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Neurotransmitter Release at the Thalamocortical Synapse Instructs Barrel Formation But Not Axon Patterning in the Somatosensory Cortex

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To assess the impact of synaptic neurotransmitter release on neural circuit development, we analyzed barrel cortex formation after thalamic or cortical ablation of RIM1 and RIM2 proteins, which control synaptic vesicle fusion. Thalamus-specific deletion of RIMs reduced neurotransmission efficacy by 67%. A barrelless phenotype was found with a dissociation of effects on the presynaptic and postsynaptic cellular elements of the barrel. Presynaptically, thalamocortical axons formed a normal whisker map, whereas postsynaptically the cytoarchitecture of layer IV neurons was altered as spiny stellate neurons were evenly distributed and their dendritic trees were symmetric. Strikingly, cortex-specific deletion of the RIM genes did not modify barrel development. Adult mice with thalamic-specific RIM deletion showed a lack of activity-triggered immediate early gene expression and altered sensory-related behaviors. Thus, efficient synaptic release is required at thalamocortical but not at corticocortical synapses for building the whisker to barrel map and for efficient sensory function.

Introduction

Genetic alterations of synaptic transmission underlie a number of developmental disorders, such as autism spectrum disorders or mental retardation (Bourgeron, 2009). However, the role of hard-wired defects of neural circuits in these affections remains unclear. Neural activity is involved in neuronal network wiring but no visible change in brain development was observed upon abolishing synaptic release (Verhage et al., 2000; Molnár et al., 2002). Since neurotransmitters and electrical activity have direct effects on morphogenetic processes such as neuronal growth (Zhang and Poo, 2001), guidance (Hanson and Landmesser, 2004; Nicoll et al., 2007), or gene transcription (Borodinsky et al., 2004), a large part of the developmental effects of neural activity could be independent of synaptic activity. However, the role of synaptic release in later activity-dependent refinement of neural circuits could not be examined because of early lethality.

Neurotransmitter release is controlled by proteins that prime vesicles to the presynaptic active zone, where they control vesicle fusion following Ca2+ rise (Sudhof, 2004). Among these proteins, the Rab3 interacting molecules (RIM) family includes seven isoforms, two of which, RIM1 and RIM2, are expressed broadly in the brain (Schoch et al., 2006). RIM1 and RIM2 are central organizers of presynaptic release, interacting with a number of active zone proteins as well as synaptic vesicle proteins (Mittelstaedt et al., 2010). Loss of function studies revealed that RIMs are required for the docking of synaptic vesicles and for the assembly of calcium channels in the presynaptic active zone (Deng et al., 2011; Han et al., 2011; Kaeser et al., 2011). RIM1/RIM2 double knock-out mice show a massive reduction in the priming and calcium-triggering of neurotransmitter release (Schoch et al., 2006; Kaeser et al., 2011).

Ablation of RIMs at selected synapses should allow determining the role of calcium-dependent neurotransmitter release on late phases of neural wiring. We used the rodent primary somatosensory barrel cortex as a model in which neural activity plays a role in sculpting barrels within the posteromedial barrel subfield (Erzurumlu and Kind, 2001; Wu et al., 2011). Barrels are columnar processing units that respond preferentially to the stimulation of individual whiskers. The development of a barrel is initiated by the clustering of thalamocortical (TC) axon terminals, followed by an arrangement of the cortical layer IV neurons...
and by the orientation of their dendrites toward incoming TC terminal arborizations (Wu et al., 2011). The formation of barrels over the first postnatal week depends on neural activity, with the involvement of both presynaptic and postsynaptic mechanisms (Iwasato et al., 2000; Hannan et al., 2001; Rebsam et al., 2002; Gheorghita et al., 2006; Lu et al., 2006; Ballester-Rosado et al., 2010).

Here we conditionally deleted all multidomain RIM isoforms in the thalamus causing a severe reduction in evoked release at the TC synapse. This did not alter the targeting and patterning of the TC afferents, but severely impaired the dendritic arbor orientation of target neurons in the somatosensory barrel cortex. In contrast, mice with a cortex-specific deletion of RIMs showed a normal barrel organization.

Materials and Methods

Animal breeding

RIM1 flox/flox -RIM2 flox/flox mice (Kaesper et al., 2008, 2011) were crossed to two different Cre mouse lines to obtain recombination in the thalamus and in the cerebral cortex. Breeding was performed on a mixed background 129sv × C57BL/6. To minimize potential variation due to background issues, littermate controls of either sex were used for all the experiments and processed simultaneously with the recombined samples.

To obtain thalamic-specific recombination, the serotonin transporter (Sert) Cre mouse line was used. This is a knock-in of nls-Cre in the 5’UTR region of Sert (Sletkung; Zhuang et al., 2005). In these mice, Cre is active in the ventrobasal thalamus (VB) from E15.5 (Narboux-Nême et al., 2008). Unexpectedly, some offspring of RIM1 flox/flox -RIM2 flox/flox breeding with Sert1/2 transgene strain gave rise to null alleles (RIM2 allele), indicating occasional germline expression of Cre that needed to be controlled in all litters. In our breeding we usually obtained: RIM1Cre1/2 -RIM2 Cre1/2, which are referred to as RIM-DKO Sert, and RIM1Cre1/2 -Sert1/2, RIM1Cre1/2 -RIM2 Cre1/2, which are referred to as Controls (Ctrl). In their home cages, RIM-DKO Sert could not be distinguished from their control littermates and showed normal survival rates.

For cortex-specific recombination, we used the Emx1-Cre mouse line, a knock-in of Cre in 3’ from the stop codon (Iwasato et al., 2000). In these mice, Cre is active in the glutamatergic cortical neurons and in glia but not in GABAergic neurons at E12.5 (Iwasato et al., 2000). Rim recombination with Emx1-Cre strain is referred to as RIM-DKO Emx1, and their littermates were used as controls. The RIM-DKO Emx1 mice were smaller than their control littermates and all died around weaning.

We used the Tau mGFP-nls-LacZ reporter mouse line to check recombination efficiency. This is a knock-in in the Tau gene of a construction containing a stop cassette flanked by loxP sites, a MARCKS sequence fused to the green fluorescent protein (GFP) and an IRES-NLS-LacZ cassette (Hippcheneyer et al., 2005). In the presence of Cre, neurons express a membrane-bound GFP and β-Galactosidase (β-Gal) in the nucleus.

Experiments were conducted in compliance with the standard ethical guidelines (European Community guidelines on the care and use of laboratory animals and French Agriculture and Forestry Ministry guidelines for handling animals).

Genotyping was done on tail lysates prepared by immersing tissue in 50 mM NaOH, at 95°C for 1 h, and then neutralizing with 1 x Tris-EDC. PCR was conducted as previously described (Kaesper et al., 2008, 2011).

RIM1 and RIM2 quantitative PCR

Brains from postnatal day 7 (P7, P0 being the day of birth) RIM-DKO Emx1, RIM-DKO Emx1 and controls were dissected out and directly frozen on liquid nitrogen for 10 s and transferred to dry ice. The VB and somatosensory cortex were microdissected on 120 μm cryostat section. RNAs were extracted from samples and genomic DNA was removed with “RNAqueous-Micro Kit” (Applied Biosystems). First-strand cDNA was synthesized by reverse transcription of 50 ng of total RNA with Superscript-Ⅱ reverse transcriptase (Invitrogen) according to standard protocols. Reverse transcriptase was omitted in some samples as a negative control. Relative expression levels of RIM1 and RIM2 mRNA were determined by real time RT-PCR using Absolute SYBR Green Mix (ABgene) and a set of primers specific for the RIM1 and RIM2 floxed sequences. RIM expression was normalized to mouse CyclophilinB mRNA expression. Data were analyzed with the 2−ΔΔCt method (Livak and Schmittgen, 2001). Values are expressed as the mean of 3–5 separate experiments, each comprising triplicates.

Tissue preparation

Mice were killed by an overdose of xylazine (1.92 mg/kg body weight, i.p.) and pentobarbital (100 mg/kg i.p.). They were perfused with 4% paraformaldehyde in phosphate buffer. Brains were dissected and either rinsed in PBS for cytochrome oxidase staining, or postfixed overnight at 4°C. Brains were cryoprotected overnight in 30% sucrose in PBS at 4°C before sectioning at 60 μm with a freezing microtome. To obtain tangential sections, cortices were dissected and flattened between two glass slides with spacers before postfixation.

Histology

The cytochrome oxidase (CO) staining of brain sections was conducted on free-floating sections. They were placed in the CO reaction solution (5 mg diaminobenzidine, 5 mg cytochrome C, 0.4 g sucrose in 0.1 mm Tris, pH 7.6) for 12–24 h at 37°C.

Immunocytochemistry was performed on free-floating sections. They were incubated overnight at 4°C in appropriate primary antibodies including: rabbit polyclonal antibody (Ab) c-Fos (1:1000, Santa-Cruz Bio-technology), rabbit polyclonal Ab CD5/Cux1 (1:500, Santa-Cruz), rabbit polyclonal Ab β-Gal (1:5000, Rockland), mouse monoclonal Ab NeuN (1:1000, Millipore), rabbit polyclonal Sert (1:1000, Calbiochem), Guinea pig polyclonal Ab vGlut2 (1:2000, Millipore Bioscience Research Agents). After several rinses, species-specific fluorescent secondary Ab (1:500 Invitrogen) were incubated for 2 h, before mounting with Mowiol (Calbiochem). vGlut2 immunohistochemistry was preceded by a 10 min incubation in 50 mg sodium citrate at 97°C. For peroxidase revelation of Sert, biotinylated anti-rabbit (1:300, Vector) followed by avidin-biotin-peroxidase complex (1:400, GE Healthcare) was used before the diaminobenzidine reaction.

In situ hybridization was performed on free-floating frozen sections as described previously (Narboux-Nême et al., 2008). NRT/BCIP was used as blue substrate for in situ revelation. The following plasmids containing full-length cDNA were used: RIM1 (IMAGE:40047877) and RIM2 (IMAGE:4505661).

Measures and cell counts

Quantification of areas. vGlut2 immunostained sections were photographed at a ×1.2 magnification using a stereomicroscope (Olympus). The total cortical area was measured on the tangential sections. The postomedial barrel subfield (PMBSF) was outlined with ImageJ software and its area measured. Within this area, the vGlut2-positive regions correspond to the barrel hollows containing the dense TC axon terminals. The vGlut2-positive patches were outlined and their areas measured. The total PMBSF area minus the sum of vGlut2-positive areas was used as an estimate of the total interbarrel area. We refer to this value as the sepal area in the rest of the manuscript, although strictly speaking, it comprises two different elements, the cellular wall surrounding the hollows and a sepal region between the barrel walls. Interestingly double immunostainings for vGlut2 and Tenascin, an extracellular glycoprotein that surrounds structural and functional developing barrels in S1 (Crossin et al., 1989; Steinidler et al., 1990) showed that tenascin-positive regions correspond precisely to the vGlut2-negative regions.

Quantification of neuronal density. Photomicrographs of tangential sections immunostained for vGlut2 and Cux1 were acquired with a 40X objective using a Leica SP2 confocal microscope. The C2 barrel was placed in the center of the field and a single confocal section was acquired. The vGlut2-positive area was delimited after automatic thresholding using a fixed threshold value (MetaMorph Software). This area was defined as the barrel hollow. A 20 μm thick belt surrounding the barrel hollow was computed using MetaMorph imaging software. This area was defined as the barrel wall (Ballester-Rosado et al., 2010). The Cux1-positive cell nuclei were counted with the cell counter plug-in.
Stereological methods were applied to avoid multiple counting. The cell counts were normalized per surface area for each compartment, providing cell density estimates for the hollow and wall compartments.

\textit{Quantification of coclustering.} Pictures of coronal sections from P10 Sert \textsuperscript{CRE} \textsuperscript{Tau} \textsuperscript{mGluR1-tdTomato} \textsuperscript{and Emx1 \textsuperscript{Cre} \textsuperscript{Tau} \textsuperscript{mGluR1-tdTomato}, immuno-

Stained for \textbeta-Gal together with NeuN, were acquired with the confocal microscope. Using the cell counter plug-in of ImageJ, the number of \textbeta-galactosidase-positive neurons within the NeuN population was measured in the VB and posterior medial (POm) nucleus of the thalamus and in the cerebral cortical layers.

\textbf{Statistical analysis}

All measures were done blind to genotype. For statistical analysis, the distributions of the results were tested with a Shapiro-Wilk test, followed by an \textit{F} test. We found a normal distribution in the experiments analyzed. In most cases, the variance between groups was similar and a nondirectional Student’s \textit{t} test was applied when variance was different, a nondirectional homoscedastic \textit{t} test was used. To compare small populations (\textit{n} < 6), Mann–Whitney tests were applied.

\textbf{Electrophysiology}

\textit{TC slice preparation.} Animals (P5–P7) were anesthetized by intraperitoneal injection of pentobarbital (15 mg/kg) and decapitated. The brain was quickly removed and placed in ice-cold (2–4°C) oxygenated (5% O\textsubscript{2}, 95% CO\textsubscript{2}) standard artificial CSF (ACSF). TC slices were cut (thickness, 400 \textmu m) using a vibratome (VT1000; Leica), as previously described (Laurent et al., 2002). The slices were first maintained 1 h at 33°C and later at room temperature (22–24°C) in oxygenated standard ACSF.

\textit{Electrophysiological recordings.} For recordings, the slices were glued on a polystyrene-coated small (1.2 mm diameter) glass coverslip, which was then placed in a small (~1 ml) chamber, perfused at 3 ml/min with ACSF at near physiological temperature (33–34°C). Recordings were obtained from layer IV neurons identified under visual control using an upright microscope (Cascade 512B, Roper Scientific) interfaced with a video camera. 

\textit{TC fiber stimulation (Laurent et al., 2002) was found by progressively increasing the stimulation intensity until a plateau unitary response was obtained (see Fig. 2a; Takahashi, 1992; Silver et al., 1996). To unambiguously define the single fiber unitary EPSC, we plotted the amplitude of the responses in relation with the stimulation intensity. A shown in our previous work at cortical (Laurent et al., 2002), and thalamic (Evrand and Ropert, 2009) synapses, it is sometimes possible to distinguish axonal failures from release failure. In most cases, there was no evidence for axonal failures and the response changes from complete failures at smaller intensity (<2.3 mA, Fig. 2a2), to a stable unitary response at higher intensities. In such cases the stimulation intensity was maintained above threshold.

The data were analyzed offline using Clampex (Molecular Devices), Excel (Microsoft), and Igor Pro 4.1 (WaveMetrics). The amplitude of the AMPA receptor (AMPAR)-mediated component of the EPSC at −70 mV was measured between the baseline and the peak of the response by averaging the signal during a short time window (40 ms). The amplitude of the NMDAR component of the EPSC was measured at +30 mV by averaging the current recorded during an identical time window (40 ms) placed 15 ms after the stimulation. The EPSC failure was measured at −70 mV and was defined as a response that was smaller than twice the SD of the baseline current. The EPSC latency was defined as the time when the EPSC reached 10% of its peak amplitude. Paired-pulse ratios (PPRs) were obtained by applying two stimulations at various time intervals and calculated at −70 mV as the second EPSC amplitude divided by the first EPSC amplitude (EPSC2/EPSC1) including failures. The EPSC kinetics was quantified by fitting their decay with a biexponential function \( y = A_{\text{fast}} e^{-t/\tau_{\text{fast}}} + A_{\text{slow}} e^{-t/\tau_{\text{slow}}} \), where \( A_{\text{fast}} \) and \( A_{\text{slow}} \) are the amplitudes of the fast and slow components respectively, \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) their time constant, \( y_0 \), the offset.

\textit{Solutions and drugs.} All chemicals were purchased from Sigma unless otherwise specified. Standard ACSF contained the following (in mM): 126 NaCl, 2.85 KCI, 1.25 KH\textsubscript{2}PO\textsubscript{4}, 1.5 MgSO\textsubscript{4}, 2 CaCl\textsubscript{2}, 2 NaHCO\textsubscript{3}, 5 Na-pyruvate and 10 glucose. The following compounds were bath applied: \textit{N}- (−)-2-amino-5-phosphonopentanoic acid (\textit{N}-APS, 50 \textmu M; Ascent Scientific), 2-3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[\textit{f}]quinoline-7-sulfonamide disodium salt (NBQX, 10 \textmu M; Ascent Scientific), [6-imino-3-(4-methoxy-phenyl)-1-(6H)-pyridazinobenzoic acid hydrobromide] (gabazine, 10 \textmu M; Ascent Scientific).

Goldi-Cox

Mice were killed and perfused with 0.9% NaCl. Brains were dissected and soaked in the Goldi-Cox filtrated solution (K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7}, 35 mM, K\textsubscript{3}CrO\textsubscript{4} 43 mM, HgCl\textsubscript{2}, 38 mM) for 2 d, rinsed in 30% sucrose for 1 week and sectioned at 200 \textmu m with a vibratome (Leica VT1000S). Sections were mounted on slides, color reacted in 30% NH\textsubscript{4}OH for 30 min, and fixed in 30% AL4 Kodak photo fixative for 30 min. They were counterstained with cresyl violet, dehydrated and coverslipped with Eukitt (Electron...
deficient synapses alter network formation/function

Microscopy Sciences). Neurons were drawn using a camera lucida with a 100× oil-immersion objective for dendritic analyses. A total of 50 Ctrl and 41 RIM-DKO<sup>sert</sup> layer IV spiny stellate cells were reconstructed from 4 mice of each genotype. Morphometric analysis of dendrites was done with NeuronJ software. Asymmetry of dendrites was measured as described previously (Datwani et al., 2002), briefly, a line was drawn between the tip of each dendrite and the center of the soma. Neurons were considered as asymmetric when 50% or more of these lines representing dendrites were oriented in a 90° angle. Otherwise, they were counted as symmetric. The proportions between genotypes were tested with a χ² test. A two dimensional Sholl analysis (Sholl, 1953) was used to analyze dendritic branching patterns. Concentric circles of 10 μm intervals were brought over each cell with the center of the circles positioned in the middle of the soma. Intersections of different dendritic orders and circles were counted.

The cell count plug-in of the ImageJ program was used to count the number of branches and bifurcation points. The same program was used to count spines on 20 μm portions of the secondary and tertiary dendrites from stacks of photomicrographs captured using a 100× oil objective on a Leica microscope. Statistical comparisons used ANOVA.

Voltage-sensitive dye imaging
Voltage-sensitive dye (VSD) imaging of the cortical activity evoked by tactile stimulation was performed on 6- to 8-week-old mice under urethane anesthesia (1.7 mg/g), as previously described (Ferezou et al., 2007). Briefly, a large fraction of mouse sensorimotor cortex was exposed unilaterally and stained for 1 h with the VSD RH1691 (Optical imaging, 1 mg/ml, in Ringer’s solution containing [in mM]: 135 NaCl, 5 KCl, 5 HEPES, 1.8 CaCl₂, 1 MgCl₂). After removing the unbound dye, the cortex was covered with agarose (0.5%) and a NaCl, 5 KCl, 5 HEPES, 1.8 CaCl₂, 1 MgCl₂). After removing the unbound dye, the cortex was covered with agarose (0.5%) and a coverslip. Cortical imaging was done through a tandem-lens fluorescence microscope (Scimda), equipped with 2 Leica PlanApx 1X objectives, a 630 nm excitation filter, a 650 nm dichroic, and a long pass 665 nm emission filter. Alternate sequences of images were acquired every 20 s (at 500 frames/s), using a CMOS-based camera (MiCam Ultima, SciMedia), with or without piezo electric stimulus (2 ms) delivered to the right C2 whisker. Data were analyzed using custom-written routines in IgorPro (WaveMetrics). Subtraction of the averaged unstimulated sequences was used to correct for photobleaching.

Sensory-mediated c-Fos expression
All whiskers of adult mice were clipped on the right side, under light anesthesia (ketamine, 100 mg/kg IP). The following day, mice were placed in a cage containing new objects (typically a cardboard tube, two plastic goblets and two balls of paper for each cage). Mice explored this new environment for 1 h, after which they were perfused. For c-Fos immunostaining, brains were sectioned at 50 μm with a vibratome (Leica VT1000). Immunohistochemistry was performed as described above.

Object exploration
Training and testing were conducted in Plexiglas boxes (white floor, gray walls) of 17 × 32 × 40 cm, in the dark. Before the test, animals were habituated to the empty boxes in 10 min sessions for two consecutive days. For the test, two objects were placed in the boxes and animals were allowed to freely explore for 10 min. Sessions were recorded and analyzed using Viewpoint software. The software determined both the position of the nose and the center of mass of the mouse. Center of mass position over time determined the speed of the mouse during exploration, and object exploration was defined when the nose was within a 2 cm large circle around the object. Eight control and 9 RIM-DKO<sup>sert</sup> mice were used for this test.

Beam walking test
The test was conducted as described previously (Carter et al., 2001). Briefly, mice were trained for 4 d to walk on an elevated 22 mm section beam to reach a platform. On the trial day, each mouse was given three trials to cross the beam and the time used to do so was noted. When mice did not succeed to reach the platform within 2 min, the trial was considered as a failure. The two best times over the three trials were averaged to determine each mouse’s crossing time. All failures per genotype were summed and averaged to estimate the probability of failure per trial. Seven Ctrl and 9 RIM-DKO<sup>sert</sup> mice were used for this test.

Results
Region-specific deletion of RIM genes in the thalamus or cortex
The presence of RIM1α and RIM1β has been reported as early as P1 in brain extracts (Kaeser et al., 2008). To determine the cellular localization of RIM1 and RIM2 expression along the thalamo-cortical pathway, we performed in situ hybridization (ISH) analyses from P1 to P14 using full-length probes that recognize either all RIM1 or all RIM2 splice variants. During the first postnatal week, RIM1 was broadly expressed in the forebrain with a stronger expression in the cerebral cortex and weaker expression in the thalamus (Fig. 1a–d). RIM2 was expressed in partly overlapping domains in the thalamus and in the cerebral cortex (Fig. 1e–h).

Cre-mediated recombination of the RIM1 and RIM2 floxed alleles removes all isoforms expressed from these genes (RIM1α, 1β, 2α, 2β, and 2γ; Kaeser et al., 2008, 2011), in contrast to the constitutive α-RIM KOs (Schoch et al., 2001, 2006). To optimize the recombination of the RIM1 and RIM2 genes, heterozygous RIM1/RIM2 floxed/null mice were used.

To ablate RIMs in the thalamus, the RIM1/RIM2 floxed mice were crossed to a mouse line expressing Cre recombinase in the serotonin transporter (Sert, Sl6a4) gene, generating RIM-DKO<sup>sert</sup> mice. Sert is strongly expressed in the major thalamic sensory relay nuclei during development (Lebrand et al., 1998), and Cre expression driven by the Sert promoter is efficient in the thalamus, occurring by embryonic day 15 (E15) (Narboux-Nême et al., 2008). Because Cre is inserted into the Sert locus, care was taken to maintain only one Cre allele. To evaluate the percentage of recombination in the different somatosensory relays, we crossed Sert-Cre mice with the Tau<sup>mgfp-nls-lacZ</sup> reporter strain (Hippneneyer et al., 2005). After recombination, neurons express a nuclear β-galactosidase (Fig. 1j). X-gal staining revealed that the somatosensory thalamus relay shows high levels of recombination: in the VB nucleus, which receives lemniscal inputs and projects to cortical layers IV and VI, 96% of the NeuN-positive neurons expressed β-galactosidase at P10 (n = 2, 1200 neurons, Fig. 1j). In the POm, which receives paralemniscal inputs and projects to the septae in layer IV and to layers Va and I, 89% of the NeuN-positive neurons expressed β-galactosidase (Fig. 1k, n = 2 mice, 540 neurons). No recombination was noted in thebrainstem trigeminal nuclei. In the cerebral cortex, some recombination was noted in the deep layers: 3.25% of layer VI neurons expressed β-galactosidase (n = 3 mice, 1050 neurons). While no recombination was observed in layers I–V.

To quantify the recombination of the RIM genes in the RIM-DKO<sup>sert</sup> mice, we performed qPCR on thalamic and cortical extracts from P7 RIM-DKO<sup>sert</sup> brains. There was a strong reduction of both RIM1 and RIM2 in the thalamus compared with control [RIM1: 12% (±1.2%) of control, n = 3–5 mice, p = 0.018; RIM2: 5% (±1.3%) of control, n = 3–5 mice, p = 0.012], and no difference was found in the cerebral cortex [RIM1: 101% (±38%) of control, n = 3–4 mice, p = 0.314; RIM2: 94% (±27%) of control, n = 3–4 mice, p = 0.5; Fig. 1k].

To ablate RIM proteins in developing cortical neurons, we used the Emx1-Cre mouse in which recombination was found to occur in all excitatory cortical neurons derived from the cortical plate by E10.5 (Iwasato et al., 2000). Mice derived from Emx1-Cre × Tau<sup>mgfp-nls-lacZ</sup> crosses confirmed the strong recombina-
tion in the cerebral cortex (Fig. 1), showing also scattered recombination in the thalamus. Quantifications of the proportion of neurons recombined by β-Gal/NeuN colocalization on confocal images revealed that 87.3% of cortical neurons express β-Gal (n = 11005, 3 mice, 1575 neurons), which corresponds approximately to the entire population of glutamatergic neurons in the cortex, while in the VB somatosensory thalamus, 22% of the neurons expressed β-Gal (n = 11006, 3150 neurons).

Deletion of RIMS in the thalamus impairs glutamate release at the TC synapse

Previous findings showed that the ablation of RIM proteins causes a massive decrease in evoked EPSC and IPSC amplitudes. The frequency of miniature EPSC and IPSC (mEPSC and mIPSC) was also reduced without changing their amplitude. The reduction of synaptic transmission was associated with a reduction of the readily releasable pool (RRP) and of the voltage-gated Ca²⁺ current due to a deficit in the coupling between Ca²⁺ channels and the RRP (Deng et al., 2011; Han et al., 2011; Kaeser et al., 2011). To test the functional properties of TC synapses in the barrel cortex, experiments were conducted in P5 to P7 control and RIM-DKO set mice from the same litters using acute TC slices (Fig. 2). The AMPA component of the TC monosynaptic EPSC was recorded at −70 mV in the presence of gabazine (10 μM), and D-AP5 (50 μM), which are GABA and NMDA receptor antagonists, respectively. In control animals, low-frequency (0.03 Hz) minimal electrical stimulation of the internal capsule (IC) evoked a stable single fiber unitary EPSC in layer IV spiny stellate neurons (Fig. 2a1,a2) with a small failure rate as previously described (Laurent et al., 2002). In RIM-DKO set littermates, EPSCs were also observed at the same latency (4.9 ± 0.2 ms, n = 9, in control; 4.8 ± 0.1 ms, n = 11, in the RIM-DKO set). However the efficacy of the TC synaptic transmission, measured as the average amplitude of the unitary responses including failures, appeared much weaker in RIM-DKO set than in control mice (34.9 ± 4.4 pA, n = 9, in control; 11.6 ± 2.6 pA, n = 11, in RIM-DKO set; p < 0.0001;
Fig. 2). This change of synaptic transmission efficacy correlated with an increased failure rate (4.8 ± 0.4%, range 0–16, n = 9 in control; 58.9 ± 5.8%, range 26–81, n = 11, in RIM-DKO	extsuperscript{Sert} mice; p < 0.0001). Based on previous evidence of unchanged mEPSC amplitude in the RIM-DKO	extsuperscript{Sert} mice (Deng et al., 2011; Han et al., 2011), the failure rate increase seen in the RIM-DKO	extsuperscript{Sert} mice should give an average EPSC amplitude of 15.1 pA, a theoretical value close to the experimental value (11.6 pA), suggesting that the reduction of synaptic efficacy at the TC synapse of the RIM-DKO	extsuperscript{Sert} mice is mostly due to a presynaptic effect on the glutamate release probability. Consistent with a presynaptic modification of the TC transmission, we also observed an increased coefficient of variation (0.40 ± 0.04, n = 9 in control; 1.33 ± 0.013, n = 11 in RIM-DKO	extsuperscript{Sert} mice), and a change of the paired pulse ratio (PPR) which was estimated by using paired IC stimulation at several intervals between 20 ms and 1 s (Fig. 2b1,b2). At 20 ms, the PPR calculated as the ratio between the second and first EPSC amplitude (EPSC2/EPSC1) was significantly increased in the RIM-DKO	extsuperscript{Sert} (0.87 ± 0.08, n = 8 in control; 2.15 ± 0.37, n = 9 in the RIM-DKO	extsuperscript{Sert} mice; p < 0.01; Fig. 2b3).

To test whether the expression of the postsynaptic ionotropic glutamatergic receptors by the layer IV neurons was affected in RIM-DKO	extsuperscript{Sert} mice, single fiber IC stimulations were used in presence of gabazine (10 μM). The NMDA/AMPA ratio, measured at the peak of the NMDA component of the EPSCs at +70 mV and the AMPA component at −70 mV (Fig. 2c1,c2). This ratio was similar in controls (2.22 ± 0.37, n = 9) and RIM-DKO	extsuperscript{Sert} mice (1.77 ± 0.27, n = 11; Fig. 2c3), indicating that the relative proportion of postsynaptic AMPA and NMDA receptors...
Figure 3. Laminar and tangential distribution of TC axons in S1 of RIM-DKO<sup>Sert</sup> and RIM-DKO<sup>Emx1</sup> mice. a–c, To analyze the TC tracts and laminar distribution of TC axons in RIM-DKO<sup>Sert</sup> and RIM-DKO<sup>Emx1</sup> mice, SERT-immunocytochemistry was performed in coronal sections of P7 brains. In the three genotypes: control (a), RIM-DKO<sup>Sert</sup> (b), and RIM-DKO<sup>Emx1</sup> (c), thalamic axons reach the cortical layers VI and IV where they arborize into well delimited clusters (a’–c’). d–f, To evaluate the topographic map formed by TC axons in a tangential plane, vGlut2 immunostaining was performed on serial tangential sections from flattened cortical hemispheres of P7 mice. The sections through layer IV were photographed and analyzed. The TC clusters corresponding to the barrels of the principal whiskers in the PMBSF are clearly delimited in control (d) RIM-DKO<sup>Sert</sup> (e) and RIM-DKO<sup>Emx1</sup> (f) mice; Increase in the septal intervals between barrel rows in the RIM-DKO<sup>Sert</sup> (g) and RIM-DKO<sup>Emx1</sup> (h) mice; Absence of septal septa, quantification method: the PMBSF area was delimited by joining the external boundaries of the vGlut2 stained patches corresponding to the five rows of the main vibrissa (delineated in pink). The area covered by the individual TC patches/barrels was measured (white areas) and the septal area was calculated as the difference between these two areas (blue area). h, k, histograms showing the summed area of each individual patch in the PMBSF, normalized to area measured in the control, in the RIM-DKO<sup>Sert</sup> (j) and the RIM-DKO<sup>Emx1</sup> (l) mice; k, l, quantification of the septal/PMBSF area ratio in RIM-DKO<sup>Sert</sup> (k) and RIM-DKO<sup>Emx1</sup> (l) mice. AL: anterolateral barrel subfield, dg: digits; PMBSF: posteromedial barrel subfields, A–E: barrel rows, 1–5: barrel columns. Scale bars: a, 500 μm; a’, 100 μm; d, 1 mm.

Deletion of RIM genes in the thalamus or cortex does not affect the somatosensory topographic map made by incoming TC axons

The development of a normal architecture of the barrel cortex comprises two coordinated events: first, the formation of periphery-related patterns by the sensory TC afferents, and second, the clustering of layer IV neurons. The emergence of TC periphery-related patterns begins by P3 in mice. Patterning is first noted in PMBSF and then by P5 in the anterior lateral barrel subfield (ALBSF) that corresponds to the large whiskers, which labels the TC axons as a population at the sinus hair representations (Senft and Woolsey, 1991; Rebsam et al., 2002).

We analyzed the general topography and laminar distribution of the developing TC axons by Sert immunohistochemistry which labels the TC axons as a population at P7 (Lebrand et al., 1998). We found that the laminar position of the TC axonal clusters in cortical layers IV and VI was unchanged in RIM-DKO<sup>Sert</sup> (n = 6) and RIM-DKO<sup>Emx1</sup> (n = 6) compared with control (n = 12) mice (Fig. 3a–c’).

To analyze the segregation of TC afferents into whisker-related patterns, vGlut2 immunostaining was performed on tangential sections from flattened P7 hemispheres in the RIM-DKO<sup>Sert</sup> and the
RIM-DKO<sup>Emx1</sup> mice (Fig. 3d–f). In all genotypes, clusters of TC terminals corresponding to the different body parts were observed. Both the principal whisker-related patterns of the PMBSF and the barrels corresponding to digits and the smaller TC axonal clusters of the ALBSF were sharply outlined (Fig. 3d–g). However, in the PMBSF of the RIM-DKO<sup>Sert</sup> mice, TC clusters appeared smaller with larger interbarrel space (called septae for convenience see Materials and Methods, Measures and cell counts; compare Fig. 3d,e). We measured the area of each TC cluster in the PMBSF as the dense patch of vGlut2-immunostaining (Fig. 3g). In the RIM-DKO<sup>Sert</sup> samples, the summed area of individual barrels was reduced by 22% compared with controls (Fig. 3h; p = 0.0077). To measure the septal area, we outlined the contours of the PMBSF and subtracted the area covered by the TC patches (Fig. 3j). The septal area was increased in the RIM-DKO<sup>Sert</sup> samples (Ctrl: 21 ± 0.6% RIM-DKO<sup>Sert</sup>; 29 ± 1% of the total PMBSF area, p < 0.001; Fig. 3k). Similar measures in RIM-DKO<sup>Emx1</sup> mice showed no change in the size of TC patches (Fig. 3i), or the size of the PMBSF, the area occupied by the TC terminals (RIM-DKO<sup>Emx1</sup>; 93 ± 3% of control, p = 0.54, Fig. 3i), or the septal regions (Ctrl: 24 ± 2%; RIM-DKO<sup>Emx1</sup>; 27 ± 1%; p = 0.29; Fig. 3i).

We further examined the maturation of TC synapses at the ultrastructural level. Because TC synapses represent only 10% of the total complement of asymmetric synapses in layer IV in the somatosensory cortex (Benshalom and White, 1986), anti-vGlut2 immunolabeling was used for their specific identification (Nahmani and Erisir, 2005). In 2 months old mice, TC vGlut2-labeled terminal boutons in layer IV were large, filled with round synaptic vesicles, and faced perforated postsynaptic densities in both control and RIM-DKO<sup>Sert</sup> mice (Fig. 4).

These results indicate that RIM function in either the thalamus or the cortex was not required for the formation byafferent TC axons of a somatosensory map with precise ordering of periphery related patterns. Lack of RIMs in thalamic neurons caused a reduction in the size of TC terminal clusters in layer IV of the barrel cortex, but no change in synapse formation and ultrastructure.

Deletion of RIM genes in the thalamus but not in the cortex causes a barrelless phenotype

Shortly after the arrival of TC axons in layer IV, spiny stellate neurons adopt a ring-like distribution around each TC axonal cluster (Rice and Van der Loos, 1977), becoming more densely packed on the external boundary of the clusters. We evaluated the organization and density of layer IV neurons with regard to the TC axonal clusters using the nuclear stains DAPI or Cux1 immunostaining that labels a vast majority of glomateric layer IV neurons (Nieto et al., 2004; Ferrere et al., 2006). In mice, where only RIM1, or only RIM2 isoforms were invalidated in the thalamus, the neurons in cortical layer IV formed normal ring-like arrangements around the TC clusters, including the smaller barrels corresponding to the sinus hair representations (Fig. 5a–c'). However, when both RIM1 and RIM2 were deleted from the thalamus, cortical neurons showed an even distribution in the barrel walls and center (Fig. 5d,d',f). To quantify these changes, the density of Cux1-positive neurons was measured in the C2 barrel (Fig. 5e–h). Neuronal density in the hollow was similar in control and recombined mice (Ctrl: 4873 ± 429 neurons/mm<sup>2</sup> RIM-DKO<sup>Emx1</sup>; 4777 ± 282 neurons/mm<sup>2</sup>), but was reduced in the barrel wall (Ctrl: 6815 ± 387 neurons/mm<sup>2</sup> RIM-DKO<sup>Sert</sup>; 5267 ± 278 neurons/mm<sup>2</sup>). The wall/hollow cell density ratio was greatly reduced in the RIM-DKO<sup>Sert</sup> mice (Fig. 5i; Ctrl: 1.42 ± 0.06 n = 6; RIM-DKO<sup>Sert</sup>: 1.1 ± 0.03, n = 8; p = 0.001). Thus, there is a barrelless phenotype in the RIM-DKO<sup>Sert</sup> mice.

Quantification in RIM-DKO<sup>Emx1</sup> mice (Fig. 5g) showed no change in the clustering of the layer IV neurons (wall/hollow cell density ratio: Ctrl: 1.3 ± 0.05 (n = 6); DKO<sup>Emx1</sup>: 1.35 ± 0.03 (n = 6); p = 0.58). In both RIM-DKO<sup>Sert</sup> and RIM-DKO<sup>Emx1</sup> mouse strains, barreloids were clearly visible in the thalamus by CO reaction (data not shown). The alterations in the organization of layer IV neurons in RIM-DKO<sup>Sert</sup> mice persisted throughout adult life, as observed with Nissl staining or by the uniform distribution of ROR<sup>B</sup>, a layer IV neuronal marker which expression level was not modified (data not shown).

These results indicated that RIM function is required in the thalamus but not in the cerebral cortex for the development of barrels in the somatosensory cortex. RIM function in this process appears to require both the RIM1 and the RIM2 genes, as deletion of either of these genes in the thalamus was insufficient to cause barrel formation defects.

Modified dendrite organization in the RIM-DKO<sup>Sert</sup> mice

The formation of barrels involves a reorganization of the dendrites of layer IV neurons that become polarized toward the center of the TC axonal patches, and acquire an asymmetric branching morphology (Rice and Van der Loos, 1977). To evaluate the dendritic organization of layer IV neurons, Golgi analyses were done in adult RIM-DKO<sup>Sert</sup> (n = 4) and control (n = 4) mice. Fifty control and 41 RIM-DKO<sup>Sert</sup> layer IV spiny stellate neurons were reconstructed and analyzed. One representative neuron for each genotype is shown in Figure 6 (Fig. 6a,b). Cells were qualified as asymmetric when >50% of the dendrites were distributed within one quadrant (Fig. 6a,d',b,b') as described previously (Datwani et al., 2002). The percentage of asymmetric spiny stellate neurons was smaller in RIM-DKO<sup>Sert</sup> than in control mice (Fig. 6c,d; 21% vs 70%; n = 4 each, p = 0.028, Mann–Whitney). The dendritic span of the spiny stellate neurons,
measured as the areas projected in a two-dimensional plane, was more than doubled in the RIM-DKOSert mice (12,205/μm²) compared with control mice (5885/μm²; p < 0.001).

This can be explained by the loss of asymmetry and by an increase in the length of dendrites visible in the Sholl analysis: dendrites of RIM-DKOSert mice cross circles up to 150 μm diameter around the cell soma, whereas in controls the limit is 120 μm. In addition, dendrites from RIM-DKOSert neurons had more intersections than controls with every circle from 50 to 90 μm (n = 4 for each genotype, p < 0.05 for 50 and 90 μm; p < 0.001 for 60, 70 and 80 μm; Fig. 6e). Moreover, a tendency for a moderate increase in dendritic branch endpoints was noted in the RIM-DKOSert mice (14.2 ± 0.7 dendrites/cell), compared with controls (13.4 ± 0.4 dendrites/cell; n = 4 each, p = 0.123, Mann–Whitney) with a reduced dendritic complexity, as the number of primary branches increased (control 3.3 ± 0.09 dendrite/cell; RIM-DKOSert 4.1 ± 0.1; n = 4 each, p = 0.029, Mann–Whitney) while the number of third and fourth degree branches was reduced (Fig. 6f). Finally, counts of the number of spines along randomly selected 20 μm segments of the secondary and tertiary dendrites of the layer IV
neurons (Fig. 6g,h) showed a significant increase in the density of spines in the RIM-DKO<sup>Sert</sup> compared with control mice [Fig. 6i, secondary: 17.4 ± 1.4 vs 11.5 ± 0.4 spine/20 μm for controls, n = 4 each, p = 0.0143; tertiary: 19.1 ± 1.5 (n = 3) vs 12.7 ± 0.7 for controls (n = 4) spine/20 μm, each, p = 0.0286; Mann–Whitney tests]. These results suggest that reduced efficacy of synaptic transmission at the TC synapse causes significant structural changes in the dendritic differentiation and organization of layer IV spiny stellate neurons, with a lack of acquisition of a polarized dendritic arborization, and an increase in branch and spine numbers.

**Sensory-evoked responses are altered in RIM-DKO<sup>Sert</sup> mice**

To evaluate the long term consequences of altered presynaptic release at the TC synapse on processing of somatosensory information, we performed in vivo voltage-sensitive dye (VSD) imaging of cortical activity evoked by tactile sensory stimulation. This visualization of cortical activity was unchanged in RIM-DKO<sup>Sert</sup> mice compared with controls (Fig. 7b,d). These results showed that although there is a significant reduction of the amplitude of the sensory driven responses in the RIM-DKO<sup>Sert</sup> mice, the spatiotemporal dynamics of the intracortical activity was unchanged.

We also tested the consequences of this reduced thalamic synaptic drive on the activity-modulated gene expression. Expression of immediate early genes such as c-Fos is triggered in the somatosensory cortex, and in layer IV in particular, by sensory exploration in naturalistic conditions (Staiger et al., 2000). Mice were placed in an environment containing several objects that differed by their textures and shape and c-Fos expression was evaluated 1 h later. To identify the response to sensory exploration versus nonspecific activation of cortical neurons, all whiskers were clipped on one side of the snout. In control mice, there was a fivefold increase in the number of c-Fos-positive neurons in the S1 region of the unclipped side relative to the clipped side (Fig. 8a,d,e). In RIM-DKO<sup>Sert</sup> mice, c-Fos expression in layer IV was strongly reduced in both the clipped and the unclipped sides (Fig. 8b,b',c), in comparison with controls, although a small de-
deflection in control and RIM-DKOSert mice. Typical responses are illustrated in the upper panel: the first images on the left show resting fluorescence of VSD-stained unilateral craniotomies. Adjacent images show the VSD fluorescence changes at different times following a single right C2 whisker deflection. Scale bar, 2 mm. Figure 7. Cortical sensory processing is altered in RIM-DKOSert mice. a, VSD imaging of cortical activity evoked by single whisker (C2) deflection in control and RIM-DKOSert mice. Typical responses are illustrated in the upper panel: the first images on the left show resting fluorescence of VSD-stained unilateral craniotomies. The squares indicate the regions of interest used to quantify the signal on the C2 barrel column of the primary somatosensory cortex (S1, red square) and in the primary motor cortex (M1, blue square). Adjacent images show the VSD fluorescence changes at different times following a single right C2 whisker deflection. Scale bar, 2 mm. b, Averaged responses from control mice and RIM-DKOSert mice, quantified from S1 (left) and M1 (right). c, Peak amplitudes of the sensory responses measured in S1. d, Ratio M1/S1 (means ± SD for each group).

gree of activation could be noted when comparing the clipped/uncropped side of the mutants, which may be related to the residual transmission in nonrecombined neurons (estimated to 4–11%, see Results, Region specific deletion of RIM genes). These results indicated that reduced transmitter release at the TC synapse profoundly impacts activity-dependent gene expression in the layer IV of the somatosensory cortex.

Overall, these experiments indicated that decreased neurotransmitter release at the TC synapse, diminished sensory-evoked responses, but had even stronger effects on the activity-dependent gene expression which was almost abolished.

Behavioral consequences in RIM-DKO<sup>Sert</sup> mice

To evaluate the functional consequences of the morphological and physiological changes of sensory processing on behavior, we began by examining motor exploratory behavior of the adult mice when placed in a familiar nonstressful context containing novel objects to explore. Total locomotor behavior, measured with video tracking, was unchanged in the RIM-DKO<sup>Sert</sup> (3.8 ± 0.4 mm/s n = 9) compared with controls (3.8 ± 0.3 mm/s n = 9; Fig. 8d), and animals whisked while exploring the novel objects. However, interestingly the time spent exploring these novel objects was increased in the RIM-DKO<sup>Sert</sup> (44 ± 16 s n = 9) compared with controls (25 ± 7 s; n = 8, p = 0.043; Fig. 8e). This increase is consistent with observations of increased thigmotaxis in rats that have undergone a lesion of the primary somatosensory cortex (Luhmann et al., 2005), and can be interpreted as indicating defective sensory integration. To further evaluate sensorimotor performance, we tested mice in the beam walking test, that has previously been noted to be disturbed in barrelless MAOA-KO mice and normalized in MAOA-5-HT1B DKO with rescued barrel formation (Salichon et al., 2001). During successful trials, the RIM-DKO<sup>Sert</sup> took a significantly longer time to traverse the beam (34.44 ± 9.3 s, n = 8, p = 0.037) compared with their control littermates (9.86 ± 4.3 s, n = 7; Fig. 8f). In addition, RIM-DKO<sup>Sert</sup> mice had a strong probability to fail crossing the beam on a given trial (29.6 ± 11.7%, n = 9, p = 0.016) compared with controls (no failure, n = 8; Fig. 8g).

These results indicated that although general motor activity was not visibly altered in the RIM-DKO<sup>Sert</sup>, sensorimotor integration was defective in these mice.

Discussion

Using regional ablation of the RIM1 and RIM2 proteins at TC or corticocortical synapses, we find that synaptic release at TC but not at corticocortical synapses is necessary for the formation of barrels as cellular specializations in the mouse neocortex. Our results also show that presynaptic control of TC transmission is dispensable for the formation of a topographic map by the incoming TC axons. Decreased evoked release at the TC synapse almost abolished sensory-evoked immediate early gene expression in the cortex.

Presynaptic release is required at TC but not corticocortical synapses for barrel formation

The importance of synaptic glutamate release for barrel development has previously been suggested in mutants with defective synaptic release at the TC synapse (Lu et al., 2006; Erzurumlu and Gaspar, 2012). However, a causal link was difficult to establish because of the pleiotropic effects of these mutations. Our observations stress the importance of the TC synaptic input for the refinement of cortical circuits in the barrel cortex, since the thalamus-specific deletion of RIM1 and RIM2 affected the cortical barrel structure whereas cortex-specific deletion did not. TC synapses have a high probability of release early in development, before barrel emergence (Laurent et al., 2002; Yanagisawa et al., 2009). This activity is driven by the thalamic afferents and the stimulation of the whisker pads (Minlebaev et al., 2011), and is therefore well suited to play a driving role in the establishment of a columnar organization in the cerebral cortex.

Because a vast majority of the glutamatergic synapses in the cerebral cortex and in the barrel field in particular, are cortico-cortical (Benshalom and White, 1986; Douglas et al., 1986) it appeared surprising that RIM deletion in excitatory neurons of the cerebral cortex in RIM-DKO<sup>Emx1</sup> had no visible effect on the normal development of the barrel architecture. This indicated that neurotransmitter release by intrinsic corticocortical barrel circuits, and subplate to layer IV connections which amplify th-
Our observations suggest that there is a functional redundancy of RIM1 and RIM2 proteins at the developing TC synapse. Indeed, both RIM1 and RIM2 genes are expressed in the VB during barrel development, and selective ablation of either RIM1 or RIM2 genes in the thalamus is insufficient to cause visible barrel alterations. This functional redundancy is consistent with previous observations investigating the role of RIM proteins in the control of key steps of presynaptic organization and function (Schoch et al., 2006; Kaeser et al., 2011).

Thalamus-specific removal of RIMs alter barrel development and dendritic maturation but not patterning of thalamic afferents

Barrel cortex development involves the coordinated maturation of the presynaptic and postsynaptic neuronal elements. Presynaptically, the TC axon terminals segregate into terminal patches, and postsynaptically, the spiny stellate neurons in layer IV become distributed around these TC patches and acquire a polarized orientation of their dendrites. The changes found in the RIM-DKO<sup>Sert</sup> indicated a dissociation of these two developmental processes: the TC patches were well segregated in contrast to the absence of cellular barrels, reminiscent of changes observed after cortical-specific deletion of glutamate receptors (review in Inan et al., 2006, Erzurumlu and Gaspar, 2012).

The observation of well segregated TC patches corresponding to whisker domains in the RIM-DKO<sup>Sert</sup> mice questions the notion that presynaptic plasticity mechanisms play a key role in the whisker-related patterning of TC afferents. This assumption was based on previous observations in mutants with defective presynaptic function (Laurent et al., 2002; Lu et al., 2006) and altered segregation of the TC axons. In MAOA KO (Cases et al., 1996; Rebsam et al., 2002) and AC1 KO mice (Welker et al., 1996; Gheorghita et al., 2006), TC axons invade the space between barrels. A proposed model for these changes was that TC afferents are selectively strengthened or destabilized based on their release probability (Lu et al., 2006). This implies that spike timing-dependent learning rules (long-term potentiation/depression) play a causal (instructive) role in TC axon segregation. However, in the RIM-DKO<sup>Sert</sup> the high incidence of synaptic failures at the TC synapses is likely to disrupt Hebbian spatio-temporal coding that strengthens coactive afferents and weakens others. Thus, it is more likely that axon guidance molecules, rather than synaptic consolidation, are the main drivers for clustering TC afferents into periphery related patterns. This interpretation is also supported by observations showing that signaling via presynaptic TC 5-HT1b receptors and AC1 modify axonal responses to guidance molecules such as the ephrins or netrin (Nicol et al., 2006; Bonnin et al., 2007).

However our observations do not completely rule out the role of neurotransmitter release in shaping terminal axons since there was a residual 30% release at the TC synapses of the RIM-DKO<sup>Sert</sup> mice. Moreover, spontaneous release may still occur, although it is strongly reduced in the absence of RIM proteins (Deng et al., 2011), and our present observations showed a reduced size of the individual TC axonal patches. A possibility would then be that either the residual glutamate release in TC axons of the RIM-DKO<sup>Sert</sup> is sufficient to enable axon growth-mediated mechanisms that form patch-like structures, or that a low synaptic activity equally affecting all TC inputs is not sufficient to perturb activity-dependent competitive interactions among all incoming afferents.

Interestingly, the space between the TC axonal patches was increased in RIM-DKO<sup>Sert</sup> mice, raising the intriguing possibility that this could result from a competition for cortical space be-
tween cortical and thalamic axons making synaptic contacts onto layer IV neurons. For instance, corticocortical afferents from callosal axons, or layer II/III neurons, occupy complementary domains to the TC afferents, in the septa of the barrel cortex (Koralek and Killackey, 1990; Sehara et al., 2010) whose maintained synaptic release properties may increase relative to the TC afferents in the RIM-DKO

The formation of barrels in the mouse somatosensory cortex involves the formation of a cylindrical array (“barrel”) of layer IV spiny stellate neurons, with neurons along the walls orienting their structural organization (Wong and Ghosh, 2002; Iwasato et al., 2008). 

Modifications of c-Fos and downstream signaling have previously been shown to be implicated in dendrite and spine structural defects observed in the barrel cortex. Together, these results implicate a major role for the neural activity imposed by the thalamic afferents rather that cortical afferents in sculpting cellular patterns and dendritic orientation of layer IV spiny stellate cells.

Functional consequences of thalamus-specific removal of RIMs

Deletion of the RIM genes from the sensory thalamus resulted in a permanent reduction of sensory-driven cortical activation, in voltage-sensitive dye experiments. However, the spatio-temporal patterning of the somatosensory-evoked responses was undisturbed. In contrast, stimulus-evoked changes in immediate early gene expression were profoundly altered, as shown by the lack of c-Fos expression in the somatosensory cortex of the RIM-DKO

Our results show that RIM1/RIM2-mediated synaptic release at the TC but not at the corticocortical synapses is necessary for the formation of barrels as cellular specializations in the mouse neocortex, but at the TC synapse. Together, these results implicate a major role for the neural activity imposed by the thalamic afferents rather that cortical afferents in sculpting cellular patterns and dendritic orientation of layer IV spiny stellate cells.

Conclusion

Our results show that RIM1/RIM2-mediated synaptic release at the TC but not at the corticocortical synapses is necessary for the formation of barrels as cellular specializations in the mouse neocortex, but that it is dispensable for the formation of a precise topographic map and for normal TC synapse development. Altogether, this indicated that the control of presynaptic release is crucial both for the development of the neuronal network structure and its function.

References


