Parvalbumin- and vasoactive intestinal polypeptide-expressing neocortical interneurons impose differential inhibition on Martinotti cells

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Disinhibition of cortical excitatory cell gate information flow through and between cortical columns. The major contribution of Martinotti cells (MC) is providing dendritic inhibition to excitatory neurons and therefore they are a main component of disinhibitory connections. Here we show by means of optogenetics that MC in layers II/III of the mouse primary somatosensory cortex are inhibited by both parvalbumin (PV)- and vasoactive intestinal polypeptide (VIP)-expressing cells. Paired recordings revealed stronger synaptic input onto MC from PV cells than from VIP cells. Moreover, PV cell input showed frequency-independent depression, whereas VIP cell input facilitated at high frequencies. These differences in the properties of the two unitary connections enable disinhibition with distinct temporal features.

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Cortical inhibitory interneurons (IN) are grouped into three major subpopulations defined by the expression of molecular markers, namely parvalbumin (PV), somatostatin (SST) and the ionotropic serotonin receptor (5HT3aR). The main, but not exclusive, cell types within these subpopulations, largely defined by morphological features, are: PV-expressing basket cells, SST-expressing Martinotti cells (MC) and cells co-expressing 5HT3aR and vasoactive intestinal polypeptide (VIP). The functions of these IN are manifold. In general, they keep excitation in check, perform gain modulation and induce synchronization and oscillations, whereas more specifically they open or close temporal or spatial windows for input control or output generation. Their functional impact is not restricted to their interaction with excitatory neurons, but direct interactions between IN seem to be essential for sensory information processing as well.

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Results

Characterization of GIN neurons. To investigate inhibitory inputs to MC, we used two triple transgenic mouse lines, namely PV-cre::tdTomato::GIN and VIP-cre::tdTomato::GIN (Fig. 1a). The cre-knock in lines have recently been reported to be highly specific and sensitive mouse models. Within the GIN line, green fluorescence protein (GFP)-expressing cells in cortical LII/LIII and LV were described as being almost exclusively MC. In agreement with previous literature, we showed that, also in triple transgenic mice, these cells often display a multipolar or bifurcated somato-dendritic configuration and, as their most prominent feature, dense axonal clustering in LI (Fig. 1b). Furthermore, MC show an adapting firing pattern during strong current injections (Fig. 1c). Our experimental data set contained 100 biocytin-labelled GIN cells, of which 82 could be morphologically recovered. Of these recovered neurons, 79 possessed extensive axonal arborizations in LI. In three cases, the ascending axon was cut off before reaching LI. Eight well-preserved GIN cells were fully reconstructed (Supplementary Figs 1 and 2). The dendrites were primarily located in LII/LIII (Supplementary Fig. 1b). Axonal density, however, peaked in LI as well as in LII/LIII (Supplementary Fig. 1b). Therefore, we will use the term MC in the following text to refer to GFP-expressing cells.

MC are inhibited by local PV- and VIP-expressing cells. As mentioned above, PV and VIP cells form the main IN subpopulations besides MC, which are not considered to interact with each other. Accordingly, they are likely candidates for providing inhibitory inputs to MC. To test this hypothesis, we expressed channelrhodopsin 2 (ChR2) in PV and VIP cells throughout the entire cortical depth of S1 by viral transfection. In order to control for the specificity of the input population, we recorded from ChR2-transduced PV and VIP IN (PV: 11 cells, 5 mice; VIP: 8 cells, 4 mice). They show their subgroup-specific firing patterns and morphology (Supplementary Fig. 3a–c,e–g). Additionally, we controlled for adequate ChR2 expression levels. Indeed, optogenetic stimulation of these cells caused

Figure 1 | LII/LIII GIN cells show typical characteristics of Martinotti cells. (a) Fluorescent staining of triple transgenic mice (left: PV-cre::tdTomato::GIN, right: VIP-cre::tdTomato::GIN) used for the present experiments. In all, 50-μm-thick frontal sections from the barrel cortex are shown. PV or VIP cells, respectively, are labelled red and GIN cells green. Layers are indicated as L-I–IV. Scale bar, 100 μm. (b) Neurolucida reconstructions of LII/LIII biocytin-filled GIN cells. Somatodendritic compartments are shown in orange and axonal arborizations in green. Note the dense axonal branching in LI, which is characteristic for MC. Layers are indicated as L-I–VI. Scale bar, 100 μm. (c) Whole-cell current-clamp recordings of GIN cells shown in b. Depolarizing current injections caused an adapting firing pattern in these cells, as it is typical for MC.

Subthreshold voltage ramps (100 ms) of +160 pA (a) and +180 pA (b) elicited an antidromic afterhyperpolarization of 10–20 mV for all GIN cells. Membrane potential was held at –64 mV (a) and –61 mV (b). (c) Whole-cell current-clamp recordings of GIN cells shown in b. Depolarizing current injections caused an adapting firing pattern in these cells, as it is typical for MC.
depolarizations sufficient to fire action potentials (APs) (Supplementary Fig. 3d,h–k).

Light-induced activation of ChR2 in either PV or VIP IN reliably evoked inhibitory postsynaptic currents (IPSCs) in every MC tested (PV: 23 cells, 12 mice, age P38–P60; VIP: 27 cells, 14 mice, age P35–P53) (Fig. 2a–c). This indicates that each MC receives input from PV and VIP cells. The minimal laser energy required to elicit IPSCs was comparable for PV and VIP inputs (PV: 76.7 ± 15.0 μW; VIP: 124.0 ± 27.1 μW; mean ± s.e.m.) (Fig. 2d). PV cells evoked multicomponent IPSC with apparently larger amplitudes (PV: 456.55 ± 43.22 pA; VIP: 275.70 ± 46.59 pA; mean ± s.e.m.) (Fig. 2a,b,e).

By means of optogenetic stimulation, we could define presynaptic IN populations; however, their precise laminar location remained unclear. Thus we localized sources of monosynaptic inhibitory input to LII/LIII MC by focal photolysis of caged glutamate (Supplementary Fig. 4). The highest proportion (~45%) of inhibitory fields was found in LII/LIII of the home column (n = 10, 10 mice).

**Figure 2 | PV and VIP cells reliably target MC in LII/LIII of S1.** (a,b) Left: schematic of recording configuration for photostimulation of ChR2-expressing PV or VIP cells while recording from LII/LIII MC. Right: Examples of photostimulation-induced inhibitory postsynaptic currents (IPSC). Arrowheads indicate photostimulation (473 nm laser, 1 ms) of PV interneurons (a) or VIP interneurons (b) at three different intensities (subthreshold, threshold and 10× threshold). (c) Proportion of MC responding to photostimulation of PV and VIP cells. In both experimental designs, the success rate was 100%. This indicates that each MC receives inhibitory input from PV and VIP cells. (d) Threshold laser energy to elicit IPSC in MC by photostimulation of PV and VIP cells. Note the same range (mean ± s.e.m.) for both groups. (e) Mean ± s.e.m. of IPSC amplitudes (at 10× threshold level) in MC for each group (PV: n = 16, age P38–P60, VIP: n = 18, age P35–P53). Optical stimulation of the PV cell population tends to result in larger multicomponent IPSC amplitudes.

**PV and VIP cell inhibition differs in synaptic properties.** These findings indicated the presence of two discrete inhibitory inputs onto MC, namely, from PV and VIP cells, which are restricted to LII/LIII. Hence, we performed simultaneous recordings of putative presynaptic PV or VIP cells and postsynaptic MCs in this compartment. In accordance with previous results, we indeed found connected pairs of both types (age: PV MC: P21–P36; VIP MC: P21–P32; Fig. 3a). All PV and VIP cells matched their group-specific firing patterns and morphological characteristics, with PV cells being fast spiking multipolar cells and VIP cells being bipolar/bitufted cells (or partly modified variations thereof) of the adapting or irregular spiking type (Fig. 3b,c; Supplementary Fig. 2). We further analysed the elementary synaptic properties of connected pairs on a unitary level (Fig. 4; Supplementary Table 1a). The connection probability was higher for PV cells (~58%, 12/21) than for VIP cells (~35%, 11/31) (Fig. 4b). Based on the observation that each MC receives input from both of the two other types, as shown by optogenetic stimulation above, the differences in connection probability might be due to a higher degree of divergence of PV cell axonal projections onto MC, as proposed for PC targets as well.23 Single presynaptic spikes reliably (PV to MC: ~90%; VIP to MC: ~80%) elicited unitary IPSCs in MC regardless of the type of presynaptic IN (Figs 3a and 4b). However, the average IPSC evoked by PV cells (n = 12, 12 mice) showed significantly larger amplitude (PV to MC: 49.74 ± 12.97 pA; VIP to MC: 12.13 ± 3.57 pA; mean ± s.e.m.), shorter latency (PV to MC: 0.60 ± 0.07 ms; VIP to MC: 1.39 ± 0.12 ms; mean ± s.e.m.), shorter 10–90% rise time (PV to MC: 1.62 ± 0.17 ms; VIP to MC: 4.59 ± 0.64 ms; mean ± s.e.m.) and higher normalized slope (PV to MC: 0.30 ± 0.05 fraction of amplitude ms⁻¹; VIP to MC: 0.12 ± 0.02 fraction of amplitude ms⁻¹; mean ± s.e.m.) in comparison to VIP cell-evoked IPSCs (n = 11, 9 mice) (Fig. 4c,d). Finally, in most of the cases we also probed for reciprocal connections. In six of the nine cases, PV cells were reciprocally connected with MC, whereas reciprocal connections between VIP cells and MC occurred only in one of the eight trials (Supplementary Fig. 5). We did not quantify these responses owing to differences in the intracellular solutions (see Methods section).

Information processing is subject to short-term dynamic changes in synaptic transmission24–27. Therefore, we triggered trains of presynaptic spikes at different frequencies (1, 8, 40 Hz) to investigate short-term plasticity for both types of pairs (Fig. 4e; for original data, see Supplementary Table 2a). The PV-to-MC connection showed significant depression in IPSC amplitude at all frequencies (1 Hz: n = 11; 8 Hz: n = 10; 40 Hz: n = 10) (Fig. 4f; Supplementary Table 3a). Although the amount of depression differed from frequency to frequency, it was apparent and substantial (19.20 ± 4.07%–42.75 ± 4.11%; mean ± s.e.m.) already in the second response in every case (Fig. 4f; Supplementary Table 3a). By contrast, repetitive firing in VIP cells neither caused synaptic depression nor obvious facilitation of inhibitory inputs to MC at lower frequencies (1 Hz: n = 11; 8 Hz: n = 11; 40 Hz: n = 10). However, the amplitudes of IPSCs evoked by VIP cells consistently facilitated at 40 Hz beginning from the third response onwards (Fig. 4f; Supplementary Table 3a).

**PV-to-MC connections also exist in V1.** The VIP-to-MC connection seems to be present in S1, as shown here and by Lee et al,3 and in the primary visual cortex (V1)14,28. However, it has been discussed recently whether or not the PV-to-MC connection does exist in V1 as well.29,30 Accordingly, we performed paired recordings of PV cells and MC in LII/LIII of V1 (age: P27–P49; Supplementary Fig. 6a and Supplementary Table 1b). PV cells target MC with a connection probability of ~35% (6/17)
similarly to S1, PV cells reliably (~97%) caused IPSCs in postsynaptic MC (Supplementary Fig. 6b). On average (mean ± s.e.m.), the IPSCs had an amplitude of 50.68 ± 13.70 pA, a latency of 0.68 ± 0.08 ms, a 10–90% rise time of 1.77 ± 0.20 ms and a normalized slope of 0.24 ± 0.03 fraction of amplitude ms⁻¹ (n = 6 and 4 mice; Supplementary Fig. 6c). Concerning short-term plasticity, we observed synaptic depression already at 1-Hz stimulations (Supplementary Fig. 6d,e; Supplementary Table 3b). Our data are thus in line with recent publications demonstrating that PV-to-MC connections are a frequent motif in V129,30. As MC are only a subpopulation of SST-expressing IN20, this might explain previous discrepant results on PV-to-SST connections6.

Discussion

The present study demonstrates that both VIP and PV cells target MC in LII/LIII of S1 and differ strongly in unitary synaptic properties as well as short-term plasticity. Although the VIP-to-MC connection has already been shown to exist in LII/LIII of different sensory cortical areas28,14,29,30, the PV-to-MC connection has largely been explored in LII/LIII of V129,30. Here we show by combining optogenetics, glutamate uncaging, paired recordings and full morphological reconstruction that in S1 these two circuit motifs (VIP to MC and PV to MC) are fundamentally different.

An important finding is that these two inhibitory inputs onto MC differ substantially in IPSC amplitude, latency and kinetics. One mechanism possibly giving rise to such differences is divergent subcellular targeting. Owing to electrotonic spread, inputs located more distantly will be attenuated and slowed31. In addition, spread along the dendrite might account for the increase in latency. As VIP inputs in our sample are smaller in amplitude, slower in rise and relatively delayed, one might assume that VIP inputs onto MC are substantially more diffuse compared with PV inputs. Indeed, such a separation of inhibitory inputs has been described for cortical PC32–35. Although dendrite-targeting inhibition is in a position to selectively control excitatory inputs, perisomatic inhibition exerts a global control of spike output. To our knowledge, however, there is no direct ultrastructural evidence for this targeting pattern on MC or any other kind of cortical IN as of yet. Alternatively, differences in the subunit composition of GABA A-receptors in MC could also account for the differences in unitary properties mentioned above6. Interestingly, these two alternatives are not mutually exclusive but might occur in parallel37.

Besides differences in size and kinetics, these two unitary connections also differ in short-term plasticity. Frequency-independent depression of PV cell input onto MC in LII/LIII in S1, as shown in the present report, is in line with previous observations that PV cell input shows short-term depression regardless of the type of postsynaptic cell in sensory cortical areas38. By contrast, reports on short-term plasticity of unitary VIP cell inputs in these areas are lacking. Although the frequency range tested here is in line with many related studies, it must be

Figure 3 | Electrophysiology and morphology of LII/LIII PV–MC and VIP–MC pairs. (a) Examples of connected pairs of presynaptic PV or VIP cells and postsynaptic MC in LII/LIII. The average of 10 individual IPSCs (grey traces, evoked by repetitive stimulation) is shown in colour (PV to MC: red, age P23; VIP to MC: blue, age P27). Presynaptic spikes reliably evoke IPSCs in both cases. (b) Whole-cell recordings of a presynaptic PV (left) and a VIP cell (right). During depolarizing current injections, the PV cell shows a fast spiking pattern, whereas the VIP cell shows an adapting firing pattern. (c) Staining of acute brain slices containing morphologically recovered and synaptically connected pairs as well as the corresponding Neurolucida reconstructions (left: PV to MC, right: VIP to MC). The connected cells are shown in white (pseudo-coloured). Asterisks mark MC somata, arrowheads somata of presynaptic cells. GIN cells are labelled green and the corresponding presynaptic population (PV or VIP) is labelled red (tdTomato-fluorescence). For clarity, connected cells are shown separately as grey-scale images in the middle. The reconstructed pairs are shown at the bottom. Soma and dendrites of GIN cells are labelled black and the corresponding axon grey. The recorded PV cell exhibits a multipolar dendritic morphology (light red) and a locally dense axon (red), as described for basket cells. The VIP cell shows an atypical tripolar dendritic configuration (light blue) and an axon (blue) descending towards the white matter. Complete reconstructions are displayed in Supplementary Fig. 4. Scale bars, 100 μm.
Figure 4 | Unitary connections of PV and VIP cells onto MC differ in their elementary synaptic properties and short-term plasticity. (a) Grand average of unitary IPSCs (red: PV to MC, \(n=12\), age P21-P36; blue: VIP to MC, \(n=11\), age P21-P32) in MC in response to single spikes, repeatedly evoked in presynaptic IN. Averages of individual pairs are shown in grey. (b) Connection (left) and success rate of synaptic transmission (right) of the two different kinds of unitary connections. Note that the connection probability of PV cells (\(\sim 58\%\), 12/21) is substantially larger than the one of VIP cells (\(\sim 35\%\), 11/31). In connected pairs, synaptic transmission is highly reliable. (c) Overlay of grand averages (from a) aligned with respect to presynaptic spike peaks. IPSCs evoked by PV and VIP cells differ substantially in size and kinetics. For clarity, the boxed initial phase of both responses is shown at higher resolution as an inset. (d) Quantification of unitary IPSCs. Amplitude, latency, 10–90% rise time and normalized slope as fraction of amplitude per ms were analysed based on averages of each individual connected pair (PV to MC: red; VIP to MC: blue). Mean ± s.e.m. was then calculated for each group separately. Asterisks indicate significant differences (\(P<0.05\)) for all those parameters. (e) Individual examples of averaged IPSCs in MC in response to trains of five spikes (1, 8 and 40 Hz) in a presynaptic IN (PV to MC: red trace; VIP to MC: blue trace). Individual traces are shown in grey. Quantification is shown in f. (f) Quantitative analysis of short-term plasticity at different frequencies (1Hz: PV to MC, \(n=11\); VIP to MC, \(n=11\); 8 Hz: PV to MC, \(n=10\); VIP to MC, \(n=11\); 40 Hz: PV to MC, \(n=10\); VIP to MC, \(n=10\). Amplitude ratio (nth response/first response) of consecutive IPSCs plotted versus successive IPSCs. At the population level, PV to MC responses show synaptic depression under all stimulus conditions, whereas VIP to MC responses show no significant changes in amplitude at low frequencies but facilitate at 40 Hz. Values represent mean ± s.e.m.
pointed out that both types of presynaptic neurons can fire at higher instantaneous frequencies. To our knowledge, the only study using presynaptic spike trains with very short interspike intervals reported substantial synaptic depression in fast spiking as well as non-fast spiking interneurons in adult rats over a wide range of interspike intervals except for 10–20 ms. Short-term plasticity of PV inputs on MC might be described as phasic in comparison to the more tonic properties of VIP inputs. Nevertheless, even depressed PV inputs still exert a stronger influence at the soma than the corresponding VIP inputs. Considering that VIP cells might target distal dendrites, their true impact on dendritic input control would be much stronger and facilitation at high frequencies could be a dominant factor in controlling activity in the postsynaptic cell. By this means, VIP and PV cells may provide different spatial and temporal windows of opportunities.

In the present study, we have shown that each MC in LII/LIII of the barrel cortex receives two different inhibitory inputs with divergent properties. Two separate input channels may, on the one hand, allow different stimulus features to control PC inhibition by MC but it may as well allow one single excitatory input to utilize different kinds of MC inhibition. Both types of MC-inhibiting IN share much of their excitatory input, namely, local LII/LIII PC and excitatory cells in LIV of the same column. Recently, however, long-range connections from primary motor cortex have been described to preferentially target VIP cells in S1 (ref. 8). Accordingly, VIP cell-mediated inhibition of primary motor cortex have been described to preferentially target primary motor cortex have been described to preferentially target VIP cells in S1 (ref. 8). 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Bregma minus 1–2 mm, medial–lateral range: 2.5–3.5 mm) via a glass micropipette (25 μm inner diameter) connected to a Toohy Spritzer Pressure System IIe (Toohy Company, Fairfield, NJ, USA). The micropipette was positioned at 2–4 different locations, guided by surface blood vessel patterns and at 3 different depths (800, 500, 250 μm below the pial surface). Small amounts of virus in sterile phosphate-buffered saline (PBS; up to 150 nl) were injected by pressure application (3 psi, 250 ms pulse duration). The micropipette was withdrawn 10–15 min after pressure application. Finally, the animals were sedated and injected with carprofen (2.5 mg per 10 g body weight, Rymadyl, Pfizer, New York City, NY, USA). For postoperative care, animals were provided with wet food and metamizol (1.33 mg ml−1, Novaminsulfon-ratiopharm, Ratiopharm, Ulm, Germany) dissolved in drinking water. In accordance with animal care guidelines, metamizol was also applied a day before surgery.

Two-to-three weeks after viral transduction, acute brain slices were prepared and whole-cell patch clamp recordings of LII/LIII MC were performed as described above. Photostimulation (diameter: 2–4 mm) was also applied a day before surgery. Consecutive IPSCs overlapped only during 40 Hz stimulations. To measure the amplitude of single responses in this case, the decay (subthreshold, threshold and 10 × threshold for IPSC occurrence in MC; range: 3–1,000 μV) at a minimum inter-stimulus interval of 5 s.

Staining. To visualize biocytin-filled neurons as well as gephyrin, gephyrin- and tdTomato-expressing cells, slices were processed as follows: biocytin was rinsed off PBS and reacted with primary antibodies (secondary antibody, anti-rabbit/anti-goat, 1:500, BioLegend, Limerick, PA, USA; goat anti-GFP, 1:2,000, Abcam, Cambridge, UK) in blocking solution (0.25% bovine serum albumin, 10% normal donkey serum and 0.5% Triton-X100, pH 7.6, in PBS) for 48–72 h at 4 °C. Then they were rinsed in PBS (5 ×), followed by 4 h of secondary antibody incubation at room temperature, rinsed in PBS (6 ×) and stained by DAPI (1:1,000, Molecular Probes, Carlstadt, NJ, USA). Secondary antibodies used were donkey anti-goat (AF488: 1:1,000, Invitrogen, Carlstadt, NJ, USA), donkey anti-rabbit (AF546: 1:500, Invitrogen, Carlstadt, NJ, USA) and streptavidin-conjugated AF633 (1:500). Slices were mounted in AquaPolyMount (Toohey Company, Fairfield, NJ, USA). The micropipette was positioned at 2–4 m (Bregma minus 1–2 mm, medial–lateral range: 2.5–3.5 mm) via a glass micropipette (250 μm inner diameter) connected to a Toohy Spritzer Pressure System IIe (Toohy Company, Fairfield, NJ, USA). The micropipette was positioned at 2–4 different locations, guided by surface blood vessel patterns and at 3 different depths (800, 500, 250 μm below the pial surface). Small amounts of virus in sterile phosphate-buffered saline (PBS; up to 150 nl) were injected by pressure application (3 psi, 250 ms pulse duration). The micropipette was withdrawn 10–15 min after pressure application. Finally, the animals were sedated and injected with carprofen (2.5 mg per 10 g body weight, Rymadyl, Pfizer, New York City, NY, USA). For postoperative care, animals were provided with wet food and metamizol (1.33 mg ml−1, Novaminsulfon-ratiopharm, Ratiopharm, Ulm, Germany) dissolved in drinking water. In accordance with animal care guidelines, metamizol was also applied a day before surgery.

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Results were given as mean ± s.e.m. for all other values, if not stated otherwise.

Digital illustrations. Confocal image stacks were exported as maximum intensity projections and stored as TIFF files. Image brightness and contrast images were adjusted using the Photoshop software (Adobe, Dublin, Ireland).

Data availability. The data that support the findings of this study are available from the corresponding author on request. Neuroumlucida reconstructions will be made available through Neuromorpho.org.

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**Author contributions**

F.W., M.W., M.F. and R.J.W. carried out experiments. F.W., M.M., J.F.S. and M.W. were involved in study design and wrote the paper. M.W. and J.F.S. supervised all experiments. F.W., M.W., M.M. and D.S. carried out data analysis.

**Additional information**

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