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Supplemental Information

Dense Inhibitory Connectivity in Neocortex

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Inventory of Supplemental Information

Supplemental Figures and Legends

Figure S1: Distinction between inhibitory true connections and excitatory false positives. It illustrates a detailed explanation of the protocol and analysis presented in Figure 2.

Figure S2: Specificity of the photostimulation of inhibitory cells. It illustrates a control experiment for the protocol and analysis presented in Figure 2.

Figure S3: Depth dependence of the connectivity between somatostatin-expressing interneurons and PCs. It is an additional analysis related to the distance-dependence illustrated in Figure 4.

Supplemental Experimental Procedures

- Biocytin histochemistry and anatomical reconstructions

- Data analysis: electrophysiological properties and synaptic transmission

Supplemental information

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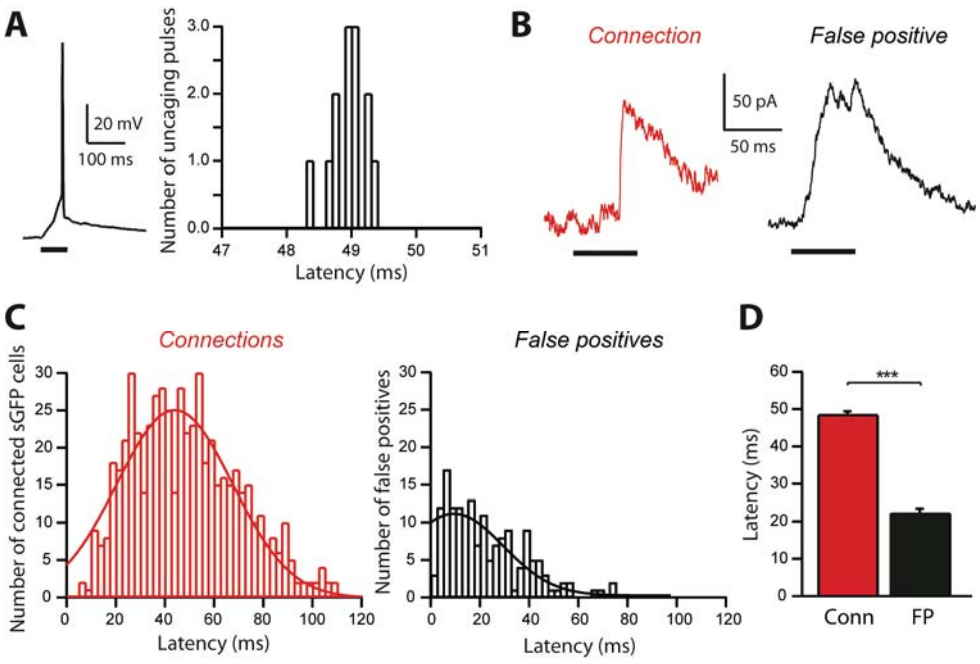
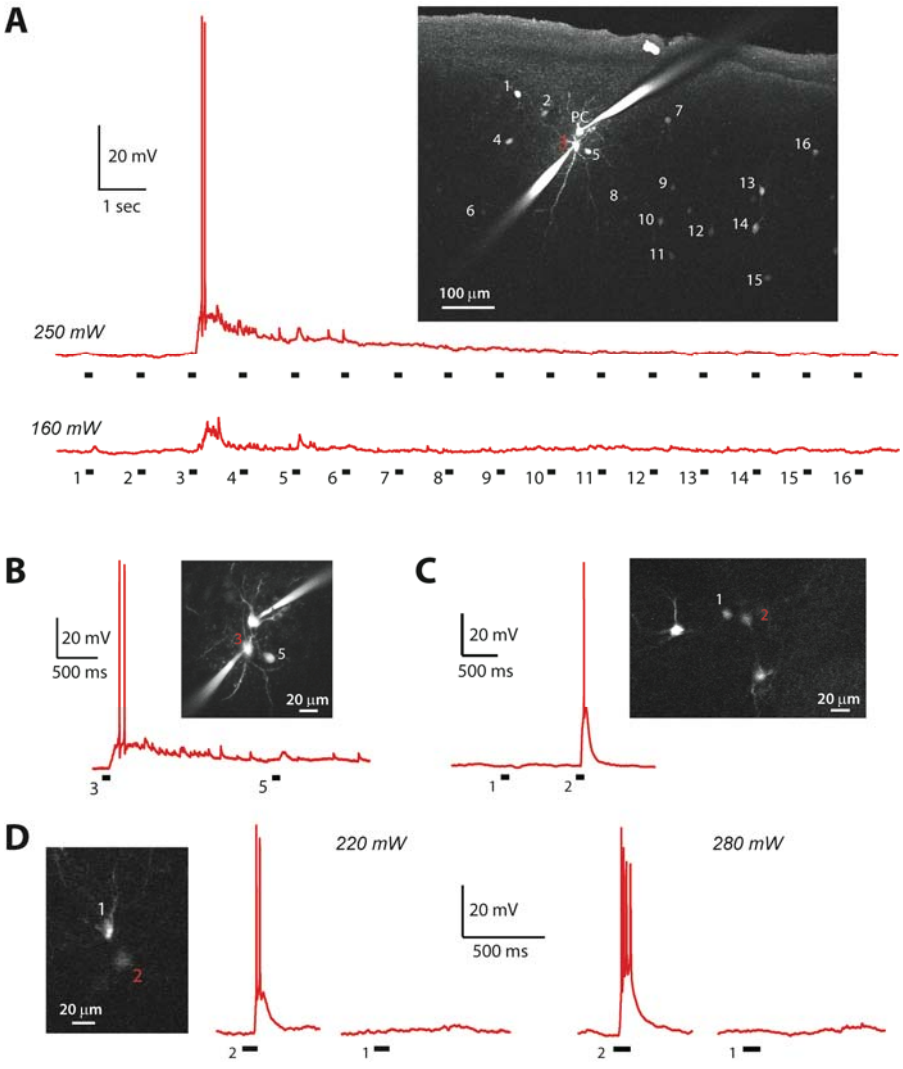
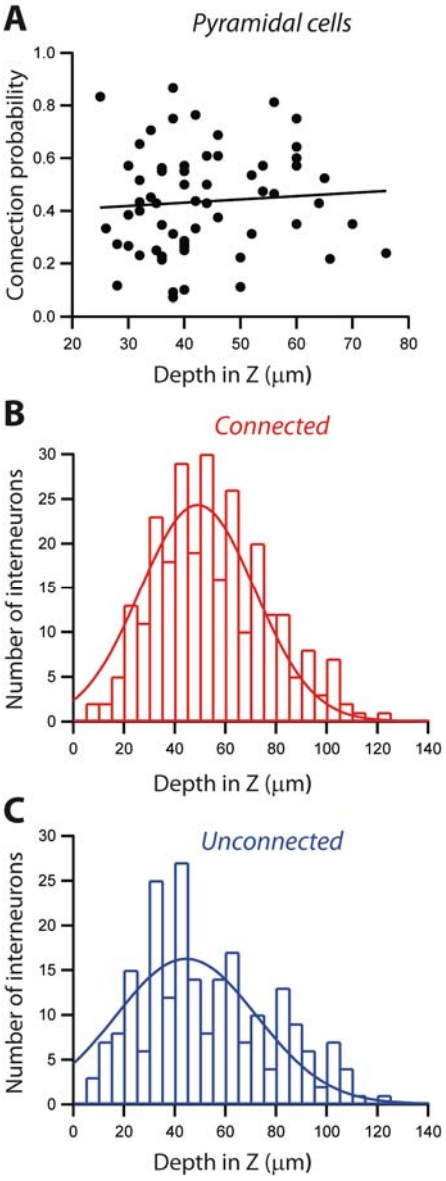
Supplemental FigureS1



Supplemental Figure S2



Supplemental Figure S3



- Supplemental Figures legends

Supplemental Figure S1: Specificity of two-photon mapping

A, Example of an action potential evoked in a sGFP cell by the uncaging protocol showed in Figure 1D. There is a delay between the onset of the laser pulse (represented by the black line). The graph shows the latency of the action potential from the beginning of the laser pulse, with the same laser power (170 mW) for 13 consecutive trials (average= 48.9±0.08 ms, n=13). **B,** Representative traces of an inhibitory connection (red) or a false positive (black) recorded in the PCs. The latency from the onset of the laser pulse of these two events are different. **C,** Distribution of the latency from the onset of the laser pulse to the start of the rising phase of the detected inhibitory connections (red) and the false positives (black). The peaks of the distributions are at 43.97±33.30 ms for the inhibitory connections (n=576) and 9.4±28.32 ms (n=164) for the false positives. **D,** The average of the latency observed for connections (48.54±0.91 ms, n=576) were significantly different ($p<0.0001$, Mann Whitney) than the latency of false positives (22.21±1.22 ms, n=164).

Supplemental Figure S2: Lack of activation of non-targeted interneurons

A, A PC and a sGFP cell were patched in a mapping experiment. The trace demonstrates a current-clamp recording of the sGFP cell #3 while uncaging over the other sGFP cells (1 to 16) in the field, this being the regular protocol of the mapping experiments. The bottom trace is

recording with 160 mW laser power, which induces a subthreshold response and the top trace, with 250 mW laser power, makes the cell fires. The patched sGFP cell only fire when the laser pulse was directed onto its soma. **B,** Zoom of the recording shown in A when stimulating either directly the recorded interneuron (#3) or stimulating a very close one (#5). We can notice that even though they are really close to each other, when the laser is stimulating cell #5, there is a slight activation of cell #3 but it remains subthreshold and really small. **B and D,** Two additional examples of the specificity of the stimulation of one single interneuron. The two neighbouring interneurons are arbitrarily named 1 and 2 and the recorded one is labeled in red and the other one in white. We can see on these two examples that even when the two GFP cells are very close to each other, action potentials are only evoked when the laser is specifically targeting the soma. In D, we observe the same thing at a power evoking 2 action potentials in the neuron #2 (left panel) or when the power of the laser is increased and evoked a burst of action potentials (right panel). With both powers, there is no activation of cell #2 when cell #1 is targeted.

Supplemental Figure S3: Analysis of depth effect

A, Relation between the depth of the recorded PCs and the connection probability from the sGFP interneurons. They were not significantly correlated (r^2= 0.0055), indicating that there is no correlation between the position of the PCs in the slice and the probability of getting inhibitory connections. **B and C,** Distribution of the depth of the photostimulated sGFP interneurons, which were found either connected (B) or unconnected (C). The peaks of the distributions, 52.2±1.7 μm for connected interneurons (n=275) and 54.0±1.3 μm (n=220) for unconnected

interneurons, were not significantly different (p=0.211, Mann-Whitney test). All the results are expressed as average±SEM.

- Supplemental Data

Depth analysis of connectivity

The two-photon photostimulation allowed us to stimulate also interneurons deep in the slice. We wondered whether our results could be dependent on the depth of the PCs or the sGFP interneurons, since the surface connections could be selectively sectioned, when compared with those located deeper in the tissue. To explore this effect, we plotted the number of connections found vs. the depth of the somatic location of the neurons. Although there was a slight trend for an increased connection probability with increased depth of the PCs, it was not statistically significant (r^2=0.005; Figure S3 A). Also, the distributions of averaged depths of connected or unconnected interneurons were not significantly different (54.0±1.3 μm, n=275 vs. 52.2±1.7 μm, n=220; p=0.211, Mann Whitney test; Figure S3 B and C). Thus, the inputs maps of sGFP cells did not appear to differ significantly as a function of these experimental conditions.

- Supplemental Experimental procedures

Slice preparation and electrophysiology

Thick coronal slices (350 μm) from P11-P41 day-old GIN transgenic mice (Oliva et al., 2000) frontal cortex were prepared using a Leica VT1000-S vibratome with a cutting solution containing (in mM): 27 $NaHCO_3$, 1.5 NaH_2PO_4, 222 Sucrose, 2.6 KCl, 2 $MgSO_4$, 2 $CaCl_2$. Slices were incubated at 32 °C in ACSF for 30 minutes and then kept at room temperature for at least 30 minutes before transferring them to the recording chamber. The recording chamber was bathed in ACSF (pH 7.4), also kept at room temperature and saturated with 95% O_2 and 5% CO_2, containing (in mM): 126 NaCl, 3 KCl, 2 $MgSO_4$, 2 $CaCl_2$, 1 NaH_2PO_4, 26 $NaHCO_3$, and 10 glucose.

For voltage-clamp recordings, neurons were either held at their resting membrane potential (sGFP cells), or at +40mV and -40mV (PCs) to distinguish inhibitory and excitatory events. Whole-cell electrodes (4 to 7 MΩ) were used. To establish whole-cell access the cells were illuminated with an oblique light and an IR-pass filter on the microscope field diaphragm, and visualized through a CCD camera (DAGE-MTI IR-1000) connected to a Sony PVM-137 black and white video monitor. Current-clamp recordings (mainly interneurons) were performed with intracellular solution (pH 7.3), containing (in mM): 135 K-methylsulfate, 10 KCl, 10 HEPES, 5 NaCl, 2.5 Mg-ATP, 0.3 Na-GTP, and 0.1 Alexa Fluor 594. Voltage clamp recordings (mainly PCs) were done with intracellular solution (pH 7.3), containing (in mM): 128 Cs-methanesulfonate, 10 HEPES, 2 $MgCl_2$, 2 $MgSO_4$, 4 Na_2-ATP, 0.4 Na-GTP, 10 Na_2-phosphocreatine and 0.1 Alexa Fluor 594. The connections between PCs were recorded in Cs-based internal solution to be able to do mapping experiments right after checking the excitatory connections. The Cs-based internal explains the broad shape of the presynaptic action potential shown in Figure 7B. Indeed, when recording in current-clamp with Cs-internal, it is hard to repolarize the neuron after a depolarizing pulse so we apply a protocol with a short depolarizing

pulse (+150 pA, 50 ms), followed by a quick and strong hyperpolarizing pulse (-300 pA, 30 ms) to help the neuron to repolarize.

Experiments were conducted at room temperature (22 to 25 °C) to prolong the life of the slices. We performed recordings from layer 2/3 neurons using MultiClamp 700B (Molecular Devices) amplifiers, and acquired the signals through a National Instruments PCI 6259 board using custom software developed with LABView (National Instruments).

Imaging and RuBi-Glutamate uncaging.

Imaging and uncaging experiments were performed using a custom-made two-photon laser scanning microscope based on the Olympus FV-200 system (side-mounted to a BX50WI microscope with a 40x, 0.8NA, or 20x, 0.5NA, water immersion objectives) and a Ti:sapphire laser (Chameleon Ultra II, Coherent, >3 W, 140 fs pulses, 80 MHz repetition rate). Fluorescence was detected with a photomultiplier tube (PMT: H7422-P40 Hamamatsu, Bridgewater, NJ) connected to a signal amplifier (Signal Recovery AMETEK Advanced Measurement Technology, Wokingham U.K.) whose output was connected to the Fluoview system. First, images of the field of view were acquired with Fluoview software (XY scan mode with 1x to 10x digital zoom), at 850 nm for Alexa-594 and 900nm for GFP, using minimal power to prevent RuBi-Glutamate uncaging. As RuBi-Glutamate is light-sensitive, the computer screens and video monitor were covered with two layers of Rosco #27 medium red filters.

A Dynamax peristaltic pump (Ranin Instruments Inc., Woburn, MA) was used to control bath perfusion to minimize total bath volume and re-circulate oxygenated media. RuBi-Glutamate (TOCRIS, St. Louis, MO) was added to the bath at 300 μM concentration. The concentration chosen for two-photon experiments, 300 μM, was the lowest concentration with which we were able to fire reliably using our stimulation protocol.

A somatic uncaging point was selected using custom software (Nikolenko et al., 2007). The electrophysiology software triggered the uncaging pulse and controlled the pulse duration. RuBi-Glutamate was excited at 800 nm for uncaging. Laser power was modulated by a Pockels cell (Conoptics). For somatic stimulations, each neuron was stimulated with a circular array of 8 subtargets, each of which was illuminated for 8 ms, giving a total duration of ~70 ms. Subtargets themselves were complex, consisting of 5 very closely spaced beamlets created by multiplexing the laser beam with a diffractive optical element (DOE) (Nikolenko et al., 2007). Typical power levels on sample were 230±80 mW (40x, 0.8NA objective) or 170±60 mW (20x, 0.5NA objective), the power was modulated depending if we wanted to evoke one or a burst of action potentials. Most of the time we evoked bursts of action potentials to be sure to detect the connections. Successive neuronal targets were stimulated every 1 second; this rapid neuron to neuron stimulation allowed us to quickly assess the connectivity of multiple neuronal pairs using the "switching test" (see Results). Occasionally, some of the responses were "mixed", composed of outward and inward currents at -40mV. Since the purpose of our study was to detect all potential inhibitory connections we tallied these mixed responses as inhibitory for our analysis, because they did reveal the existence of an inhibitory connection. All maps with paired or triple recordings were acquired with a 20x objective (0.5 NA); the investigated fields represented around 600 x 800 µm, including therefore the whole layer 2/3 and layer 1.

Biocytin histochemistry and anatomical reconstructions.

Neurons were filled with biocytin (2mg/mL) by the patch pipette. At the end of the experiment, the slices were fixed and kept overnight in 4% paraformaldehyde in 0.1M phosphate buffer (PB) at 4ºC. The slices were then rinsed three times for five minutes per rinse on a shaker in 0.1M PB. They were placed in 30% sucrose mixture (30g sucrose dissolved in 50ml ddH_20 and 50 ml 0.24M PB per 100 ml) for 2 hours and then frozen on dry ice in tissue freezing

medium. The slices were kept overnight in a -80ºC freezer. The slices were defrosted and the tissue freezing medium was removed by three twenty minute rinses in 0.1M PB while on a shaker. The slices were kept in 1% hydrogen peroxide in 0.1M PB for thirty minutes on the shaker to pretreat the tissue, then were rinsed twice in 0.02M potassium phosphate saline (KPBS) for twenty minutes on the shaker. The slices were then kept overnight on the shaker in Avidin-Biotin-Peroxidase Complex. The slices were then rinsed three times in 0.02M KPBS for 20 minutes each on the shaker. Each slice was then placed in DAB (0.7 mg/ml 3,3"-diaminobenzidine, 0.2 mg/ml urea hydrogen peroxide, 0.06M Tris buffer in 0.02M KPBS) until the slice turned light brown then immediately transferred to 0.02M KPBS and transferred again to fresh 0.02M KPBS after a few minutes. The stained slices were rinsed a final time in 0.02M KPBS for 20 minutes on a shaker. Each slice was observed under a light microscope and then mounted onto a slide using crystal mount.

Successfully filled and stained neurons were then reconstructed using Neurolucida software (MicroBrightField). The neurons were viewed with 100x oil objective on an Olympus IX71 inverted light microscope or an Olympus BX51 upright light microscope. The Neurolucida program projects the microscope image onto a computer drawing tablet. The neuron's processes were traced manually while the program recorded the coordinates of the tracing to create a digital three-dimensional reconstruction. The x and y axes form the horizontal plane of the slice, while the z-axis is the depth. The user defined an initial reference point for each tracing. The z coordinate was then determined by adjustment of the focus. In addition to the neuron, the pia was drawn.

Data analysis: electrophysiological properties and synaptic transmission

Neuronal input resistance was calculated from voltage responses obtained after injecting a hyperpolarizing current (~ -20 pA, 1 sec duration). The amplitude of action potentials was estimated as the potential difference between their voltage threshold (the abrupt increase in slope depolarization) and the peak of the spike waveform. Instantaneous firing frequency was calculated as the reciprocal of the interspike interval. The spike frequency adaptation (SFA) was calculated using the formula: adaptation ratio = $F_{final}/F_{initial}$, where $F_{initial}$ is the initial spike frequency (1/first interspike) and F_{final} is the average frequency calculated from the last interspike intervals. The potentiation, depression and summation were measured using paired-pulse stimulation by evoking 2 action potentials in the sGFP interneuron and recording the IPSC evoked in the PC. The amplitude of the second IPSC is divided by the amplitude of the first one to determine the potentiation or depression. For summation, the amplitude of the peak of the current of IPSC2 compared to the baseline is divided by the amplitude of the first one, IPSC1. The latency of a connected pair was measured from the peak of the action potential to the start of the IPSC rising phase.