The Guidance Molecule Semaphorin3A is Differentially Involved in the Arealization of the Mouse and Primate Neocortex

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The visual cortex is organized into discrete domains characterized by their specific function, connectivity, chemoarchitecture, and cytoarchitecture. Gradients of transcription factors across the anteroposterior and mediolateral axes of the neocortex have previously been demonstrated to specify the main sensory regions. However, they do not account for the establishment of multiple areas in the primate visual cortex, which occupies approximately 50% of the neocortical surface. We demonstrate that the guidance molecule Semaphorin3A (Sema3A) is initially secreted in the cortical plate of the embryonic marmoset monkey and acts as an intrinsic cue to control the migration of subpopulations of neuronal progenitors and projection neurons expressing the receptor Neuropilin 1 (Npn1). During the first 2 postnatal weeks, Sema3A expression becomes primarily associated with ventral visual cortical areas, leading to the specific migration of Npn1+ neurons in the late maturing visual areas. In the mouse, Sema3A distribution is not arealized, but Npn1 expression becomes restricted to the posterior neocortex at embryonic day 16.5. The selective reduction in the striate cortex we observe in Sema3A−/− animals potentially results from the differential distribution of Npn1+ cells. Therefore, the Sema3A/Npn1 pathway participates to the parcellation of the visual neocortex in both the mouse and the marmoset, however, through different regulatory processes.

Keywords: development, gene expression, marmoset monkey, migration cues, Neuropilin 1, visual cortex

Introduction

The neocortex is divided into functionally specialized and anatomically distinct areas, delineated by sharp variations in the cytoarchitecture and the cellular composition of the 6 cortical layers (O’Leary and Nakagawa 2002; Sur and Rubenstein 2005; Rash and Grove 2006). Each area is functionally unique within the sensory system it belongs to and form species-specific connectivity maps. However, the general neocortical organization is similar across mammalian species (Manger 2005), suggesting conserved mechanisms of specification. With the evolutionary expansion of the neocortical surface (Rakic 2009) to include more cortical areas in higher species (Kaas 1997), cortical maps have become more complex. Therefore, nonhuman primate models provide a more correlative insight into the neurodevelopment and arealization of a complex neocortex, whereas rodent models enable us to look at the gene modification of neocortical development, and at an earlier stage of evolution. The marmoset monkey (Callithrix jacchus), which neocortical surface has been fully mapped by extensive electrophysiological and cytoarchitectonic studies (Rosa and Schmid 1995; Fritsches and Rosa 1996; Rosa and Tweedale 2000, 2005; Rosa et al. 2000; Bourne et al. 2007; Mashiko et al. 2012), has become a prominent model for neocortical studies. The smooth surface and isotropic growth of the marmoset neocortex, and its protracted period of corticogenesis, make it an ideal model for developmental studies. In addition, the germinal zones comprise more compartments than the mouse to generate the larger neocortex (Garcia-Moreno et al. 2012). Cortical lamination occurs in a sequence spanning over several weeks, from embryonic day (ED) 60 to birth (ED145), compared with days in the mouse (Angevine and Sidman 1961; Bayer and Altman 1991; Missler et al. 1993). Analysis of the expression of the maturation marker nonphosphorylated neurofilament during arealization of the marmoset visual cortex demonstrated that areas form sequentially, following the hierarchical of functional processing (Bourne and Rosa 2006). However, the exact genetic and molecular regulators underlying cortical patterning and parcellation remain largely unknown, in both primate and rodent models (Rash and Grove 2006).

Loss of function studies in the mouse demonstrated that gradients of morphogens and transcription factors control the initial steps of neocortical parcellation by conferring regional identity (Bishop et al. 2000; O’Leary and Nakagawa 2002; Lukaszewicz et al. 2006). Guidance molecules of the Eph/ephrin family are also differentially expressed between the primary visual area (V1; striate cortex) and the adjacent secondary visual area (V2) in the developing macaque and marmoset neocortex (Donoghue and Rakic 1999a,b; Teo et al. 2012). These data suggest that cortical parcellation occurs by differential regulation of neuronal migration during lamination, leading to the establishment of the discrete cytoarchitectonic characteristics observed in each area.

Members of the Semaphorin family of guidance molecules are key regulators of cortical development, in particular, the secreted Semaphorin3A (Sema3A; Kolodkin et al. 1993). Sema3A is expressed in the mouse ventricular (VZ) and subventricular zones (SVZ) and the outer cortical layers from ED14 to postnatal day (PD) 0 (Giger et al. 1996; Polleux et al. 2000). Sema3A binds to Neuropilin 1 (Npn1) and forms a holoreceptor complex with the Plexins (Kolodkin et al. 1997) to promote pyramidal neuron (Chen et al. 2008) and interneuron (Zimmer et al. 2010) migration to layer 2 by inducing dendritic elongation and axonal growth cone collapse (Polleux et al. 2000; Shelly et al. 2011). Knocking down elements of the receptor complex in neural progenitors targeted to layer 2, including Npn1, PlexinA1-4 or -D1, has been demonstrated to result in aberrant migration patterns and thinning of layer 2 (Chen et al. 2008).

In the present study, we use a combination of data from the mouse and marmoset to define the involvement of Sema3A in neocortical area specification, and how this has continued through expansion of the neocortex. We demonstrate that Sema3A switches from a homogenous distribution in the marmoset embryonic cortical plate to an area-specific profile in the early postnatal visual neocortex, selectively guiding late-born Npn1+ neurons.
neurons into specific maturing areas, before being downregulated. We reveal that, in the mouse, deficiency of Sema3A results in a reduction in the volume of the visual neocortex and enlargement of anterior cortical fields. Therefore, we conclude that Sema3A contributes to neocortical area specification in a mechanism that is conserved throughout evolution and argue for an additional species-specific role of Sema3A to support the genesis of additional visual neocortical areas in higher-order mammals.

Materials and Methods

Animals

Marmosets

Cerebral tissues of 13 marmoset monkeys (C. jaccus) of either sex were collected for the study. All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the Monash University Animal Ethics Committee.

Mice

Brains of 7 semaphorin III/D (lacZ) targeting mice (No RBRC01105) (Taniguchi et al. 1997) comprising 4 homozygotes and 3 littermates controls aged PD 28 were purchased from the Riken BioResource Center (Ibaraki, Japan). Brains of 12 embryonic and 2 newborn C57BL/6 mice were used for the expression profile analyses.

Tissue Preparation

Marmosets received a lethal dose of sodium pentobarbital (100 mg/kg) and were transcardially perfused with 0.1 M heparinized phosphate buffer (PB, pH 7.2) containing 0.1% sodium nitrite (vasodilator), warmed to 37°C, followed by a fixative solution of 4% paraformaldehyde (PFA) in 0.1 M PB. Mouse embryos harvested from time-mated females and newborn pups were quickly decapitated and the heads collected in ice-cold PBS. All brains were postfixed in 4% PFA for 24 h, cryoprotected in increasing concentrations of sucrose (10–30%), frozen in isopentane cooled to −45°C, and subsequently stored at −80°C. Parasagittal and coronal sections (40 µm) were cut on a cryostat, collected in a serial order and stored at −20°C in a cryoprotectant solution (50% 0.05 M PB, 30% ethylene glycol, and 20% glycerol) for immunolabeling, and in methanol for in situ hybridization. Embryonic brains were cut at 20 µm, the sections collected on Superfrost slides and kept at −80°C.

Histology

For each animal, one series was reacted for Nissl substance staining using a 0.05% cresyl violet solution to enable the demarcation of the neocortical layers. Sections were stained until layer 4 was discernible from other layers.

Immunolabeling

Free-floating serial sections were washed in 0.1 M sodium phosphate buffer (PBS; Dulbecco), and slide-mounted sections were rehydrated in PBS, before being incubated at room temperature with 4% Normal Horse Serum (Gibco, Invitrogen), 0.15% bovine serum albumin (BSA) (fraction V; Sigma), and 0.1% Triton X-100 (Sigma) for extracellular epitopes and 0.3% Triton X-100 for intracellular epitopes. For peroxidase immunostaining, sections were treated with 0.3% hydrogen peroxide in 50% methyl alcohol made up with PBS for 30 min before being incubated in the blocking solution. The following primary antibodies were incubated overnight at 4°C in the blocking solution: Mouse anti-rat βIII Tubulin (1:500; Millipore), mouse anti-mouse NeuN (1:500; Millipore), mouse anti-mouse nonphosphorylated neurofilament (NNF; 1:1000; Covance), sheep anti-human Neuroplelin1 (1:400; R&D Systems), rabbit anti-mouse Pax6 (1:300; Covance), rabbit anti-human Sema3A (1:400; Abcam), rabbit anti-mouse Tbr1 (1:200; Abcam), rabbit anti-mouse Tbr2 (1:200; Millipore), and mouse anti-mouse TuJ1 (1:200; R&D Systems). Sections were then washed with 0.1% Tween-20 (Sigma) in PBS prior to incubation with appropriate fluorescently conjugated secondary antibodies: Alexa Fluor 594 and 488 goat anti-rabbit IgG, Alexa Fluor 488 donkey anti-sheep IgG (H+L), Alexa Fluor 594 and 488 goat anti-mouse IgG (H+L) (1:800; Molecular Probes), or with a biotinylated goat anti-rabbit secondary antibody (1:500; Dako). Fluorescently stained sections were subsequently incubated with Hoechst solution (Dako) to stain the nuclei, washed in PBS, and mounted with Dako mounting media and coverslipped. Sections processed for peroxidase immunostaining were incubated with avidin–biotin–horseradish peroxidase (1:200; Amersham Pharmacia Biotech) in PBS. Immunoreactivity was revealed using a metal-enhanced chromogen, 3,3′-diaminobenzidine (DAB), and a stable peroxide buffer (Pierce Biotechnology Inc.) for 2–10 min for visualization. Sections were then mounted onto Superfrost slides, dehydrated in graded alcohols, defatted in xylene, and coverslipped with DPX (British drug house). Negative controls were performed routinely by omitting the primary antibody, for which no staining was observed.

The specificity of the rabbit anti-human Sema3A antibody was validated on 3-month-old marmoset whole-brain lysate. The western blot revealed a single band at the predicted size of 95 kDa. Preincubating the antibody with a 10-fold molar excess of recombinant protein abolished the staining on marmoset PD3 sections.

Probes

The marmoset Sema3A (NCBI gene ID : XM_002807042.1) riboprobe (length: 785 bp) was designed to overlap the probe designed for mouse homologous gene used in the Allen Developing Mouse Brain Atlas (http://developingmouse.brain-map.org/) using the forward primer: AACAAGGCTTCTGCTAG and reverse primer: TTGCTTTGCGATGGT. An adjacent series of sections were systematically hybridized with the sense probe to validate the specificity of the signal observed. Mouse Sema3A and Sema6A probes were gifts from Dr Alain Chedotal, and Sema3C and Npn1 probes gifted from Prof. Andreas Puschel.

In Situ Hybridization

For single-color nonradioactive in situ hybridization (ISH), all solutions were treated with diethylyl pyrocarbonate (Sigma). Slide-mounted and free-floating marmoset and mouse sections were rehydrated in PBS, postfixed in 4% PFA 10 min, rinsed in PBS, treated with Proteinase K (2 µg/ml) in 0.1 M Tris–HCl pH 8, 50 mM ethylenediaminetetraacetic acid (EDTA) at 37°C for 10 min, followed by 5 min in 4% PFA, washed in PBS, acetylated for 10 min, and rinsed in PBS. Sections were then preincubated in hybridization solution: 50% formamide, 10% dextran sulfate (Amresco), 500 µg/ml polyadenylic acid potassium salt (Sigma), 50 µg/ml yeast t-RNA (Sigma), 50 mM dithiothreitol (Sigma); and a salt solution containing 300 mM NaCl, 10 mM Tris base (Sigma), 10 mM sodium phosphate (Sigma), 5 mM NaEDTA, 0.2% Ficol 400 (Sigma), and 0.2% polyvinylpyrrolidone (Sigma). The probes (250 ng/ml in the solution) were hybridized overnight at 62°C. The next day, slides were washed in 50% formamide (Sigma), 2x SSC, 0.1% Tween 1 h at 62°C; in maleic acid buffer containing tween 20 (MABT) (0.1 M maleic acid, 0.15 M NaCl, 0.2 M NaOH, 0.1% Tween 20, pH 7.5) for 3 h at room temperature, blocked in MABT, 20% normal goat serum for 2 h at room temperature, and incubated with anti-digoxigenin-alkaline phosphatase Fab fragment (1:2000; Roche) overnight at 4°C. On the third day, the slides were washed for 4 h in MABT at room temperature, equilibrated in staining buffer (0.1 M Tris–HCl, 0.1 M NaCl, 10 mM MgCl2, 0.1% Tween 20, pH 9.5) for 10 min and then incubated with nitroblue tetrazolium 5-brom-4-chloro-3-indolylphosphate solution (NBT-BCIP) (1:50; Roche) at room temperature in the dark for up to 24 h.

Stripe Assay

Neural progenitor cells isolated from marmoset neonatal V1 as previously described (Homman-Ludiy et al. 2012) were dissociated and plated (2 x 10⁴ cells/ml) on glass coverslips precoated with polylysine (0.5 mg/mL, Sigma). Human recombinant Sema3A-fraction crystallizable (Fc) (100 µg/ml, R&D Systems); native or heat inactivated for 30 min at 75°C and BSA-Alexa488 (20 mg/ml, Invitrogen); was injected into a silicone matrix (obtained from Martin Bastmeyer; Karlsruhe University, Germany) to generate 50 µm wide alternating
stripes. The entire surface of the coverslip was subsequently coated with laminin (20 µg/mL, Sigma). Cells were cultured for 18 h in Neurocult NS-A differentiation medium (StemCell Technologies) supplemented with 3% normal goat serum (Gibco), fixed with 4% PFA, and labeled with an antibody against β-III Tubulin as previously described. Fluorescent photomicrographs of 6 nonoverlapping fields (2.25 mm²) per coverslip were acquired; the numbers of nuclei on the stripe and in the interstripe space were counted using the cell counter tool of the software Fiji (http://fiji.sc/wiki/index.php/Fiji). Nuclei that were both on the stripe and the interstripe were excluded from the counting. Statistical analyses of the data were performed using GraphPad Prism 5.0b for Mac OS X.

**RT-PCR**

Total mRNA were extracted from the neural progenitors used in the stripe assay, with the RNAqueous®-Micro Kit (Ambion). The cDNA library was generated using the SuperScript® III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer’s recommendations and used as a template to amplify the transcripts of interest by polymerase chain reaction using specific primers (Table 1) and the GoTaq® Green Master Mix (Promega).

**Image Processing**

Sections were examined using a Zeiss Axioplan imaging microscope. Low-power photomicrographs (1300 × 1030 dpi) were taken using a Zeiss Discovery V20 stereomicroscope with an Axioacam HRc digital camera connected to the Axiovision 4.7.1 software (Zeiss). High-power photomicrographs (1300 × 1030 dpi) were taken using a Zeiss Imager. Z1 microscope fitted with the Apotome system allowing 3-dimensional analysis, with an AxioCAM HRm digital camera connected to the Axiovision 4.8.2 software (Zeiss). The Z-stacks (Fig. 1G, H) were acquired with a Leica SP5 confocal microscope. Adobe Photoshop CS3 10.01 was used to crop, adjust contrast and assemble images, which were subsequently compiled and annotated using Adobe Illustrator CS3 13.0.2.

**Volume Estimation**

Total cortical and area volume were estimated according to the Cavalieri principle using the software Fiji. A grid comprising of cross markers was applied to the region of interest, each marker representing a surface. The number of markers was then counted for each region of interest using the cell counter tool, and the total volume was calculated by multiplying the total surface (the number of markers × individual surface area) by the interval between sections. The volume of the total neocortex was greater than that of individual neocortical areas and required different parameters to be used. Therefore, a surface of 250 000 mm² and 400 µm interval between sections were used to estimate the total neocortical volume, and a surface of 35 105 mm² and 160 µm interval were used for individual neocortical areas. Statistical analyses of the data were performed using the GraphPad Prism 5.0b for Mac OS X.

**Results**

**Expression of Sema3A and Npn1 in the Early Developing Marmoset Visual Cortex**

At ED90, the marmoset neocortex is still in the preliminary stages of development; the cortical plate is uniform and does not exhibit the sharp borders delineating the cortical areas in the adult brain (Fig. 1A). High magnification of the foveal region of the primary visual cortex (V1; Fig. 1A, boxed region) illustrates the absence of lamination in the cortical plate and broad VZ/SVZ indicative of intense neurogenesis (Fig. 1A). In this region, *Sema3A* transcripts, revealed by in situ hybridization, were distributed from the VZ to the outer SVZ with less-intense labeling detected in the internal fiber layer (Fig. 1C). Similar to previous reports in the mouse, *Sema3A* transcripts also accumulated in the emerging cortical plate. The protein exhibited a more restricted distribution profile and was only found in the cortical plate and the subplate (Fig. 1D) in a high pial to low ventricular gradient, similar to previous reports in the mouse (Chen et al. 2008). The ligand was associated with TuJ1+ fibers (Fig. 1F), suggesting that Sema3A is secreted through the cell body as well as their neurites.

Npn1, the binding domain of Sema3A receptor complex, was also present in the presumptive V1 region, expressed by radially and tangentially oriented cells confined to the SVZ (Fig. 1E) where expression of the transcription factors Pax6 (Fig. 1G) and Tbr2 (Fig. 1H) indicate intense neurogenesis. Using confocal Z-stacks, we characterized the presence of Npn1-Pax6 and Npn1-Tbr2 double-positive cells, demonstrating that Npn1 is expressed by radial glia (Fig. 1G) and neural progenitors (Fig. 1H), respectively, in the embryonic marmoset inner SVZ.

To ensure consistency and reproducibility across observations, the data presented correspond to the region outlined in Figure 1A; however, the distribution profile of the ligand and the receptor was uniform throughout the anteroposterior axis of the neocortex and did not exhibit a region-specific distribution.

**Conserved Function of Sema3A in the Marmoset Visual Cortex**

In the rodent neocortex, Sema3A is bifunctional, capable of attracting the apical dendrite of migrating pyramidal neurons while repelling their axon (Polleux et al. 2000; Shelly et al. 2011). The function of Sema3A has not previously been investigated in the nonhuman primate, and therefore, we tested in vitro if Sema3A properties were conserved between orders. Neuronal precursor cells isolated from the neonatal marmoset V1 and maintained in culture as neurospheres (Homman-Ludiiye et al. 2012), were dissociated to single cells and plated onto coverslips coated with stripes of human recombinant Sema3A-Fc, native or heat inactivated (control) (Fig. 2A, B). We confirmed by reverse-transcription polymerase chain reaction [reverse transcription polymerase chain reaction (RT-PCR)] that the cells expressed the principal elements of Sema3A receptor complex, *Npn1, Plexin-A1*, and *Plexin-A4*, prior to plating (Fig. 2E). After 18 h, culture on coated coverslips, the distribution of β-III Tubulin+ undifferentiated precursors did not appear to be affected by stripes of heat-inactivated Sema3A (Fig. 2D), and the number of nuclei on or off the stripes was not significantly different (11.3 ± 1.4 ON and 8.2 ± 0.4 OFF;
In vitro data combined with the receptor and ligand expression profile suggest that Npn1+ neurons, born in the SVZ, migrate into the cortical plate, attracted by Sema3A secreted by earlier neurons.

**Sema3A and Npn1 Expression Persists Once Layers of Marmoset V1 Are Apparent**

At ED110, the 6 cortical layers have emerged as revealed by nuclear staining (Fig. 3A). However, the layers have not yet reached their full span or cellular density, which combined with a broad SVZ indicates ongoing migration of cells into the supragranular layers at this stage. Sema3A transcripts were uniformly distributed throughout all areas of the neocortex. In the radial dimension, a gradient was observed with a more intense signal observed in the superficial layers.
excluding layer 1, which was mostly devoid of staining, and a reduced signal in the deeper layers, 4–6, and in the subplate (Fig. 3B). The profile of the protein distribution across the cortical layers correlated with that of the transcripts (Fig. 3C), but the protein was not detected in the subplate. Sema3A punctate staining decorated cell processes extending from...
NeuN+ cell bodies and outlined the already dense network of basal and apical dendrites in layer 5 (Fig. 3C′, arrowhead). The population of cells expressing the receptor Npn1 in both the SVZ and the subplate extended long processes radially, into the developing cortical layers (Fig. 3D), and were positive for Tbr1 (Fig. 3D′), a transcription factor transiently expressed by neurons committed to a pyramidal fate (Englund et al. 2005).

For the remainder of the marmoset study, we focused on the distribution of the protein Sema3A revealed by immunolabeling, as the distribution of the protein is more informative and representative of the localization of ligand activity than that of its transcript.

Sema3A Adopts a Layer-Specific Pattern Following Lamination of the Marmoset V1

At ED130, when the lamination of the marmoset V1 is complete (Missler et al. 1993), the neocortical layers are readily observed by nuclear staining (Fig. 4A, Hoechst). Sema3A distribution became restricted to discrete layers with depletion of the ligand in layer 4 and accumulation in layer 5 (Fig. 4A). In layer 5, Sema3A was associated with the dense network of basal dendrites and the large apical dendrite emerging from Sema3A+ pyramidal neurons (Fig. 4A′). Sema3A+ apical dendrites extended through the Sema3A-negative layer 4 toward the supragranular layers (Fig. 4A).

The border between V1 and V2 became apparent at ED130, as revealed by the characteristic change of layer 4 thickness (Fig. 4B). Despite the acquisition of area identity, Sema3A remained consistently distributed across the neocortex and did not exhibit area-specific profile (Fig. 4B′).

Npn1+ fibers extended from the SVZ as described at earlier stages (Fig. 4C). Npn1+ cell bodies distributed from the SVZ across to the infragranular layers. Tbr1+ cells were restricted to layer 6 as previously described for the mouse (Englund et al. 2005) and Npn1+ cells that migrated beyond layer 6 into layer 5 did not co-label for Tbr1 at this stage (Fig. 4C′).

Sema3A Is Progressively Downregulated in the Postnatal Marmoset Visual Cortex

The sequence of postnatal maturation of the marmoset visual areas has been recently established using the expression profile of NNF, which specifically labels a subset of mature pyramidal neurons (Bourne and Rosa 2006; Bourne 2010). We analyzed the expression profile of Sema3A at PD 3, 7, and 14 in the: primary visual area (V1); secondary visual area (V2); middle temporal area (MT); and, dorsomedial area (DM), MT and DM forming part of the early maturing dorsal visual processing stream; and area V4, which is part of the ventral visual processing stream (Fig. 5; schematic).

At PD3 and 7, Sema3A expression was restricted to a few cells in layers 5 and 6 (Fig. 5A, B). At PD14, Sema3A was absent from V1 (Fig. 5C). In adjacent V2, the expression pattern revealed stronger labeling at PD3 compared with V1 (Fig. 5D). The density of Sema3A+ cell bodies and neuropil was greater in layers 2/3 compared with layer 5. At PD7, Sema3A+ cell bodies were distributed across layers 2, 3, and 5, but the density of neuropil staining in the supragranular layers was reduced when compared with PD3 (Fig. 5E). Large apical dendrites emerging from layer 5 could be observed crossing the...
Sema3A-negative layer 4 to reach layer 3. As with V1, at PD14, no labeling was observed in V2 (Fig. 5).

Previous research has demonstrated that area MT exhibits cellular characteristics of a primary cortical area in terms of its development (Bourne and Rosa 2006). At PD3, Sema3A immunolabeling in area MT revealed expression in layers 5 and 6 and intense cellular staining in layers 2 and 3 (Fig. 5G). Sema3A+ pyramidal neurons in layer 5 were darkly stained and exhibited a large cell body, extending a dense network of basal dendrites as well as a thick apical dendrite that could be traced all the way to layer 2. At PD7, Sema3A expression was downregulated in area MT (Fig. 5H), with only a few large

Figure 5. Area-specific expression of Sema3A in the marmoset visual cortex at PDs 3, 7, and 14. Sema3A was restricted to layers 5 and 6 in the primary visual cortex (V1) at PD 3 (A) and 7 (A–C), and downregulated at PD14 (C). The V2 exhibited a bilaminar profile at PD3 (D, E, F) and PD7 (E) with a dramatic loss of expression at PD14 (F). In area MT, the ligand was distributed in layers 2, 3, and 5 at PD3 (G, H, I), restricted to layer 5 at PD7 (H) and downregulated at PD14 (I). Area DM did not express Sema3A at the 3 studied stages (J, K, L). Area V4 strongly expressed Sema3A at PD3 and 7 (M, N, O), subsequently downregulated at PD14 (O). DM: dorsal medial area; MT: middle temporal area; V1: primary visual area; V2, V4: secondary visual area. At PD7, the border between V1 and V2, easily discernible in the Hoescht-stained sections (P), delineated a sharp boundary of Sema3A expression, revealed by immunolabeling (Q). Npn1+ cells selectively accumulated in the ligand enriched V2 (R). Expression of the receptor Npn1 was also detected in the white matter. Npn1+ cells in the cortical layers expressed the transcription factor NeuN, confirming their neuronal identity (S, S′). Scale bar (A–O) (in L) 300 µm, (P, R) 400 µm, (S, S′) 5 µm
pyramidal neuron cell bodies observed in the deep layer 5 and their apical dendrite extending into layer 2. In addition, Sema3A expression was not observed at PD14, (Fig. 5I), similar to areas V1 and V2.

Unlike the other visual cortical areas examined, the dorsal visual stream associated area DM did not exhibit any Sema3A expression following birth apart from negligible expression detected on cell bodies in layer 5 and their apical dendrite (Fig. SJ,K,L).

Visual area V4, which belongs to the ventral visual processing stream, revealed intense Sema3A staining at both PD3 and 7 (Fig. 5M,N). The pattern was similar to that described in area V2, associated with large pyramidal neurons in layer 5 and smaller cell bodies, densely distributed in the supragranular layers 2 and 3. The intensity of the staining was sustained at PD7, where thick darkly labeled apical dendrites could be observed emerging from layer 5 and extending into layers 2 and 3 (Fig. 5N). As with the other visual areas analyzed, Sema3A expression was downregulated in V4 by PD14 (Fig. 5O).

In summary, before birth and during cortical lamination, there was no distinction between the presumptive visual cortical areas in terms of their Sema3A expression, but a distinct change in the laminar profile of Sema3A occurred at birth. Following birth, Sema3A was differentially expressed across the maturing marmoset visual cortex, exhibiting strong staining in late maturing ventral stream areas and weaker staining in early maturing primary areas, while some areas were totally devoid of expression. Sema3A was downregulated in all areas analyzed at PD14.

We next investigated whether the area-specific distribution of Sema3A correlated with the route of Npn1+ neuron migration. At PD7, the border between area V1 and V2 is easily demarcated, by changes in nuclear staining (Fig. 5P) and minimal Sema3A labeling in V1 (Fig. 5Q). Npn1-expressing neurons revealed by coexpression of NeuN and Npn1 (Fig. 5S, S’), were distributed differentially across the V1/V2 border where they specifically accumulated in the Sema3A-enriched V2, and were absent from V1, except for cells located in the white matter (Fig. 5R). These observations are consistent with a model in which late Npn1+ neurons are recruited to V2 by selectively downregulating Sema3A expression in the early maturing areas. Sema3A did not appear to specify areal borders in the early cortical plate, but contributed to area formation in the postnatal cortex. To validate this hypothesis, we proposed to analyze the consequences of the loss of Sema3A on cortical area specification. There are currently no primate models available for Sema3A loss of function. Consequently, we analyzed the parcellation of the visual cortex in a Sema3A−/− mutant mouse line (Taniguchi et al. 1997).

Loss of Sema3A Alters Cortical Patterning in the Mouse Model

Few homozygous mutants survived until adulthood and therefore, analyses were performed at PD28, which allows for the maturation of the visual areas and the upregulation of the marker NNF. NNF labels a subpopulation of mature pyramidal neurons and has been successfully used to demarcate cortical areas in numerous species including the mouse (Van der Gucht et al. 2007), cat (Van der Gucht et al. 2001), ferret (Homman-Ludiy et al. 2010), and marmoset (Bourne et al. 2005, 2007; Bourne and Rosa 2006).

The entire neocortex of Sema3A KO was compared with wild-type littermates. We confirmed that the lamination of V1 was similar in Sema3A−/− mice compared with control littermates (data not shown), and there was a similar distribution of layer markers Ctip2 and Tbr1. Quantification of the number of NeuN+ neurons per mm² of V1 surface did not reveal significant difference between mutant and wild-type animals (data not shown).

In wild-type animals at PD28, NNF staining profile in V1, medial portion of V2 (V2M), and lateral V2 (V2L) was similar to that previously described (Van der Gucht et al. 2007). In brief, in V1, NNF was detected in layers 3 and 5 (Fig. 6A), labeling large pyramidal cell bodies surrounded by a dense neuropil. Small immunolabeled neurons were also present in layer 4, which were crossed by large apical dendrites emerging from layer 5 (Fig. 6C). The V2M, located dorsal to V1, was characterized by a decrease in the density of darkly stained multipolar neurons in layer 3 as well as a less dense network of NNF+ fibers compared with V1 (Fig. 6C). This enabled the delineating of the border between the 2 areas. The border between V1 and more ventrally located V2L was readily demarcated by the dramatic reduction of NNF staining in layer 3 (Fig. 6A).

In the Sema3A mutant mice, NNF laminar distribution was not affected (Fig. 6B); however, the intensity of the labeling was markedly reduced compared with wild-type animals (Fig. 6A). Observation at higher magnification revealed that NNF+ neurons in layer 5 of V1 were less abundant in the mutant (Fig. 6D) than in wild-type animals (Fig. 6C), but their morphology was normal with darkly stained cell bodies extending a broad apical dendrite through layer 4 and a ramified neuropil network. However, NNF+ cell bodies in layer 3 of V1 failed to extend their basal dendritic tree in the mutant mice, leading to a dramatic reduction in the neuropil labeling in mutants when compared with wild-type animals. NNF expression in the adjacent area V2M was strongly affected by the loss of Sema3A. The density of immunoreactive cell bodies, as well as neuropil staining, was dramatically reduced in both layers 3 and 5 when compared with wild-type animals (Fig. 6C, D). Area V2L demonstrated a lighter expression of NNF in wild-type animals, mostly concentrated in layer 5 (Fig. 6A), while mutant animals demonstrated a reduced number of NNF+ neurons in layers 3 and 5 of V2L (Fig. 6B) associated with a lighter neuropil staining. This suggested a marked reduction in dendritic extension in the absence of Sema3A. The reduction of NNF staining was restricted to the posteriorly located visual areas and did not affect anterior cortical fields, including the representation of the barrel field of the primary somatosensory cortex (S1BF), which demonstrated similar distribution and intensity in wild-type and Sema3A−/− animals (Fig. 6E,F).

In addition to the altered cellular morphology within the visual cortex, investigation of the areal volume suggested a reduction in the size of each visual cortical area. The overall volume of the neocortex did not alter between the wild-type and mutant animals (Fig. 6G). However, the volume of V1 was significantly decreased by >26% (n = 4, P < 0.02, Mann–Whitney test) in mutant animals compared with their wild-type littermates (Fig. 6H). Comparatively, the volume of S1BF was significantly increased by 16% (n = 4, P < 0.01, Mann–Whitney test) in the mutant compared with wild-type animals (Fig. 6H). These results (modeled in Fig. 6I) suggest that loss of Sema3A causes a reduction in the neocortical...
surface dedicated to visual processing and a consequential enlargement of the representation of the somatosensory cortices, with the total neocortical volume remaining constant. Sema3A might, therefore, act as a patterning factor required for the adequate development of posterior visual domains. To characterize how Sema3A exerts its patterning function, we next analyzed the expression profile of Sema3A and Npn1 at 3 key stages of mouse corticogenesis.
Regionalization of Npn1 Expression, but not Sema3A, During Mouse Corticogenesis

First, we analyzed the distribution of Npn1 and Sema3A transcripts at ED14.5, the onset of neuronal migration in the neocortex. Coronal sections taken at 2 different levels along the anteroposterior axis of the brain (Fig. 7; schematic, left hemisphere) reveal similar levels of Npn1 expression in the presumptive visual and somatosensory cortices (Fig. 7A, B, D, E, arrow). The ligands Sema3A (F), Sema3C (G), and Sema6A (H) are homogenously distributed along the anteroposterior axis. Sema3A transcripts accumulate in the VZ and the CP (Fig. 7F). Sema3C is distributed between the SVZ and the SP as well as the hippocampus and the EC. Sema6A is found in the subplate (V, arrow) and in a discrete band in the deep CP (V, open arrow). At P0, Npn1 is downregulated in the cortex (Fig. 7I, I'), but expression is sustained in the hippocampus. Sema3A is strongly expressed in the striatum and in the neocortex, (J, J') concentrated in the supragranular layers and large cell bodies in layer 5 (J'). At PD0, Sema3A exhibits strong signal in the hippocampus, the CC, and the EC and also labels cells in the supragranular layer 3 (K). Sema6A is strongly expressed in the hippocampus and the EC. Signal is also detected in the cortical layers with higher intensity observed rostrally (L). Section levels are illustrated on the schematic. CC: corpus callosum; CP: cortical plate; EC: external capsule; Hipp: hippocampus; MZ: marginal zone; SC: somatosensory cortex; SP: subplate; SVZ: subventricular zone; V: ventricle; VC: visual cortex; VZ: ventricular zone. Scale bar 600 µm.

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Previous reports suggest that Npn1+ neurons targeted to layer 2 are born at that stage (Chen et al. 2008). Npn1 expression was strong in the hippocampus and the SVZ located directly above the ventricle, with a marked decrease of the intensity of the signal in more rostral regions of the SVZ (Fig. 7E, E', arrow). Thus, at E16.5, neurons destined for the visual cortex strongly expressed Npn1 compared with that destined to the somatosensory cortex, which expressed low or no Npn1. The differential distribution of Sema3A target cells between the somatosensory and visual cortices could explain the phenotype we described in Sema3A−/−mice. At P0, Npn1 was totally downregulated in the neocortex (Fig. 7I), but maintained strong expression in the hippocampus (Fig. 7J') and the olfactory bulbs (data not shown).

At E16.5, Sema3A was expressed in the VZ and the cortical plate and did not present regional accumulation (Fig. 7F). The
signal was distributed throughout the cortical plate with higher intensity detected in the outer layer (Fig. 7F). At P0, Sema3A expression remained strong in the VZ and across the neocortex (Fig. 7I), concentrated in the supragranular layers but large cell bodies distributed in layer 5 also exhibited intense staining (Fig. 7I). This bilaminar pattern is similar to what we describe in the marmoset; however, the distribution of the transcripts coding for Sema3A failed to reveal an area-specific pattern. This suggests a significant modification of Sema3A expression regulation between the mouse and marmoset.

**Alternative Ligands to Sema3A Receptor Complex Expressed in the Mouse Developing Neocortex**

We analyzed the distribution of 2 other members of the Semaphorin family, the secreted Sema3C and the membrane-bound Sema6A, that act as alternative ligands for members of the Npn1/ Plexin receptor complex (Runker et al. 2008; Ruediger et al. 2013) that could potentially affect Npn1+ neurons migration in Sema3A−/− animals, contributing to the phenotype we describe. At ED16.5, Sema3C was absent from the cortical plate and essentially restricted between the SVZ and the subplate (Fig. 7G). Strong expression was detected in the hippocampus, the corpus callosum, and the external capsule and persisted at PD0 (Fig. 7K). At this stage, Sema3C was upregulated in the cells located in layer 3.

At E16.5, Sema6A transcripts were strongly expressed in the hippocampus and the VZ (Fig. 7H), but also accumulated in the deep layers of the cortical plate (Fig. 7H, open arrow) and formed a thin band in the subplate (Fig. 7H, arrow). At P0, Sema6A expression was sustained in the hippocampus and the corpus callosum, and expanded throughout the cortical layers (Fig. 7L).

Sema3C and -6A are present in the developing mouse cortex; however, they remain weakly expressed or absent from the cortical plate until PD0, at which stage Npn1 expression is already downregulated in the neocortex. Therefore, it is unlikely that neither ligand is responsible for the phenotype observed in the Sema3A−/− animals, which is most likely due to the arealization of Npn1 expression at ED16.5.

**Discussion**

While the interaction between Sema3A and Npn1 has previously been demonstrated to be required for the appropriate migration and polarization of cortical neurons (Polleux et al. 2000; Chen et al. 2008; Shelly et al. 2011), the role Sema3A in defining cortical area identity has never been investigated to the best of our knowledge. In the present study, we reveal that Sema3A contributes to patterning the visual cortex in both the nonhuman primate and the mouse, although the temporal mechanism differs between the 2 species. In the mouse, Sema3A is broadly expressed across the developing cortex, while Npn1+ cells are restricted to the posterior region of the neocortex.

Npn1+ cells become preferentially accumulated in the posterior region of the neocortex at E16.5, when neurons begin their migration toward the outer cortical layers. The loss of Sema3A perturbs the patterning of the mouse neocortex, particularly the posterior areas, including reduction in the primary visual neocortex (V1) volume and abnormal dendritic arborization in all visual areas. In the larger marmoset neocortex, Sema3A expression transitions from a homogenous embryonic distribution to an area-specific profile. This pattern appears to be associated with the maturation sequence of the visual cortex, with the later maturing areas retaining Sema3A expression longer than early maturing regions. These results suggest that, during the expansion of the visual cortex in primates, the regulation of Sema3A expression has been modified to support the formation of a larger neocortex.

**Sema3A Is Expressed in the Ventricular Zone, but Its Activity Is Restricted to the Cortical Plate**

Sema3A is a chemotactant for neocortical neurons, guiding them to the appropriate layer (Chen et al. 2008) by promoting apical dendrite outgrowth and axonal growth cone collapse (Shelly et al. 2011). Apical dendrites of neurons in the VZ preferentially turn toward an exogenous source of Sema3A or the marginal zone of a wild-type mouse explant, but are not affected by a Sema3A−/− cortical explant (Polleux et al. 2000), suggesting that Sema3A polarizing activity in the neocortex is achieved through the accumulation of the diffusible ligand close to the marginal zone. This mode of action appears to be conserved in the marmoset in which the protein is restricted to the cortical plate; however, our analysis also detected Sema3A transcripts in the VZ in addition to the cortical plate, suggesting a disparity between the location where Sema3A is expressed and where its activity is required.

One possibility is that neurons upregulate Sema3A expression in the VZ, but do not secrete the protein until they reach the cortical plate and acquire functional activity as secretion of Sema3A is activated by depolarization (de Wit et al. 2006). The immunolabeling protocol we used comprises low concentration of detergent and therefore, we only detect the protein present in the extracellular environment, where Sema3A interact with proteoglycans of the extracellular matrix (de Wit et al. 2005). Another possibility is that differences in the composition of the extracellular matrix lead to the inability of the secreted ligand to be retained in the VZ. Neural stem cells in the VZ express the extracellular matrix molecules laminin and their integrin receptors (Lathia et al. 2007), establishing a physical framework regulating cell cycle progression (Miranti and Brugge 2002). The cortical plate makes up a different extracellular environment, enriched in hyaluronan, heparin, and chondroitin sulfate proteoglycans decorated with a large combination of residues (Zaremba et al. 1989; Guimarães et al. 1990; Hockfield et al. 1990). These enwrap cortical neurons as they mature (Giamanco and Matthews 2012) and contribute to the maturation of projections and synapses (see Wang and Fawcett 2012). Microenvironments that confer different adhesive properties and ability to retain Sema3A could be the responsible for the accumulation of Sema3A in the cortical plate, where its activity is required, despite early onset of expression in the VZ.

**Sema3A Contributes to Patterning the Visual Cortex**

The analysis of Sema3A null mice revealed no defects in neocortical lamination, as was previously observed (Behar et al. 1996; Catalano et al. 1998). However, one study did demonstrate thinning of the mutant neocortex at PD12 (70% compared with controls), with more defects being observed in the medial rather than the lateral portion of the cortex (Sasaki...
et al. 2002). The discrepancy compared with our observation could be explained by the difference in developmental stage analyzed; the loss of cells leading to a thinning of the neocortex at PD12 is possibly compensated later by increased interneuronal migration (Zimmer et al. 2010). Alternatively, the phenotype of the animals surviving until PD28 is potentially less severe as the mutation of Sema3A has been associated with embryonic and postnatal lethality, with remarkably few homozygotes surviving into adulthood (Behar et al. 1996). Our study is the first to analyze the arealization of Sema3A−/− neocortex, using the marker NNF and criteria previously established in adult mice (Van der Gucht et al. 2007). The intensity of NNF immunostaining was markedly decreased due to altered morphology of pyramidal neurons and reduced dendritic branching in layers 3 and 5 in Sema3A−/− mice. Similar defects have been previously described at PD0 and 7 and are rescued by the addition of exogenous Sema3A (Fenstermaker et al. 2004). The reduction in pyramidal NNF labeling was limited to the visual cortical areas, particularly those examined in the current study (V1, V2M, and V2L), while the NNF profile appeared normal in anterior domains such as S1BF. This observation, combined with the reduction in the volume of area V1 and the compensatory enlargement of S1BF in Sema3A−/−, argues in favor of a specific role for Sema3A in patterning the visual cortex.

The somatosensory neocortex, where Npn1 and Sema3A are expressed at levels comparable with the visual neocortex at ED14.5, is not affected to the same extent by the loss of Sema3A. Therefore, our results suggest that the specific accumulation of Npn1+ cells in the posterior SVZ is the principal mediator of Sema3A specification function. The expansion of S1 implies that compensatory mechanisms operate in this region in the mutant, providing an advantage to somatosensory neuron identity. This effect does not appear to be elicited by Sema3C or −64, but other Semaphorins or factors such as vascular endothelial growth factor A could intervene.

In contrast to our observations in the mouse, the ligand and the receptor are uniformly distributed in the marmoset neocortex at the onset of lamination but also later, as areal borders begin to emerge. In fact, it is not until after birth that the Sema3A distribution becomes area specific. However, other guidance molecules exhibit a more precocious area-specific expression profile and delineate the prospective borders of visual areas in the primates during embryonic development. For example, the Eph/ephrins are discretely expressed in presumptive visual cortical areas in the embryonic macaque (Donoghue and Rakic 1999a,b). Sema3A is, therefore, unlikely to be responsible for establishing areal boundaries in the marmoset neocortex, but is involved in directing subpopulations of neurons expressing Npn1 to particular visual areas at different temporal points of development. Thus, the Sema3A/Npn1 pathway contributes to cortical arealization in the mouse and the marmoset by differential regulation of Npn1 and Sema3A expression at different stages of neocortical development.

The ultimate step of area specification is the maturation of cells and the establishment of intra- and interareal connectivities, which have previously been demonstrated to occur in a defined sequence in the visual cortex. Areas such as V1, which are the recipient of direct connections from the thalamic relay nuclei mature first, and higher-order areas mature later following the hierarchy of visual processing (Felleman and van Essen 1991). Studies in the marmoset demonstrate that V1 and area MT mature simultaneously, followed by V2, and the other extrastriate areas (Bourne and Rosa 2006). In addition, areas belonging to the dorsal stream mature before ventral stream areas (Conde et al. 1996; Distler et al. 1996; Bourne 2010).

After birth, various Sema3A expression profiles can be distinguished across the marmoset visual cortex. V1 and MT switch from the embryonic bilaminar pattern to expression restricted to layer 6, while the bilaminar profile is maintained in extrastriate areas V2 and V4 1 week postpartum before disappearing at 2 weeks. This observation suggests a correlation between the spatiotemporal expression and maturation of the visual cortical area. Alternatively, the expression could be regulated by the position of the area within the hierarchy of visual processing, with a “primary area” specific profile and a distinct profile for the extrastriate areas. However, the lack of expression in the extrastriate area DM, which defines the third “tier” challenges these postulations as DM and V4 have comparable maturation and hierarchical order (Felleman and van Essen 1991). Areas V4 and DM belong to the dorsal and ventral processing streams, respectively, and therefore, we propose that Sema3A is differentially expressed postnatally in early maturing areas, as well as across the 2 processing streams. The arealization of Sema3A postnatally is likely not to be only involved in the migration of cells, but also the establishment of connectivity between areas, which is occurring at this stage of development.

The transition to a different expression profile during the first 2 weeks postpartum is almost certainly under the control of transcription factors regulating Sema3A expression. The identification of the regulatory interactions acting upstream of Sema3A will help elucidate the mechanisms of cortical arealization. However, remarkably little is known about the mechanisms regulating Sema3A expression. Studies in muscle satellite cells report that Sema3A expression is activated by fibroblast growth factor 2 and repressed by transforming growth factor β 2 and -3 (Do et al. 2011). A transcriptomic approach characterizing genetic programs activated specific regions of the marmoset neocortex similar to what has been achieved in the mouse (Ayoub et al. 2011; Belgard et al. 2011; Oeschger et al. 2012) is required to identify potential candidates.

A Mechanism for Sema3A in the Migration of Neurons to the Neocortex

In the developing mouse cortex, the transcription factors Pax6 and Tbr2 domains of expression are mutually exclusive; Pax6+ radial glia cells are restricted to the VZ and Tbr2+ neural progenitors to the SVZ (Englund et al. 2005). Colocalization of Pax6 and Tbr2 in the SVZ is characteristic of higher species, including humans (Bayatti et al. 2008; Reillo et al. 2011) where Pax6+ is expressed by intermediate radial glia cells (IRGcs). IRGcs constitute a population of progenitors believed to account for the tangential expansion of the cortical surface, characterized by a radial process contacting the pial surface but not the ventricle (Reillo et al. 2011). In the marmoset cortex at ED90, Npn1+ cells form a mixed population of Pax6+/Npn1+ and Tbr2+/Npn1+ progenitor cells, restricted to the
SVZ. The presence of Sema3A in the cortical plate suggests that the ligand is involved in orienting IRGC processes, confirmed in vitro by the stripe assay experiment. Our observations are consistent with a working model in which the mixed population of Npn1+ progenitors gives rise to Npn1+/Tbr1+ neurons committed to a pyramidal fate (Hevner et al. 2003; Englund et al. 2005), which are found in the subplate 20 days later at ED110, and which settle in the upper cortical layers at ED130. Therefore, neurons are targeted to Sema3A+ enriched domains from the progenitor stage and form a lineage characterized by the expression of Npn1.

In the present study, we demonstrate that both Sema3A and its receptor are expressed by NeuN+ neurons, with the ligand sublocalized on TuJ1+ fibers, suggesting that the migration of Npn1+ neurons in the marmoset neocortex is regulated in a neuron-mediated fashion. Projection neurons migrate along radial glia fibers to reach their specific position in the neocortex (Rakic 1972), a process that requires neuron–glia interaction via expression of adhesion molecules (Anton et al. 1999). Upon reaching the appropriate layer, neurons switch from a gliophilic to neuropilic adhesive activity in order to detach from the radial glia fibers and to settle among other neurons (Anton et al. 1999). Other examples of neurophilic migration are rare, except in the human telencephalon (Letinic and Rakic 2001). The secretion of Sema3A by established neurons could contribute to the polarization of subsequent waves of migrating neurons while preventing them from adhering too firmly. This hypothesis is supported by the results of the stripe assay experiments where undifferentiated precursors adopt polarized morphology in the presence of Sema3A, with the cell bodies settling away from the source of Sema3A.

During late embryonic and early postnatal development, Sema3A adopts a bilaminar profile in the marmoset, becoming strongly expressed in the upper layer 2/3, similar to the description in the rodent (Polleux et al. 2000; Chen et al. 2008), but also in layer 5 around pyramidal neuron cell bodies and dendritic projections. The typical inside–out pattern of cortical lamination predicts that neurons destined to layer 2 migrate through layer 5 (Angevine and Sidman 1961) and therefore, we predict that supragranular neurons in the marmoset migrate through the Sema3A-enriched layer 5. One interpretation is that the additional Sema3A region observed in the primate is necessary as neurons have to migrate greater distances as a consequence of the evolutionary thickening of the neocortex. Therefore, the local accumulation of Sema3A in the infragranular layer acts as an intermediate target for supragranular neurons, which travel greater distances in the expanded marmoset neocortex, and forms more numerous connections.

Altogether, our results reveal for the first time that the guidance cue Sema3A, through interaction with Npn1, is implicated in patterning posterior structures of the mouse and the marmoset monkey neocortex. This role is conserved during evolution, but has been modified with the emergence of larger and more complex brains. The patterning role is achieved through regionalization of the distribution of the receptor in the mouse and of the ligand in the larger primate brain, where a greater number of cortical areas have to be specified.

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