)** Dot plot visualization of marker gene expression across transcriptomic clusters identified in L4-6, highlighting the molecular diversity within these excitatory neuron populations.

**Fig. S1. Electrophysiological and morphological classification of L5 and L6 neurons with FISH validation.**

**(a)** UMAP plot of Layer 5 neurons based on electrophysiological features, showing three distinct clusters. L5 IT1 (n=69), L5 IT2 (n=60), L5 ET (n=11).

**(b)** Morphological classification of Layer 5 neurons using dendritic branch points, tips, and tuft endings. L5 IT1 (n=27), L5 IT2 (n=24), L5 ET (n=6).

**(c)** UMAP plot of Layer 6 neurons based on electrophysiology, revealing four distinct clusters. L6 IT1 (n=20), L6 IT2 (n=7), L6 CT (n=18), L6b (n=13).

**(d)** Morphological classification of Layer 6 neurons based on dendritic branch points, apical dendrites, and asymmetry. L6 IT1 (n=8), L6 IT2 (n=4), L6 CT (n=14), L6b (n=6).

**(e-f)** K-means clustering of Layer 5 and Layer 6 neurons integrating electrophysiological and morphological data.

**(g)** Fluorescence in situ hybridization (FISH) validation of marker genes across layer 4 to layer 6.

**Fig. 2. Diversity of L2/3 excitatory neurons in the monkey DLPFC.**

**(a)** UMAP plots derived from transcriptomic data (n = 262) based on the projection of principal components, showing the distribution of the five identified clusters: *C1QL2*, *RHAG*, *CD53*, *SPON2*, and *FRMPD2*.

**(b)** UMAP plots based on the projection of principal components derived from electrophysiological and morphological data (n = 134) that reveal the distribution of the same clusters.

**(c)** Anatomical distribution of the five Patch-seq clusters within cortical layers, overlaid on NeuN staining as a reference to show localization in the DLPFC.

**(d)** Dot plot visualization of marker gene expression across transcriptomic clusters identified in L2/3. The expression of marker genes is shown for each of the five transcriptomic clusters.

**(e)** Fluorescence in situ hybridization (FISH) validates the identified marker genes in five L2/3 clusters.

**(f)** Confusion matrices illustrating the classification of cells into the five transcriptomic clusters using a random forest classifier with three feature sets: transcriptomics, electrophysiology, and morphology. Each row of the matrix indicates the fraction of cells from a given family classified into the five families, with each row summing to 100%.

**Fig. S2. Electrophysiological and morphological properties of L2/3 IT.**

**(a-e)** Electrophysiological recordings and morphological reconstructions display the firing patterns and structural features of five transcriptome clusters in L2/3 neurons: *C1QL2* (a), *RHAG* (b), *CD53* (c), *SPON2* (d), and *FRMPD2* (e)

**Fig. 3. Diversity of interneurons in the monkey DLPFC.**

**(a)** Patch-seq mapping of interneuron populations in the DLPFC onto transcriptomically defined clusters, revealing distinct interneuron subtypes, including *PAX6*, L1 IN, *VIP*, *LAMP5*, *LAMP5 LHX6*, *PVALB*, *ChC*, *SST*, and *SST CHODL*.

**(b)** UMAP plots showing interneuron clustering based on principal components derived from electrophysiological data (n = 291).

**(c)** Violin plots depicting the normalized cortical depth of patch-seq-mapped neurons across different transcriptomic interneuron subtypes.

**(d)** Electrophysiological properties and morphological features of CGE-derived transcriptomic interneurons.

**(e)** Electrophysiological and morphological characteristics of MGE-derived transcriptomic interneurons.

**(f)** Dot plot visualization of marker gene expression across transcriptomic interneuron clusters.

**Fig. S3.** **Electrophysiological and morphological properties of interneurons.**

**(a, b)** Box plots illustrate the distribution of selected electrophysiological (a) and morphological (b) features across different interneuron transcriptomic types (t-types). Individual data points are overlaid as scatter plots, and horizontal bars above indicate statistically significant pairwise comparisons (P < 0.05, FDR-corrected Mann–Whitney U-test). Each box plot represents the median (center line), interquartile range (box edges), and whiskers extending up to 1.5× the interquartile range.

**(c)** Random forest classifier performance for interneuron classification based on different feature sets. The left panel shows a classifier trained on electrophysiological features, which demonstrates strong predictive performance. The middle panel presents a classifier trained on morphological features, also achieving high accuracy. The right panel combines both electrophysiological and morphological features, resulting in perfect classification (100% accuracy), highlighting the importance of morphology in distinguishing interneuron subtypes.

**Fig. 4. Disynaptic inhibition in the monkey DLPFC.**

**(a)** Schematic representation of connections among six simultaneously recorded neurons, including three L2/3 excitatory (L2/3 Exc) neurons, one L2/3 interneuron, one L5 excitatory (L5 Exc) neuron, and one L5 interneuron.

**(b)** Connection diagram with reconstructed morphologies of the six neurons.

**(c)** Action potentials (APs) elicited in presynaptic neurons and the corresponding inhibitory postsynaptic potentials (IPSPs) recorded in postsynaptic neurons for each identified connection. Disynaptic inhibition, induced by presynaptic excitatory neurons, shows longer latency and greater amplitude compared to monosynaptic inhibition.

**(d)** Comparison of uIPSP latencies (n = 10) and uIPSP amplitudes (n = 16) between monosynaptic and disynaptic inhibition. Statistical analysis: Student’s t-test (p < 0.01).

**(e)** Comparison of uIPSP amplitudes in monosynaptic (n = 16) and disynaptic inhibition (n = 5) before and after CNQX (5 μM) application. Statistical analysis: Student’s t-test (p < 0.01).

**(f)** Connectivity probability of disynaptic inhibition triggered by superficial L2/3 excitatory neurons across layers and cell types: L1 (3/75), L2/3 Exc (13/79), L2/3 interneuron (14/83), L4 Exc (14/81), L4 interneuron (11/69), L5 Exc (11/72), L5 interneuron (11/70), L6 Exc (8/130), L6 interneuron (6/101).

**(g)** UMAP mapping of L2/3 excitatory neurons based on electrophysiological and morphological clustering. Neurons that triggered disynaptic inhibition (triangles) were consistently mapped to the *RHAG* cluster of L2/3 neurons (n = 27).

**(h)** Two representative *RHAG* L2/3 neurons and horsetail interneurons.

**(i)** Immunostaining of recorded neurons (biocytin) with PAX6, showing that horsetail interneurons are PAX6-positive.

**(j)** Unitary excitatory postsynaptic currents (uEPSCs) recorded in *PAX6*-positive horsetail interneurons triggered by presynaptic *RHAG* L2/3 neurons.

**(k)** Postsynaptic spikes observed in *PAX6*-positive horsetail interneurons in response to presynaptic *RHAG* L2/3 neurons.

**(l)** uEPSC amplitudes recorded in different interneuron subtypes in response to presynaptic *RHAG* L2/3 neurons: *PAX6*-positive (n = 8), PV-positive (n = 7), *SST*-positive (n = 6), PC-positive (n = 7). Statistical analysis: one-way ANOVA (p < 0.01).

**(m)** Percentage of *PAX6*-positive interneurons (26/30) and non-*PAX6* interneurons (0/0) exhibiting postsynaptic spikes in response to presynaptic *RHAG* L2/3 neurons.

**(n)** Left: Connections among six simultaneously recorded neurons, including one PAX6 interneuron, two L2/3 Exc neurons, one L2/3 interneuron, one L5 Exc neuron, and one L5 interneuron. Right: Connection diagram with reconstructed morphologies.

**(o)** APs elicited in presynaptic *PAX6* interneurons and the corresponding IPSPs recorded in postsynaptic neurons.

**(p)** Probability of connections mediated by *PAX6*-positive horsetail interneurons across layers: L1 (5/37), L2/3 Exc (20/56), L2/3 interneuron (15/42), L4 Exc (14/41), L4 interneuron (18/49), L5 Exc (10/30), L5 interneuron (11/35), L6 Exc (7/42), L6 interneuron (5/33).

**(q)** Left: *PAX6*-positive interneurons received synaptic input from *RHAG* L2/3 neurons (20/43) and non-*RHAG* L2/3 neurons (5/37). Middle: Comparison of uEPSC amplitudes elicited in *PAX6*-positive interneurons by *RHAG* L2/3 neurons (n = 10) versus non-*RHAG* L2/3 neurons (n = 17). Right: Percentage of *PAX6*-positive interneurons exhibiting postsynaptic spikes in response to *RHAG* L2/3 neurons (31/34) versus non-*RHAG* L2/3 neurons (0/0).

**Fig. S4. Comparison of monosynaptic and disynaptic inhibition in L2/3 circuits.**

**(a)** Left: Morphological reconstruction of two simultaneously recorded neurons, including one L2/3 IT neuron and one L2/3 interneuron (In). Middle: Schematic diagram illustrating monosynaptic excitatory and inhibitory connections, with excitatory neurons represented by a black triangle and interneurons by a blue circle. Right: Electrophysiological traces showing action potentials (APs) elicited in presynaptic neurons and corresponding excitatory postsynaptic potentials (EPSPs) (top) and inhibitory postsynaptic potentials (IPSPs) (bottom) recorded in postsynaptic neurons.

**(b)** Left: Morphological reconstruction of recorded neurons. Middle: Schematic showing the circuit involved in disynaptic inhibition. Right: Electrophysiological traces depicting disynaptic inhibition in a postsynaptic neuron, with the inhibitory response completely abolished following the application of CNQX (5 μM) in the recording chamber.

**Fig. 5. Synaptic mechanisms underlying disynaptic inhibition mediated by *RHAG* and *PAX6* interneurons.**

**(a)** Schematic diagram showing the whole layer and whole cell type inhibition mediated by *RHAG* L2/3 pyramidal cells.

**(b)** Schematic diagram comparing monosynaptic inhibition and disynaptic inhibition pathways.

**(c)** Dot plot depicting *SYNPR* and *NECTIN3* gene expression across different L2/3 IT subtypes. The dot size represents the fraction of cells expressing each gene, and color intensity indicates mean expression levels. These two genes are markers of detonator synapses.

**(d, e)** Differences in synapse characteristics, including vesicle release, synaptic, and postsynaptic receptor features across the five L2/3 IT clusters (panel d) and comparing these features in L2/3 interneurons (panel e). This highlights the unique properties of disynaptic inhibition mediated by *RHAG* L2/3 pyramidal cells and *PAX6* interneurons.

**(f)** Left: Sample traces of spontaneous excitatory postsynaptic currents (sEPSCs) recorded in L2/3 excitatory neurons with or without disynaptic inhibition. Right: Cumulative probability plots of sEPSC amplitude and frequency from L2/3 pyramidal cells (non-disynaptic inhibition neuron vs. disynaptic inhibition neuron). The inset bar graphs show the average sEPSC amplitude and frequency, with sample sizes: non-disynaptic L2/3 pyramidal cells, n=25, disynaptic L2/3 pyramidal cells, n=21. Statistical analysis: Student's t-test, p < 0.01.

**(g)** Local input of non-disynaptic L2/3 pyramidal cells (blue) from other L2/3 pyramidal cells (black). Electrophysiological traces show action potentials (APs) elicited in presynaptic neurons and corresponding excitatory postsynaptic potentials (EPSPs) recorded in postsynaptic non-disynaptic L2/3 pyramidal cells.

**(h)** Local input of disynaptic L2/3 pyramidal cells (pink) from other L2/3 pyramidal cells (black). Electrophysiological traces show action potentials (APs) elicited in presynaptic neurons and corresponding excitatory postsynaptic potentials (EPSPs) recorded in postsynaptic disynaptic L2/3 pyramidal cells.

**(i)** Connection probability of local L2/3 pyramidal cells to non-disynaptic and disynaptic L2/3 pyramidal cells. A significant reduction in connectivity was observed between local L2/3 pyramidal cells and disynaptic L2/3 pyramidal cells. Connection rates: local L2/3 pyramidal cells to non-disynaptic L2/3 pyramidal cells: 8/76; local L2/3 pyramidal cells to disynaptic L2/3 pyramidal cells: 2/106. Statistical analysis: p < 0.05.

**Fig. S5. Functional classification of synaptic genes shown in Fig. 5D-E.**

Schematic representation of the molecular functions and synaptic localization of the genes analyzed in Fig. 5d-e. Genes are grouped by their roles in synaptic processes, including synaptic vesicle cycling, presynaptic signal transduction, synaptic active zone components, synaptic vesicle release, presynaptic adhesion, postsynaptic receptors (AMPA, NMDA, kainate), postsynaptic scaffold and adhesion proteins, and postsynaptic signal transduction.

**Fig. 6. Cross-species analysis of disynaptic inhibition.**

**(a)** Disynaptic inhibition in the monkey temporal cortex (TC). Left: Schematic representation of connections among five simultaneously recorded neurons, including L2/3 excitatory (L2/3 Exc) neurons, L2/3 interneurons, and L5 interneurons. Middle: Connection diagram illustrating the reconstructed morphologies of recorded neurons. Right: Disynaptic inhibition responses, showing Aps elicited in presynaptic excitatory neurons and corresponding IPSPs recorded in postsynaptic neurons.

**(b)** Disynaptic inhibition in the human temporal cortex (TC). Left: Schematic representation of connections among five simultaneously recorded neurons, including L2/3 excitatory neurons, L2/3 interneurons, and L5 interneurons. Middle: Connection diagram with reconstructed morphologies. Right: Disynaptic inhibition responses, showing Aps elicited in presynaptic excitatory neurons and IPSPs recorded in postsynaptic neurons.

**I** Probability of disynaptic inhibition connectivity across species and cortical regions. Disynaptic inhibition was detected in the monkey dorsolateral prefrontal cortex (DLPFC) (15/86), monkey TC (14/80), and human TC (15/90), targeting neurons in layers 2–5. No disynaptic inhibition was observed in monkey V1, monkey S1, mouse PFC, mouse TC, mouse V1, or mouse S1.

**(d)** Representative morphological characteristics of L2/3 presynaptic excitatory neurons involved in mediating disynaptic inhibition in the monkey DLPFC, monkey TC, and human TC. For comparison, the morphology of presynaptic L2/3 neurons that do not mediate disynaptic inhibition is shown in Fig. S5.

**I** Scatter plot illustrating the distribution of monosynaptic excitation and disynaptic inhibition in L2/3 neurons. The Y-axis represents the distance from the pia, while the X-axis represents the apical dendrite proximal segment angle. Monosynaptic excitation-only clusters, which lack disynaptic inhibition, are on the left, whereas clusters with disynaptic inhibition are on the right (n = 179 in monkey DLPFC, n = 80 in monkey TC, n = 69 in human TC).

**(f)** Schematic representation of neuronal connections in the monkey TC, showing one disynaptic-eliciting L2/3 excitatory neuron and one horsetail interneuron.

**(g)** Identification of disynaptic-eliciting neurons in the monkey TC. Left: Double staining of two recorded neurons with biocytin (red), PAX6 (green), and colocalization (yellow). Middle: Disynaptic-eliciting L2/3 excitatory neurons induce large uEPSCs in PAX6-positive interneurons. Right: uEPSC amplitudes across different interneuron subtypes: PAX6-positive (n = 7), PV-positive (n = 6), SST-positive (n = 6), and PC-positive (n = 5). Statistical analysis: one-way ANOVA (p < 0.01).

**(h)** Recruitment of PAX6 interneurons in the monkey TC. Left: Aps elicited in presynaptic disynaptic-eliciting L2/3 excitatory neurons and corresponding Aps recorded in postsynaptic PAX6 interneurons. Right: Percentage of interneurons exhibiting AP responses, comparing PAX6-positive (35/40) vs. non-PAX6-positive interneurons (0/0).

**(i)** Schematic representation of neuronal connections in the human TC, showing one disynaptic-eliciting L2/3 excitatory neuron and one horsetail interneuron.

**(j)** Identification of disynaptic-eliciting neurons in the human TC. Left: Double staining of two recorded neurons with biocytin (red), PAX6 (green), and colocalization (yellow). Middle: Disynaptic-eliciting L2/3 excitatory neurons induce large uEPSCs in PAX6-positive interneurons. Right: uEPSC amplitudes across different interneuron subtypes: PAX6-positive (n = 7), PV-positive (n = 6), SST-positive (n = 6), and PC-positive (n = 5). Statistical analysis: one-way ANOVA (p < 0.01).

**(k)** Recruitment of PAX6 interneurons in the human TC. Left: Aps elicited in presynaptic disynaptic-eliciting L2/3 excitatory neurons and corresponding Aps recorded in postsynaptic PAX6 interneurons. Right: Percentage of interneurons exhibiting AP responses, comparing PAX6-positive (37/40) vs. non-PAX6-positive interneurons (0/0).

**Fig. S6. Cross-species comparison of non-disynaptic L2/3 IT neuron morphology and spatial localization of disynaptic inhibition.**

**(a)** Representative morphologies of non-disynaptic L2/3 IT neurons are shown for the monkey DLPFC (blue), monkey TC (green), and human TC (purple), with a scale bar of 0.1 mm.

**(b)** Frequency histograms show the distribution of presynaptic disynaptic-eliciting L2/3 IT neurons relative to their cortical depth (distance from pia in μm) across species. Data are presented for monkey DLPFC (blue, n = 40), monkey TC (green, n = 40), and human TC (purple, n = 40).

**(c)** A scatter plot compares the detected disynaptic inhibition events across species, showing their cortical depth distributions in monkey DLPFC (blue), monkey TC (green), and human TC (purple). Additionally, *PAX6* interneuron axonal projections are mapped relative to their depth.

**Fig. 7. Cross-species analysis reveals association cortex specificity and disease relevance of *RHAG* and *PAX6* neurons.**

**(a)** Spatial transcriptomic mapping in the monkey cortex shows the distribution of *RHAG*-expressing L2/3 pyramidal neurons (upper panel) and *PAX6*-expressing interneurons (lower panel) across cortical regions (DLPFC, TC, S1, and V1), with red dots indicating presence.

**(b)** Neighborhood enrichment analysis performed using Squidpy demonstrates the local interaction and spatial association between *RHAG*-expressing neurons and other interneuron subtypes in the monkey DLPFC.

**(c)** Quantification of the number of interneurons in the immediate neighborhood surrounding each *RHAG*-expressing pyramidal neuron.

**(d)** In the mouse cortex, *RHAG*-expressing L2/3 PCs are absent, as shown in the left dot plot, while spatial mapping of *PAX6* interneurons (right) reveals no expression across PFC, TC, S1, and V1.

**(e)** In the human cortex, dot plots depict the distribution of *RHAG*-expressing L2/3 PCs (left) and *PAX6*-expressing interneurons (right) across DLPFC, TC, S1, and V1. The dot size represents the fraction of cells in the group (%), while color intensity reflects mean expression levels.

**(f)** Dot plots depict the association between risk genes for major neuropsychiatric and cognitive disorders and specific neuronal clusters, including *RHAG* and *PAX6* clusters. The left-hand annotation bar indicates gene associations with various disorders using color coding. Disorder abbreviations: BD: Bipolar Disorder; MDD: Major Depressive Disorder; SCZ: Schizophrenia; AD: Alzheimer's Disease; ADHD: Attention-Deficit/Hyperactivity Disorder; INT: Intelligence (cognitive function).

**(g)** Bar graphs showing the enrichment of risk genes for each disorder in distinct neuronal cell types.