Experience-dependent regulation of NG2 progenitors in the developing barrel cortex

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We found that, during the formation of the mouse barrel cortex, NG2 cells received glutamatergic synapses from thalamocortical fibers and preferentially accumulated along septa separating the barrels. Sensory deprivation reduced thalamocortical inputs on NG2 cells and increased their proliferation, leading to a more uniform distribution in the deprived barrels. Thus, early sensory experience regulates thalamocortical innervation on NG2 cells, as well as their proliferation and distribution during development.

NG2-expressing oligodendrocyte progenitor cells (NG2 cells) represent the largest population of neural progenitors in the developing postnatal and adult brain1. NG2 cells receive functional glutamatergic synapses2 whose function is unknown. Because NG2 cells are still able to proliferate and migrate while receiving functional synapses3–5, it has been proposed that neuron–NG2 cell synapses could regulate NG2 cell development in an activity-dependent manner2. To define the role of neuron-NG2 cell synapses in a physiological system, we investigated functional and developmental interactions between thalamocortical axons and NG2 cells in the mouse somatosensory cortex.

We performed patch-clamp recordings in thalamocortical slices from CNP-EGFP mice between postnatal days 3 and 5 (P3–5; Supplementary Fig. 1). Ventrobasal stimulation resulted in evoked excitatory postsynaptic currents (eEPSCs) in layer IV CNP-EGFP+ NG2+ cells with average amplitude and decay time of 48 ± 82 pA and 1.6 ± 0.3 ms, respectively (n = 16 cells; Fig. 1a). The average stimulation-to-response delay was 6.8 ± 0.4 ms (n = 16 cells), as would be expected for unmyelinated thalamocortical fibers6. eEPSCs were blocked by the AMPA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 10 μM, n = 5 cells; Fig. 1b). Serotonin (5-HT) inhibits presynaptic release of glutamate in synapses between thalamocortical axons from the ventrobasal nucleus and layer IV cortical neurons7. 5-HT (20 μM) inhibited eEPSCs in layer IV CNP-EGFP+ NG2+ cells by 63 ± 7% (n = 5 cells; Fig. 1c,d), but had no effect on AMPA receptor–mediated currents evoked by application of 1 mM glutamate (Supplementary Fig. 2). These results confirm that EPSCs evoked in NG2 cells by ventrobasal stimulation depend on the synaptic release of glutamate from thalamocortical axons7.

We investigated the distribution of CNP-EGFP+ NG2+ cells in somatosensory cortex layer IV (Fig. 1e,f and Supplementary Fig. 3). At P6, barrels are structurally well defined, with a central core of VGLUT2+ thalamocortical synapses, surrounded by a divider of NeuN+ neurons8. Glutamine synthetase+ astrocytes are accumulated in barrel cores9. CNP-EGFP+ NG2+ cells exhibited a low density in barrel cores, but accumulated in septa separating neurons that likely belong to different barrels. This distribution was observed by intensity profile plot and cell counting perpendicular to barrel walls (Supplementary Fig. 3). The specific distribution of CNP-EGFP+ NG2+ cells was detectable as soon as barrels emerged around P4 (Supplementary Fig. 4). At P2, we were already able to detect a specific decrease in CNP-EGFP- and

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Fig. 1 NG2+ progenitors are functionally innervated by thalamocortical glutamatergic synapses and accumulate at the border between cortical barrels. (a) Stimulation of the ventrobasal nucleus evoked EPSCs in layer IV CNP-EGFP+ NG2+ cells, which exhibited a gaussian distribution of their delays (average delay = 7 ms). (b–d) EPSCs were blocked by 10 μM of CNQX and reduced by 20 μM serotonin. (e) Barrel field from P6 CNP-EGFP mice immunostained for VGLUT2, NG2, NeuN and glutamine synthetase (GS). Scale bars represent 50 μm. (f) Average fluorescence intensity plots (n = 3 mice, 3 barrel unit per mouse, bin size = 5.5 μm) of VGLUT2, CNP-EGFP, NG2 and GS across barrels. Average NG2 and CNP-EGFP fluorescence were significantly more intense in barrel septa than in cores, whereas average glutamine synthetase fluorescence was significantly more intense in barrel cores (***P < 0.001, error bars represent s.e.m.)
NG2-associated immunofluorescence in emerging layer IV and layer VI of the somatosensory cortex, where thalamocortical axons begin to accumulate (Supplementary Fig. 5). The layer-specific distribution of NG2 could still be observed at P15 (Supplementary Fig. 6), when a large number of oligodendrocytes were myelinating.

Early sensory experience is known to regulate barrel formation, but its influence on NG2 cells has not been investigated. We removed and cauterized the central row of mystacial whiskers at birth and analyzed NG2 cell distribution in the barrels corresponding to deprived whiskers (Fig. 2a–c). At P6, the distribution of CNP-EGFP+ NG2+ cells was more uniform in the central row barrels of deprived mice, as compared with controls; the CNP-EGFP+ NG2+ cell density increased by 26 ± 6% (n = 4 mice, P < 0.05) and the average fluorescence intensity for CNP-EGFP and NG2 were both higher in deprived barrel central row than in the surrounding barrel rows (P < 0.001).

We performed a deprivation of all whiskers on the right-side whisker pad at birth and compared the density of CNP-EGFP+ NG2+ cells in all layers between the control hemisphere ipsilateral to the lesion, where the intact whiskers are represented, and the deprived hemisphere contralateral to the lesion, where the deprived whiskers are represented (Fig. 2f). The density of CNP-EGFP+ NG2+ cells was increased by 34.9 ± 7.3% in layer IV of the deprived hemisphere (36,360 ± 1,750 cells per mm²) compared with the control hemisphere (27,200 ± 1,100 cells per mm², n = 4 mice; paired t test, P < 0.01). The density in layer II–III (control = 30,400 ± 800 and deprived = 29,800 ± 1,100 cells per mm², P > 0.05) and V (control = 38,700 ± 1,200 and deprived = 37,100 ± 1,300 cells per mm², P > 0.05) was not significantly affected, and a small increase in layer VI failed to reach significance (control = 29,800 ± 1,200 and deprived = 32,600 ± 1,500 cells per mm², P > 0.05).

We then determined whether the strength of thalamocortical glutamatergic inputs onto layer IV NG2 cells could vary according to location of these cells in the barrel and whether these inputs could be regulated by early sensory deprivation. We compared the glutamatergic innervation of layer IV CNP-EGFP+ NG2+ cells at P4–5 in the barrel cores and septa of control barrel cortex with the innervation of layer IV CNP-EGFP+ NG2+ cells of deprived barrel cortex. To minimize the variability in evoked EPSC amplitudes, we recorded glutamatergic EPSCs in layer IV CNP-EGFP+ NG2+ cells in response to stimulation of the subcortical white matter (SCWM) below each recorded cell. EPSCs evoked in CNP-EGFP+ NG2+ cells were blocked by CNQX and inhibited by 20 μM-5-HT (57 ± 12%, n = 4 cells), confirming that, at this age, they mostly arise from thalamocortical glutamatergic axons. The average amplitude of evoked EPSCs was significantly higher in layer IV CNP-EGFP+ NG2+ cells located in barrel cores (93.2 ± 7.8 pA, n = 8 cells) than in cells located in septa (9.7 ± 8.3 pA, n = 9 cells, P < 0.01) or in layer IV CNP-EGFP+ NG2+ cells recorded in the deprived side (20.1 ± 17.8 pA, n = 8 cells, P < 0.05) (Fig. 3a–e).

We also determined how central row deprivation alone would affect the strength of thalamocortical input in CNP-EGFP+ NG2+ cells located in the deprived central row barrel cores and in cells located in the septa surrounding the deprived row, as compared with surrounding control barrels septa and cores. We used a slice preparation across barrel row A to E, which allows the identification of each row of barrels individually (Supplementary Fig. 7a,b). Although we found that CNP-EGFP+ NG2+ cells located in deprived barrel C core received significantly weaker inputs (38.3 ± 22.3 pA, n = 8 cells; Supplementary Fig. 7b) than cells located in surrounding barrel cores (108.1 ± 78.5, n = 10 cells, P < 0.05), we found no significant differences between cells located in septa separating control barrels (14.2 ± 12.7 pA, n = 9 cells) and septa separating deprived and nondeprived barrels (16.6 ± 13.0 pA, n = 9 cells, P < 0.05), with the average input strength remaining low in both cases.

Differences in the strength of thalamocortical innervation detected between these distinct groups of CNP-EGFP+ NG2+ cells correlated with differences in their proliferation rate. We performed dual immunostaining for Ki67, a marker of proliferating cells, and VGLUT2 to visualize barrel cores in P4 CNP-EGFP mice, and analyzed CNP-EGFP+ cell proliferation. At this age, virtually all of the CNP-EGFP+ cells were NG2+ (Fig. 1). The proliferation rate of CNP-EGFP+ cells was significantly higher in barrel septa (27.3 ± 1.9%) than in barrel cores (19.5 ± 1.3%, n = 3 mice, paired t test, P < 0.05; Fig. 3f,g). By comparing the percentage of CNP-EGFP+ cells expressing Ki67 in the somatosensory cortex between control and deprived hemisphere at P3, we found that this was significantly increased in the deprived hemisphere (21.8 ± 3.2%) compared with the control hemisphere (16.3 ± 0.9%, n = 4 mice, paired t test, P < 0.05; Fig. 3h–k).
To analyze a different sensory system, we investigated the distribution of NG2 cells in the primary visual cortex during development and after dark rearing. NG2 cells exhibited a lower density in layer 4 of the visual cortex (Supplementary Fig. 8). However, this decrease appeared at least 2 weeks later than in the somatosensory cortex, as it was detectable at P14 using intensity profile plot, but only detectable around P21 by cell counting. Visual deprivation using a dark-rearing protocol did not alter NG2 cell distribution and density (Supplementary Fig. 9).

We found that NG2 cells in cortical layer IV receive glutamatergic synapses from somatosensory thalamocortical axons during the formation of the barrel cortex. As thalamocortical fibers start innervating their target areas in cortical layer IV and VI around 2PND, NG2 cells tended to be initially excluded from these layers, but subsequently accumulated in the septa separating layer IV barrels as they emerge around P4. This partial results from a higher proliferation rate of NG2 cells in the septa, where they receive weaker thalamocortical inputs, than in barrel cores, where they are more strongly innervated by thalamocortical axons. The specific location of NG2 cells, their proliferation rate and their innervation by thalamocortical fibers all depended on normal sensory experience after birth. Although the location and proliferation rate of NG2 cells may not be directly regulated by their thalamocortical innervation, but instead result from a secondary effect of this innervation, our findings are consistent with previous findings suggesting that glutamate inhibits NG2 cell proliferation and promotes their migration. Changes in glutamate-induced proliferation and motility, combined with distinct effects on NG2 cell proliferation in barrel cores and septa, could lead to the preferential accumulation of NG2 cells in barrel septa. Given that adult barrel septa are more heavily myelinated than barrel cores and sensory deprivation abolishes the distribution of myelinated fibers, experience-dependent regulation of NG2 accumulation could constitute one of the mechanisms that control the number and positioning of myelinated fibers in the adult somatosensory cortex.

METHODS

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

Materials. The CNP-EGFP mouse has been described previously. Animal procedures complied with US National Institutes of Health and Children’s Research Institute guidelines. All reagents were from Sigma, unless otherwise stated; CNQX, QX-314 and SR 95531 (gabazine) were from Tocris Cookson. For primary antibodies, we used antibody to NG2 (rabbit, 1:200, mab5502), antibody to NeuN (mouse, 1:500, mab377) and antibody to VGLUT2 (rabbit, 1:200, ab16802) from Chemicon, antibody to glutamine synthetase (rabbit, 1:200, ab16802) from Abcam, antibody to Ki67 (rabbit, 1:500, NCL-Ki67p) from Novacraft, and antibody to parvalbumin (rabbit, 1:2,000, PV 25) from Swant. Secondary antibodies (1:200) were obtained from Jackson ImmunoResearch Laboratories.

Immunocytochemistry and confocal microscopy. Biocytin-filled, patch-clamp–recorded CNP-EGFP+ cells were visualized by fluorescence microscopy in brain slices and underwent immunocytochemical characterization with antibody to NG2 (ref. 17). CNP-EGFP mice at P2–28 were anesthetized with isoflurane, intracardially perfused with 4% paraformaldehyde (wt/vol), and the brains were removed. Dark-reared mice (n = 11) had their eyes covered throughout the anesthetization and perfusion procedures. Tangential, thalamocortical and coronal tissue sections (50–100 µm) were prepared using a vibratome (floating sections) and immunostained. Images were acquired using a Zeiss LSM 510 (Zeiss). For all images, confocal sections and z-stacks ranged between 2 and 3 µm, respectively. Images were processed using ImageJ (NIH) and Adobe Illustrator (Adobe Systems).

Cell counting and fluorescence profile plot. To measure the density of CNP-EGFP+ NG2+ oligodendrocyte progenitor cells and glutamine synthetase+ astrocytes, we counted the number of CNP-EGFP+ or glutamine synthetase+ soma colocalizing with DAPI nuclear staining in confocal z stack (30-µm thickness, z step = 2 µm) in the region of interest (ROI; barrel row, cortical layer, etc.). Densities were obtained by dividing the number of cells counted by the volume of the ROI (surface of the section analyzed × thickness = 30 µm), and expressed as the number of cells per mm². The surface analyzed was identical between conditions (control and after deprivation).

To quantify the density of CNP-EGFP+ NG2+, NeuN+ neurons and glutamine synthetase+ astrocytes across barrel walls, we aligned barrels as vertically as possible on the basis of DAPI staining, and then measured the x coordinate of each type of cells using the same rectangular ROI for each barrel wall. Measurements were limited to a group of smaller barrels representing the small frontal whiskers and exhibiting a similar diameter and either circular or hexagonal shape. Measurements were made separately for group of cells across each barrel wall and then pooled together. The x coordinates were plotted for each section analyzed (bin size = 5 µm). Relative densities were obtained by dividing the number of cells for each bin by the total number of cells counted in the ROI. Density values were expressed as percentages, with 100% being the sum of all bins. Final histograms represent the average of normalized density distribution histograms from three different animals.

Fluorescence intensity profile plots were obtained using the plot profile in ImageJ (NIH). We either show individual or average image profile plots at a single pixel resolution or average profile plots in which intensity value of several adjacent pixels are averaged (9–10 pixels per bin).

Slice preparation. Brain hemispheres were dissected from P3–5 CNP-EGFP mice on ice and cut into 500-µm-thick thalamocortical sections on a Leica VT1000S vibratome, and then placed in an ice-cold oxygenated solution (87 mM NaCl, 2.5 mM KCl, 1.25 mM NaH2PO4, 7.5 mM MgCl2, 0.5 mM CaCl2, 25 mM NaHCO3, 25 mM glucose, 75 mM sucrose; 347 mMol at pH 7.4). Slices were stored in the same solution at 35 °C for 30 min and then transferred into artificial cerebrospinal fluid (124 mM NaCl, 3 mM KCl, 2.5 mM CaCl2, 1.3 mM MgCl2, 26 mM NaHCO3, 1.25 mM NaHPO4, 15 mM glucose; saturated with 95% O2/5% CO2 at 20–25 °C).

Patch-clamp recordings. Slices were transferred to a recording chamber and perfused with ACSF at a rate of 1–2 ml s–1. Patch electrodes had resistances between 3 and 6 MΩ when filled with an intracellular solution containing 130 mM potassium gluconate, 20 mM KCl, 2 mM MgCl2, 0.1 mM EGTA, 10 mM HEPES, 0.4 mM Na-GTP, 2 mM Na-ATP and bicyno (3 mg ml–1), solution adjusted to pH 7.3, 275 mM Osm. Whole-cell recordings from CNP-EGFP+ cells were obtained using a Multiclamp 700B (Molecular Devices) and monitored via a personal computer running pClamp 9.2 (Molecular Devices). Barrel cores could be visualized by infrared video microscopy, based on their low light absorption compared to the surrounding tissue. Between P3 and P4, the region where thalamocortical fibers terminate could similarly be identified as a continuous band of cortex exhibiting a lower light absorption. In addition, layer IV of the somatosensory cortex could be recognized based on its lower density in CNP-EGFP+ cells compared to layer V and III. The exact anatomical location of each recorded cell was determined after patch-clamp recording by defining the position of the patch pipette tip relative to the low light absorption territories corresponding to barrel cores on the control sides, or relative to the continuous layer of thalamocortical fibers before P4, or in deprived sides. Cells that were recorded in the incorrect layers, mostly upper layer V and lower layer III, were routinely discarded. The cellular and physiological identity of the recorded EGFP+ cells was routinely confirmed using current–voltage steps to test for the presence of transient inward K+ current on depolarization and by filling all cells with biocytin during patch-clamp recording, followed by labeling with antibody to NG2 and biocytin. The ventrobasal nucleus or SCWM were stimulated using small bipolar electrodes (FHC). Stimulations were performed at a low frequency (0.07 Hz) to evoke a stable response. For each slice, to identify the region of the barrel cortex that was still functionally connected to the ventrobasal thalamic nucleus, we first performed extracellular current recordings at different locations in response to ventrobasal stimulation. The region connected by monosynaptic thalamocortical synapses specifically exhibited a transient hyperpolarization 6–7 ms after ventrobasal stimulation, a delay that would be expected for unmyelinated thalamocortical fibers at this age. Field potential recordings were obtained by using a glass electrode (tip resistance = 0.5 MΩ) filled with ACSF and connected to a multiclamp 700B amplifier (Axon Instruments). Although ventrobasal stimulation was used to demonstrate without ambiguity that NG2 cells are innervated by thalamocortical fibers from the ventrobasal somatosensory cortex, this method could not be reliably used to quantify and compare the degree of innervation of layer IV NG2 cells. In a given slice, the amplitude of EPSCs evoked by ventrobasal stimulation mostly depends on the number of thalamocortical axons that are preserved in the slice preparation and that are able to propagate an action potential from the ventrobasal to the somatosensory cortex. As the number of preserved axons greatly varies between different slices, as well as between adjacent barrels, comparing data of ventrobasal stimulation in separate slices, or even between different barrels in the same slice, would result in a high degree of variability in the amplitude of evoked EPSCs. To minimize the variability in evoked EPSC amplitudes when comparing thalamocortical inputs between different groups of layer IV NG2 cells, we recorded their response to a stimulation of the SCWM below each recorded cell. Pulses were generated using pClamp 9.2 (Molecular Devices) and applied with a constant current stimulus isolator (DS3, Digitimer). The stimuli used to elicit evoked EPSCs varied between 10 and 500 µA in intensity and were 100 µs in duration. Average EPSC amplitudes were calculated by averaging 20–50 responses to a maximal intensity stimulus (500 µA, 100 µs). Field potential recordings were obtained by using a glass electrode (tip resistance = 0.5 MΩ) filled with ACSF and connected to a multiclamp 700B amplifier (Axon Instruments). Each of the different drugs was applied via the bath solution. Off-line analysis was performed using Clampfit 9.2 (Molecular Devices) and Mini Analysis (Synaptosoft). In all experiments, data were filtered at 10 kHz during capacitance compensation and 5 kHz during subsequent data recording. The traces were digitized at 10 kHz. All voltage measurements and steps were corrected for a junction potential offset.

Whisker cautery. Whisker lesions were performed as described previously. Neonatal mice were anesthetized by cooling and then kept on ice during the entire surgical procedure. The central row of facial whiskers was identified under a surgical microscope and, after plucking, whiskers were ablated with a surgical cautery device (Bovie). After cautery, the mice were revived, returned to their mothers and killed at the indicated time points.
Dark rearing. Control group mice were raised in a normal lighted environment (12-h on/12-h off light cycle) and perfused at P7, P14, P21 and P28. Dark-reared mice were born and raised in complete darkness until P28, with short periods of dim red light for animal husbandry. The efficiency of the dark rearing paradigm was confirmed by parvalbumin interneuron staining, which exhibited a lower density in the dark-reared group compared to control18.

Statistical analysis. All statistical analysis was performed with unpaired t test or paired t test when indicated. Results were deemed significantly different if \( P < 0.05 \).

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