



# Neural cell adhesion molecule, NCAM, regulates thalamocortical axon pathfinding and the organization of the cortical somatosensory representation in mouse

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To study the potential role of neural cell adhesion molecule (NCAM) in the development of thalamocortical (TC) axon topography, wild type, and NCAM null mutant mice were analyzed for NCAM expression, projection, and targeting of TC afferents within the somatosensory area of the neocortex. Here we report that NCAM and its  $\alpha$ -2,8-linked polysialic acid (PSA) are expressed in developing TC axons during projection to the neocortex. Pathfinding of TC axons in wild type and null mutant mice was mapped using anterograde Dil labeling. At embryonic day E16.5, null mutant mice displayed misguided TC axons in the dorsal telencephalon, but not in the ventral telencephalon, an intermediate target that initially sorts TC axons toward correct neocortical areas. During the early postnatal period, rostralateral TC axons within the internal capsule along the ventral telencephalon adopted distorted trajectories in the ventral telencephalon and failed to reach the neocortex in NCAM null mutant animals. NCAM null mutants showed abnormal segregation of layer IV barrels in a restricted portion of the somatosensory cortex. As shown by Nissl and cytochrome oxidase staining, barrels of the anterolateral barrel subfield (ALBSF) and the most distal barrels of the posteromedial barrel subfield (PMBSF) did not segregate properly in null mutant mice. These results indicate a novel role for NCAM in axonal pathfinding and topographic sorting of TC axons, which may be important for the function of specific territories of sensory representation in the somatosensory cortex.

**Keywords:** thalamocortical axons, axonal pathfinding, axonal tract tracing, barrel cortex, adhesion and signaling molecules, NCAM

## INTRODUCTION

Establishment of topographic connectivity between thalamic nuclei and neocortical areas is fundamental to sensory function, yet the molecular signals that govern thalamocortical (TC) axon guidance and synaptic targeting are incompletely understood. In the developing TC pathway, axons from motor, somatosensory, and visual thalamic nuclei project topographically to primary motor and sensory cortical areas along strict rostro-caudal and medio-lateral axes. Once arriving in the neocortex, TC axons are guided to area- and laminar-specific synaptic targets by poorly defined cortical cues, and then synapses are formed and further refined by activity (reviewed in López-Bendito and Molnár, 2003; Molnár et al., 2012). One emerging concept is that cell adhesion molecules guide proper TC axon guidance. The L1 family cell adhesion molecules, L1, CHL1, and NrCAM, are key determinants of guidance for specific subpopulations of TC axons at the ventral telencephalon and in the cortex (Ohyama et al., 2004; Wiencken-Barger et al., 2004; Wright et al., 2007; Demyanenko

et al., 2011a,b). L1 family cell adhesion molecules are structurally related to the neural cell adhesion molecule (NCAM). However, a role for NCAM has not been established in TC axon guidance.

NCAM is a pivotal regulator of axon growth, cell adhesion, and migration. NCAM polymorphisms and altered expression have been associated with schizophrenia, bipolar disorder, and Alzheimer's disease. In addition, the entire extracellular region of NCAM is excessively released by proteolysis as a soluble fragment in affected brain regions in schizophrenia. NCAM null mutant mice lacking the three principal NCAM isoforms (NCAM180, 140, and 120) exhibit impaired learning, working memory, and hippocampal long-term potentiation (LTP). Mice expressing the soluble NCAM extracellular domain demonstrate impaired sensory gating (Pillai-Nair et al., 2005), working memory, and prefrontal cortical LTP (Brenneman et al., 2011). NCAM is also highly glycosylated, and is the principal carrier of  $\alpha$ -2,8 polysialic acid (PSA). PSA is highly expressed during embryonic

and early postnatal development and persists at low levels in the adult for plasticity (Bonfanti, 2006; Gascon et al., 2007; Rutishauser, 2008). PSA decreases NCAM–NCAM homophilic and heterophilic adhesion (Johnson et al., 2005). Mice lacking the polysialyltransferases that attach PSA to NCAM, ST8SiaII or ST8SiaIV, have decreased social interaction and motivation, impaired fear conditioning, increased exploration and aggression and impaired hippocampal LTP (Angata et al., 2004; Calandreau et al., 2010). Similar to NCAM, changes in PSA expression or polymorphisms in ST8SiaII have been observed in schizophrenia (Barbeau et al., 1995; Arai et al., 2006; Tao et al., 2007; Isomura et al., 2011), bipolar disorder (Lee et al., 2011), and autism (Anney et al., 2010). Interestingly, mice lacking PSA but not NCAM exhibit TC pathfinding defects, such that TC axons defasciculate and fail to enter the ventral telencephalon or cortex (Schiff et al., 2011). Additional deletion of NCAM partially restored TC axon guidance, suggesting that NCAM may have distinct roles from PSA in this trajectory.

Here, we report that NCAM null mutant mice exhibited aberrant TC axon guidance and an altered somatosensory map. However, projection through the ventral telencephalon was normal prenatally and demonstrates selective roles for NCAM vs. PSA in TC axon guidance.

## MATERIALS AND METHODS

### MICE

All procedures were conducted in accordance with the National Institutes of Health and European Union guidelines and with the approval of the institutional animal care and use committees of University of North Carolina and the Instituto de Neurociencias. Homozygous-null NCAM mutant mice (C57Bl/6 background) were provided by Dr. H. Cremer (Developmental Biology Institute of Marseille Luminy, Marseille, France). NCAM deficient (KO) mice and wild type (WT) littermates were generated by crossing heterozygotes. Midday of the vaginal plug was considered as E0.5, and postnatal day 0 (P0) as day of birth.

### IMMUNOHISTOCHEMISTRY

Timed-pregnant dams were anaesthetized by intraperitoneal injection of ketamine (110 mg/kg) and xylazine (10 mg/kg) and the embryos were extracted by caesarean section. Embryos (E13.5, E14.5, E16.5, and E18.5) were fixed in 4% paraformaldehyde in PBS. Postnatal (P0, P2, P4, and P6) and adult (P30) mice were anaesthetized similarly and were fixed by intracardiac perfusion. Vibratome sections, 100  $\mu$ m-thick, were blocked in 4% bovine serum albumin (BSA), 3% NHS, 0.2% Triton X-100 in PBS at room temperature for 1 h and incubated with primary antibodies diluted in blocking solution at room temperature overnight. Primary antibodies were: rat monoclonal antibody P61 to an intracellular epitope of the 140/180 isoforms of NCAM (gift of Drs. C. Goridis and H. Cremer, 1:50), mouse monoclonal antibodies 12E3 to  $\alpha$ 2-8 linked neuraminic acid (NeuAc- $\alpha$ 2-8)<sub>n</sub> with  $n = 5$  (oligo/polysialic acid; gift from Dr. T. Seki; 1:1000); rabbit polyclonal antibodies to L1 (gift from Dr. F. G. Rathjen, 1:1000) and serotonin (DiaSorin, Stillwater, IL, USA; 1:2000). The specificity of these antibodies in mouse

forebrain from E13.5 through adulthood has been demonstrated (Gennarini et al., 1984; Rathjen and Schachner, 1984; Seki and Arai, 1991; Nadanaka et al., 2001). Secondary antibodies were as follows: ImmunoPure biotinylated goat anti mouse IgM (Pierce, Rockford, IL, USA; 1:100); Cy3 conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, USA; 1:500); AlexaFluor 488 anti-mouse IgM, AlexaFluor 555 goat anti rat IgG, AlexaFluor 546 anti-rabbit IgG (Molecular Probes–Invitrogen; 1:500). Sections were mounted on microscope slides and covered with AF2 (Citifluor Ltd., London, UK) or Vectashield (Vector Labs) mounting media.

### DiI INJECTIONS AND ANALYSIS

Fixed brains of E14.5, E16.5, E18.5, P0, P2, and P4 wild type and NCAM-deficient mice were vibratome sectioned from the caudal end of the cerebrum. Once the dorsal thalamus was reached, the brains were removed from the vibratome and a crystal of DiI (Molecular Probes–Invitrogen) was inserted at mid rostrocaudal levels of the dorsal thalamus, comprising the primordium of the ventrobasal complex, to anterogradely label TC axons that project to the somatosensory cortex.

Mice remained in 0.05% azide in PBS for 3–4 weeks. Brains were then sectioned at 100  $\mu$ m in a vertical plane oriented 45° with respect to the coronal plane, in order to recover the maximum number of TC axons in each single section (Agmon and Connors, 1991; see schematics in **Figure 4**). For combined DiI–immunofluorescence staining, selected sections from DiI injected brains were processed without detergents. Sections were mounted onto glass slides with Citifluor.

The geometry of individual TC axons in the early postnatal cortex was studied in P2 and P4 wild type and null-mutant brains implanted with small DiI crystals in the dorsal thalamus. DiI labeled axons in obliquely oriented sections were imaged in a confocal microscope traced using NeuroLucida 7.0 and analyzed using NeuroExplorer software (MicroBrightField, Inc.). For the analysis we considered the following parameters: number of nodes, branch order, and planar angle of bifurcations. We used the unpaired Student's *t*-test with statistical significance set at  $p < 0.05$ .

### NISSL STAINING AND CYTOCHROME OXIDASE HISTOCHEMISTRY

For Nissl staining, vibratome sections from adult mouse brains were mounted on gelatin-coated slides and dried overnight. Sections were defatted in an ascending series of ethanol and xylene, rehydrated again and stained in 1% aqueous cresyl violet.

For cytochrome oxidase histochemistry, adult wild type and null-mutant mice were perfusion fixed with 4% paraformaldehyde, 0.2% glutaraldehyde and 4% glucose in 0.1 phosphate buffer, stored in fixative overnight and sectioned at 100  $\mu$ m in the coronal plane or in a plane parallel to the pial surface in flattened tissue blocks. Sections were processed to reveal cytochrome oxidase activity as described (Kageyama and Wong-Riley, 1982).

Sections were analyzed in a Leica DM5000 epifluorescence microscope and in confocal microscopes Leica TCS-SL and Olympus FV500.

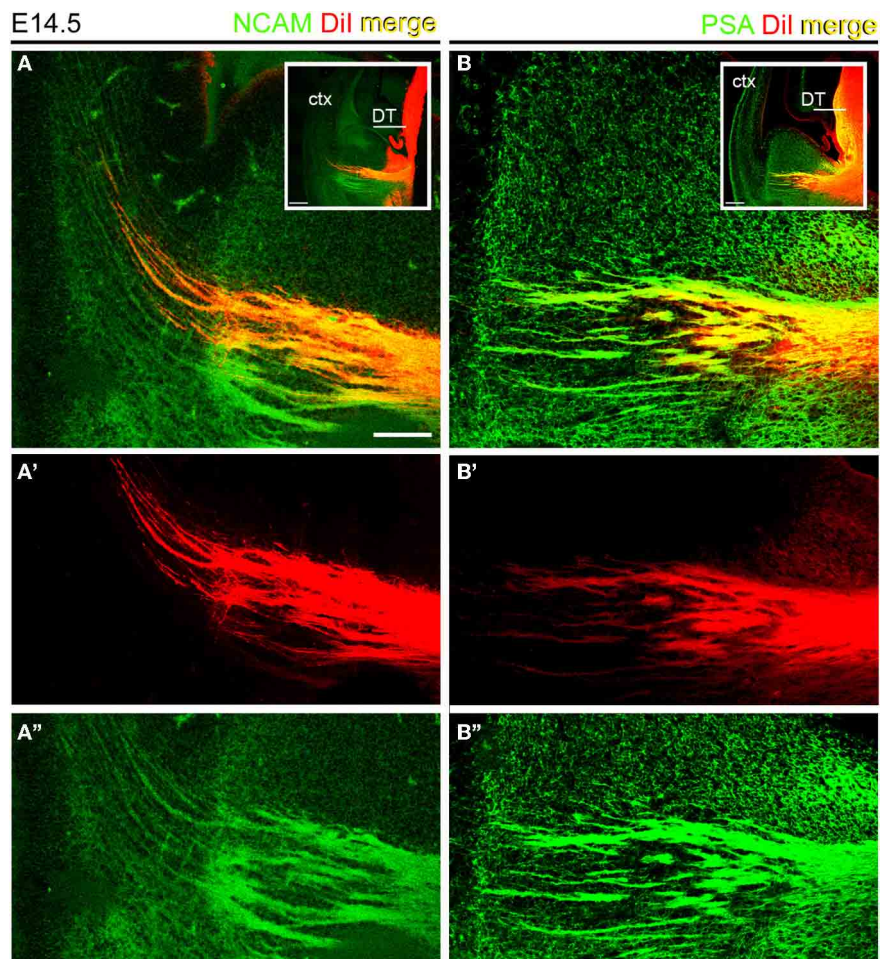
## RESULTS

### EXPRESSION OF PSA-NCAM AND NCAM IN THALAMOCORTICAL AXONS

We assessed the presence of PSA and NCAM in thalamocortical (TC) axons during prenatal development using axonal tract-tracing and immunohistochemistry. As a first step to verify that TCAs expressed NCAM and its polysialylated form PSA-NCAM, small crystals of DiI were inserted in the dorsal thalamus of E14.5 wild type mice. DiI is a lipophilic tracer that diffuses within the lipid bilayer of the plasma membrane and accurately labels axonal projections in brain tissues fixed with aldehydes (Godement et al., 1987). DiI-labeled TC axons were immunofluorescently labeled with antibodies to the cytoplasmic domain of transmembrane NCAM isoforms (140 and 180 kDa) and PSA. DiI labeled TC axons displayed both NCAM and PSA (Figures 1A,B). DiI was excited with a HeNe 543 nm laser, while an Ar/Kr 488 nm laser excited AlexaFluor 488 used to detect NCAM and PSA immunoreactivities. Comparison of Figures 1A,A',B',B'' shows

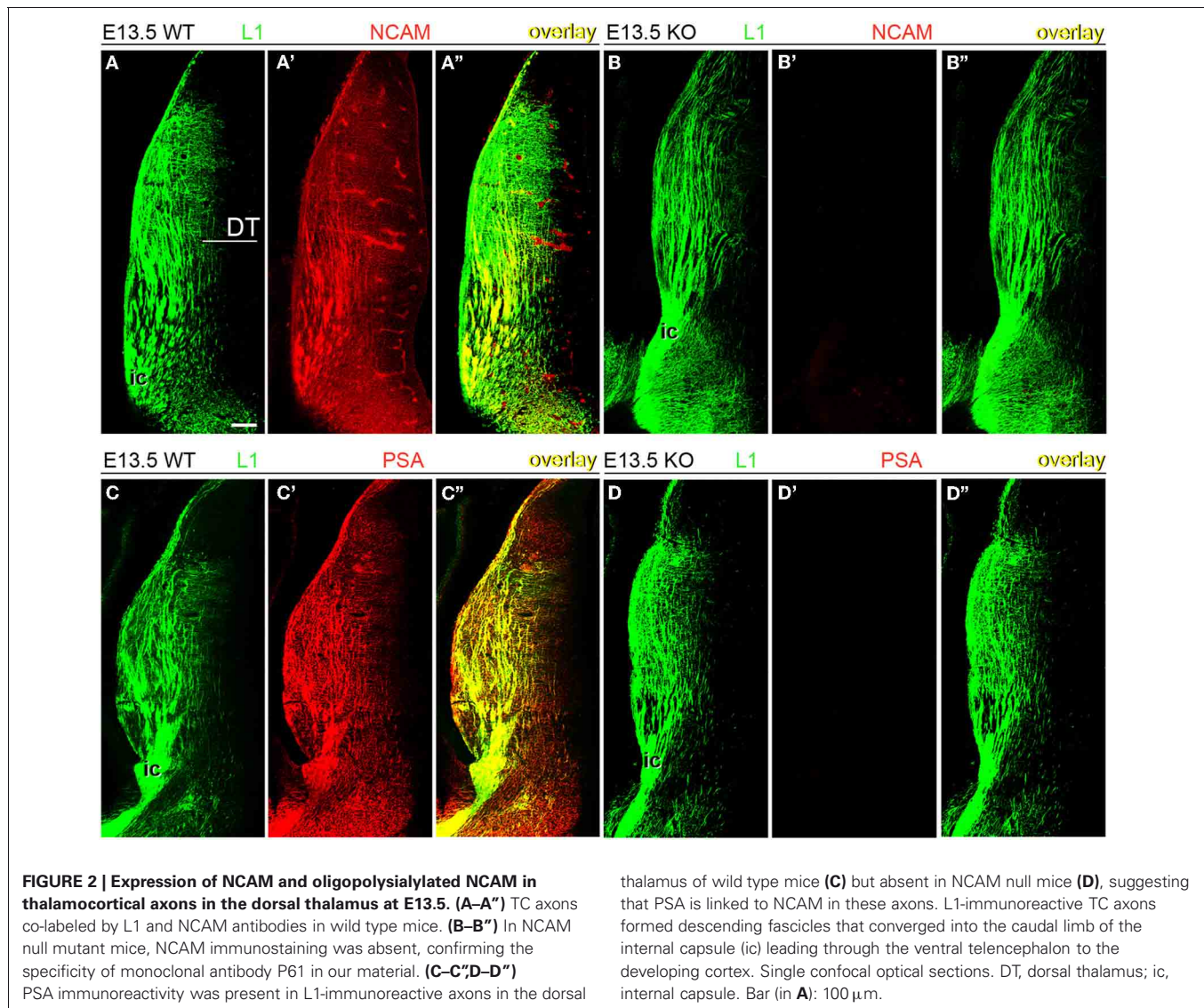
that no bleeding occurred between the two detection channels in the confocal microscope, thus confirming the localization of NCAM or PSA detection signals in DiI-labeled TC axons.

Next, wild type and NCAM null mutant E13.5 and E15.5 mice (Cremer et al., 1994) were immunolabeled with antibodies to NCAM or PSA along with L1, which labels TC axons (Chung et al., 1991; Fukuda et al., 1997; Demyanenko et al., 2011b). L1-immunoreactive axons expressed transmembrane NCAM isoforms, as detected with a monoclonal antibody (mAb P61) that recognizes the cytoplasmic domain of both NCAM140 and NCAM180 (Figures 2A–A'' and 3A–A''; Gennarini et al., 1984), but not NCAM120. NCAM immunoreactivity was absent in NCAM null mutant mice (Figures 2B–B'' and 3B–B''), demonstrating the specificity of this antibody. Using monoclonal antibody 12E3 that reacts with small chains of PSA (Seki and Arai, 1991; Nadanaka et al., 2001), we found that L1-expressing TC axons displayed immunoreactivity for PSA of low sialic acid content in wild type mice (Figures 2C–C''



**FIGURE 1 | Thalamocortical axons expressed NCAM and PSA at E14.5.** Sections containing DiI-labeled TC axons were labeled with antibodies to the intracellular domains of large NCAM isoforms (A) and PSA (B). Insets show the regions analyzed. A', A'' and B and B'' show the detection channels separately. Note that the red signal of

DiI labeled axons in the ventral telencephalon coincided in all cases with the green signal corresponding to NCAM (A–A'') or PSA (B–B'') immunoreactivities. Maximum projections of confocal microscope stacks. DT, dorsal thalamus; ctx, cortex. Bars in A, B, 200  $\mu\text{m}$ ; insets, 100  $\mu\text{m}$ .



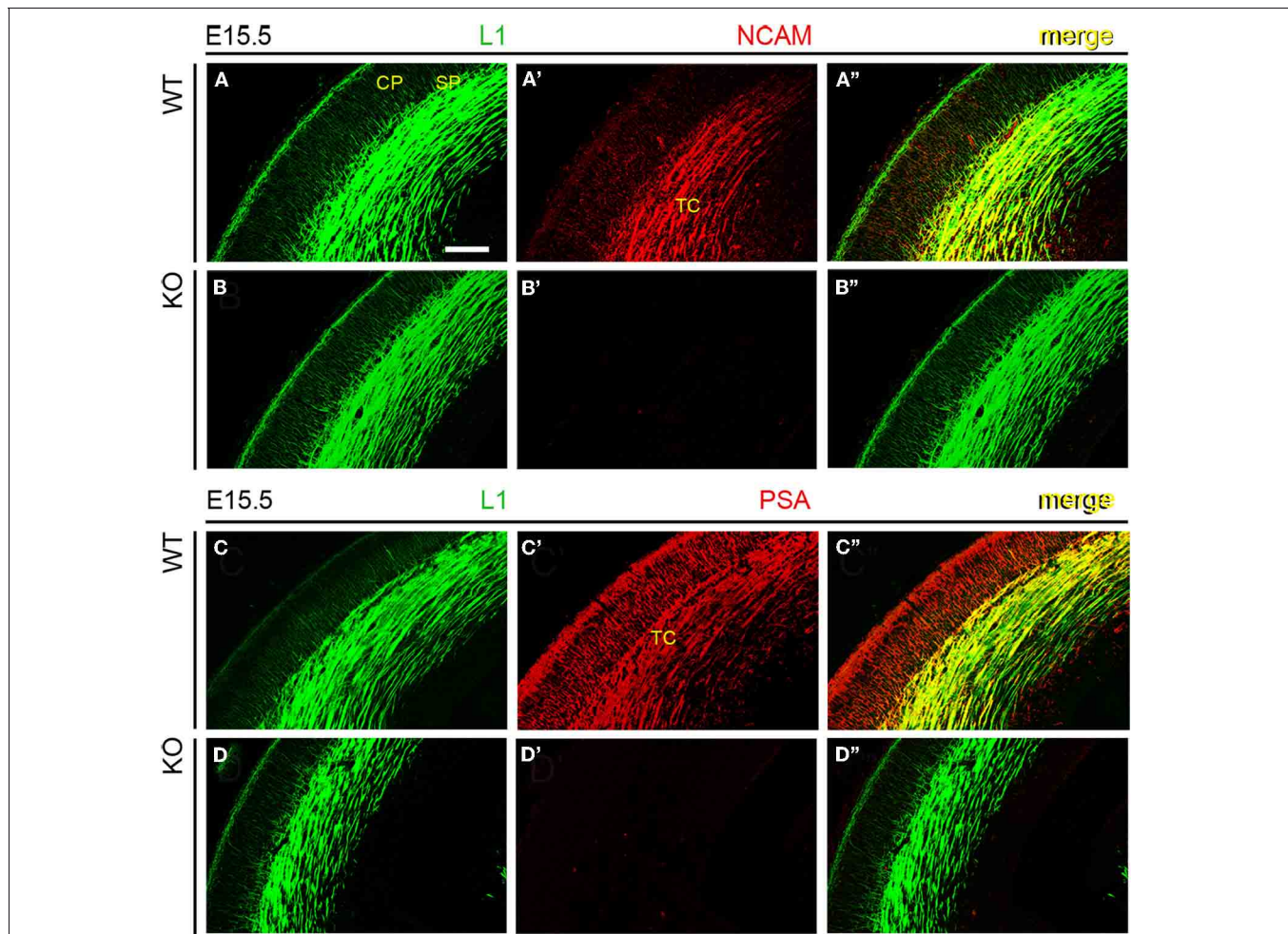
and 3C–C''). In NCAM null mutant mice, 12E3 immunoreactivity was abolished (**Figures 2D–D''** and **3D–D''**), suggesting that NCAM was the only carrier of PSA in TC axons at this stage (Seki and Arai, 1993; Cremer et al., 1997; Dityatev et al., 2004). NCAM and PSA exhibited similar patterns of immunoreactivity. PSA showed more extensive labeling than NCAM, which may be due to antibody sensitivity or PSA linkage to NCAM120, secreted NCAM, or other splice variants not recognized by our NCAM antibody. However, very little NCAM120 is present at this stage (Edelman and Chuong, 1982; Brennaman and Maness, 2008).

#### THE GENETIC DELETION OF ALL THREE PRINCIPAL NCAM ISOFORMS CAUSES TOPOGRAPHICALLY LOCALIZED DEFECTS IN THALAMOCORTICAL AXONAL GUIDANCE

In the mouse, the TC axonal projection courses through the ventral telencephalon within a narrow vertical parallelepiped oriented at 45° from the midsagittal plane (Agmon and Connors,

1991). Thus, to further study possible defects in axonal fasciculation and pathfinding in the TC axonal pathway of NCAM null mutant mice, we examined the anatomical distributions of Dil labeled TC axons in tissue sections oriented to include the maximum rostral-to-caudomedial extent of this axonal projection. The sections analyzed were controlled for similar injection size and location to minimize artifacts. In these sections (**Figures 4A,B**, see inset), the laterally located axons were rostral and medial axons were caudal in comparative terms.

The analysis of wild type and null mutant mouse embryos during the prenatal period revealed no major changes in the anatomical trajectory of TC fibers along their subpallial course through the ventral telencephalon. At E16.5 (**Figure 4**), the TC axonal projection along the subpallium was sparser in null mutant mice as compared to wild type mice (**Figures 4A,B**), and fewer TC axons were detected in the cortical subplate impinging into the lower levels of the cortical plate (**Figures 4C,D**).



**FIGURE 3 | NCAM and PSA expression in thalamocortical axons.**

An antibody to adhesion molecule L1 labeled TC axons approaching the subplate in E15.5 mouse embryos, irrespective of phenotype. **(A–A'')** Monoclonal antibody P61, directed against an intracellular epitope of NCAM, showed the co-expression of L1 and NCAM in TC axons. **(B–B'')** Absence of NCAM immunoreactivity in NCAM null mutant mice. **(C–C'')** Monoclonal antibody 12E3 recognized oligopolysialylated

chains of PSA in L1-immunoreactive TC axons. **(D–D'')** Absence of PSA-NCAM immunoreactivity in NCAM null mutant mice. The absence of NCAM **(B'')** and PSA **(D'')** immunoreactivities in null mutant mice suggested that, under our experimental conditions, NCAM is the sole carrier of polysialic acid in the developing cortex. Single confocal optical sections. CP, cortical plate; SP, subplate; TC, thalamocortical axons. Bar: 200  $\mu\text{m}$ .

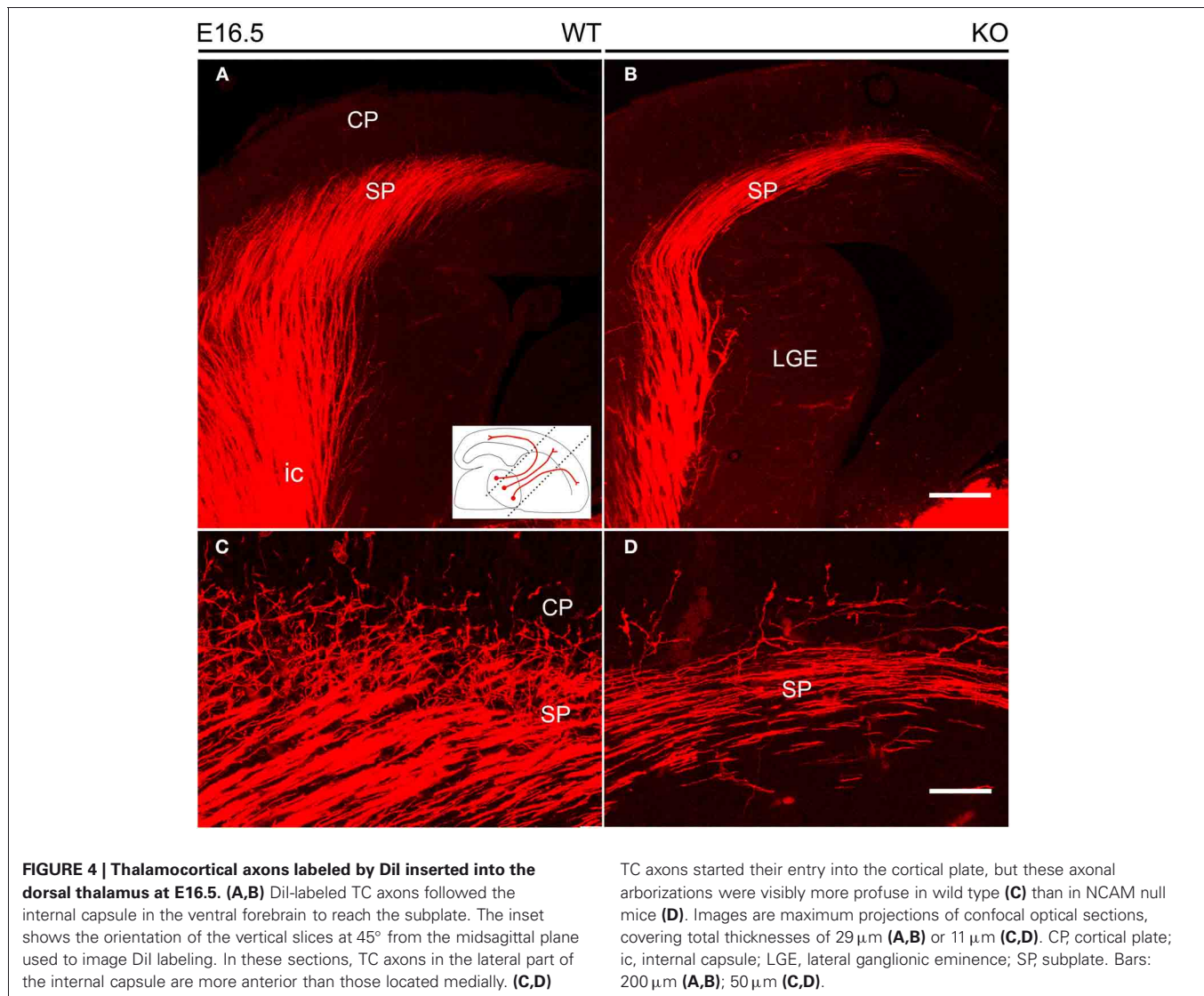
### DEFECTS IN TC AXONAL GUIDANCE THROUGH THE VENTRAL TELEENCEPHALON IN EARLY POSTNATAL NULL MUTANT MICE

NCAM removal caused a more visible phenotype of altered guidance of TC axons during early postnatal development. As TC axons course through the ventral telencephalon, their characteristic fan-like distribution as seen in the obliquely oriented vertical sections (**Figure 5A**) was altered in early postnatal NCAM null mice with respect to wild type animals. In null mutant P0 mice, TC axons navigated in a disorderly manner and, unlike in wild type mice, gave rise to local axon collaterals in the ventral telencephalon (**Figures 5A,B**). In addition, NCAM null TC axons in the rostromedial tier of the internal capsule became disoriented within the ventral telencephalon, bent abruptly at right angles (**Figure 5B**, arrows), and failed to reach the cortex unlike wild type mice (**Figure 5A**). The packing density of

TC axons in the cortex in null mutant mice (P0) was also visibly decreased, consistent with the observed disorientation (**Figures 5C,D**).

The localized anatomical changes in the subpallial course of TC axons persisted by P2 (**Figures 5E,F**), at the time TC axons start to achieve their final distribution within the cerebral cortex, and cortical layers start their cytoarchitectonic differentiation (Agmon et al., 1993; López-Bendito and Molnár, 2003). Thus, in P2 null mutant mice, the most medially located axons seemed unchanged. On the contrary, TC axons located in the rostromedial sector of the internal capsule dispersed laterally at difference to wild type axons (**Figures 5C,D**).

Dil crystals were inserted into the middle third of the dorsal thalamus where the ventrobasal complex of the thalamus that projects to the somatosensory cortex will develop, and



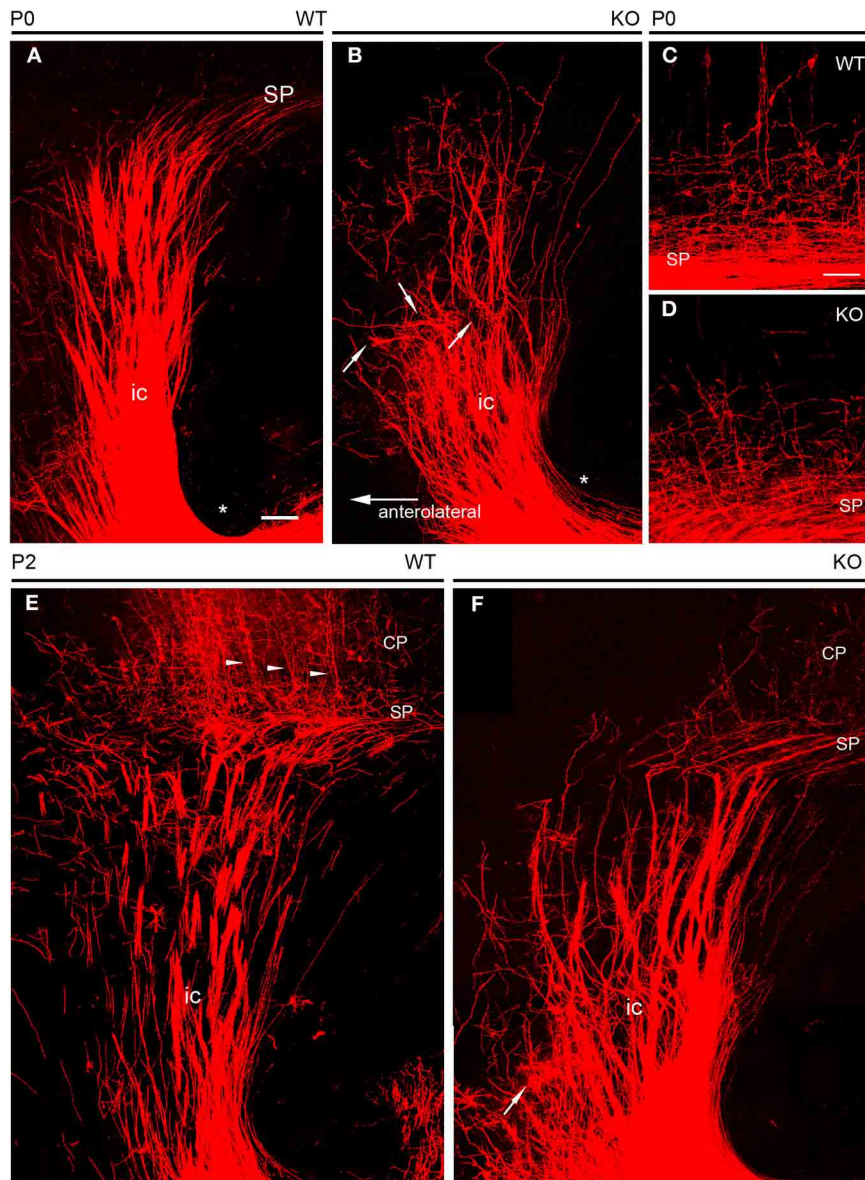
avoided the lateral geniculate primordium located more caudally. Thus, the somatosensory cortex was the probable cortical area of destination of the DiI labeled TC axons. This prompted the question as to whether localized changes in the TC axons as they course through the ventral telencephalon during the early postnatal period might result in alterations in the distribution of TC axons within the cerebral cortex and localized alterations in the cytoarchitectonic organization of this target cortical area.

#### INTRACORTICAL ARBORIZATIONS OF THALAMOCORTICAL AXONS IN NCAM NULL MUTANT MICE

NCAM null mutant mice consistently showed reduced packing density of DiI labeled TC axons in the developing cortex (**Figures 6A,B**) in spite of the variability of DiI labeling. However, it was unclear whether this was accompanied by changes in the TC axon arborizations within the postnatal cerebral cortex. Prompted by the finding by Yamamoto et al. (2000) that PSA removal alters the orientation and layer targeting of TC axons in

slice culture experiments, we analyzed whether NCAM deletion likewise causes alterations in the branching patterns of preterminal TC axons labeled anterogradely with DiI. We studied two different ages corresponding to the first week after birth when segregation into barrels occurs, at P2 (WT,  $n = 6$  hemispheres; KO,  $n = 6$  hemispheres) and at P4 (WT,  $n = 7$  hemispheres; KO,  $n = 10$  hemispheres).

For the analysis of the geometry of individual TC axons in the cortex, we considered the following parameters: number of nodes, branch order, and planar angle of preterminal TC axons. The number of nodes and the branch order give idea of the complexity of the axonal trees. The Student's *t*-test detected no differences between wild type and null mutant mice. At P2 (**Figure 6C**), the values for number of nodes were  $3.21 \pm 0.22$  per axon for wild type mice vs.  $2.79 \pm 0.21$  for null mutant mice (mean  $\pm$  s.e.m.),  $p = 0.09$ . At P4 (**Figure 6D**), values were  $3.04 \pm 0.25$  for WT mice vs.  $3.15 \pm 0.24$  for null mutant mice,  $p = 0.38$  (**Figure 6D**). Branch order was also unchanged: at P2—WT,  $3.96 \pm 0.19$  vs. null mutant,  $3.54 \pm 0.18$  (**Figure 6C**),

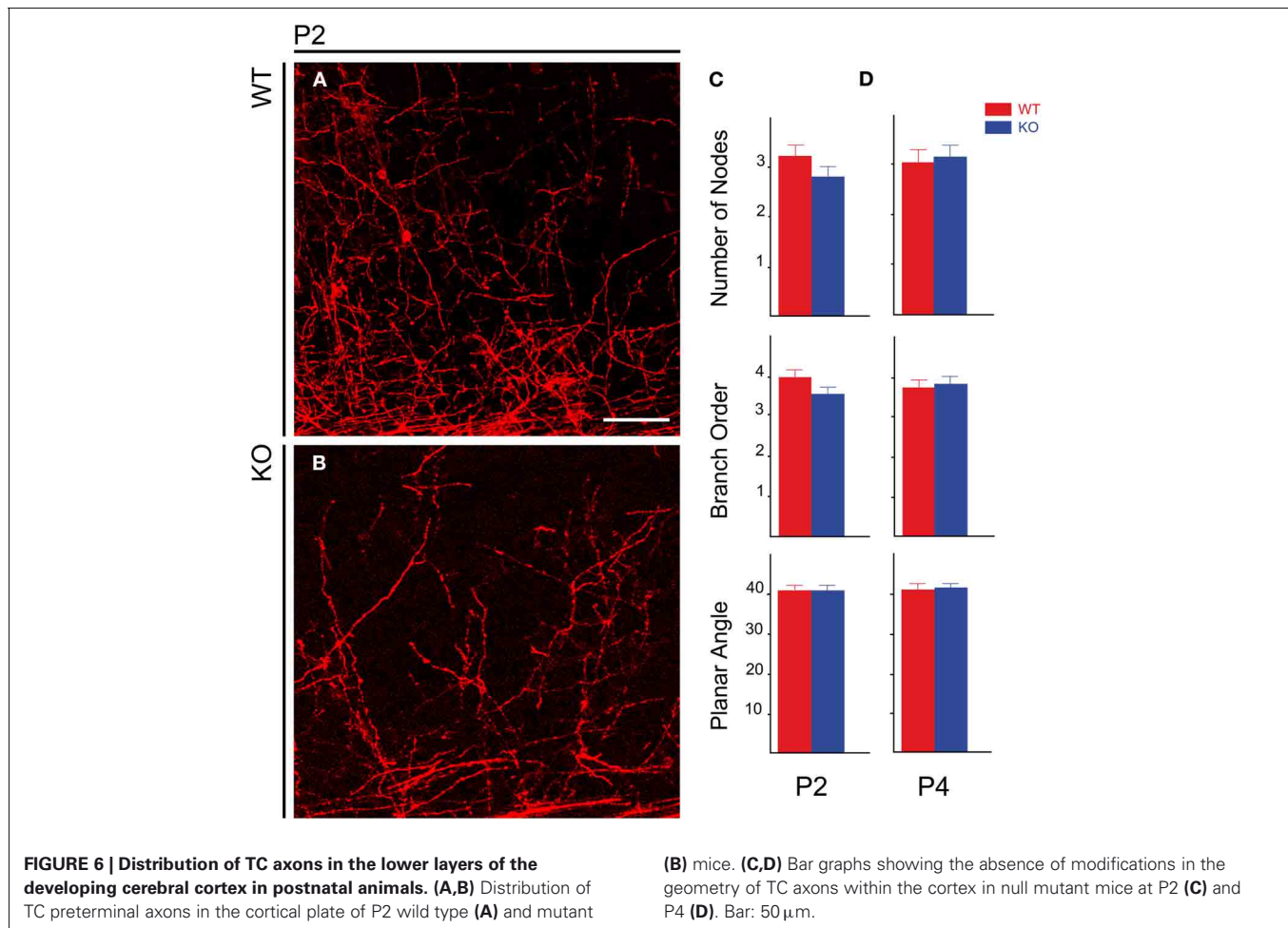


**FIGURE 5 | Dil tracing of thalamocortical fibers at early postnatal ages.** (A,B) At P0, thick fascicles of Dil-labeled TC axons traversed the ventral telencephalon in their way to the developing cortex. The course of TC axons along the internal capsule was straight in wild type embryos (A), where few axon collaterals dispersed laterally. In null mutant mice (B), TC axon fascicles in the lateral parts of the pathway bent abruptly, failing to reach the cortex; arrows point at selected examples of dispersed axon fascicles. Axons at more medial locations, although less dispersed, navigated in a disorderly manner, giving rise to abundant axon collaterals in the ventral telencephalon. Asterisks in A and B mark the inferior level of the internal capsule in the

ventral telencephalon. (C,D) TC axons entering the lower tier of the cortical plate in wild type and knockout mice at P0. Null mutant mice showed less densely packed axonal arborizations than wild type mice. (E,F) At P2, compared to wild type mice (C), TC axons in the ventral telencephalon of null mutant mice (D) dispersed laterally; one extreme example is marked with an arrow. Null mutant mice showed sparser TC axons in the cortex than wild type mice. Arrowheads in E point to retrogradely Dil labeled neurons in the cortical plate. The subplate was identified as the level TC axons bend medially to adopt trajectories parallel to the palial surface. Maximum confocal projections. Bar: 100 μm.

$p = 0.06$ ; at P4—WT,  $3.74 \pm 0.21$  vs. null mutant,  $3.83 \pm 0.18$ ,  $p = 0.38$  (Figure 6D) The planar angle is that formed in the section plane by the two segments uniting an  $n$  order node with the next order ( $n + 1$ ) node in an arborization, and measures a characteristic of the branching pattern of the axons disregarding the axonal trajectory. Planar angles were also

unchanged at P2 and P4: P2—WT,  $40.97^\circ \pm 1.17$  vs. null mutant,  $40.69^\circ \pm 1.45$ ,  $p = 0.44$  (Figure 6C); P4—WT,  $41.22^\circ \pm 1.55$  vs. null mutant,  $41.48^\circ \pm 1.31$ ,  $p = 0.45$  (Figure 6D). Together, these results indicated that, contrary to PSA removal, NCAM removal does not alter the branching patterns of TC axons in the cortex.



### THE GENETIC DELETION OF NCAM ALTERS THE SOMATOSENSORY MAP IN THE CEREBRAL CORTEX

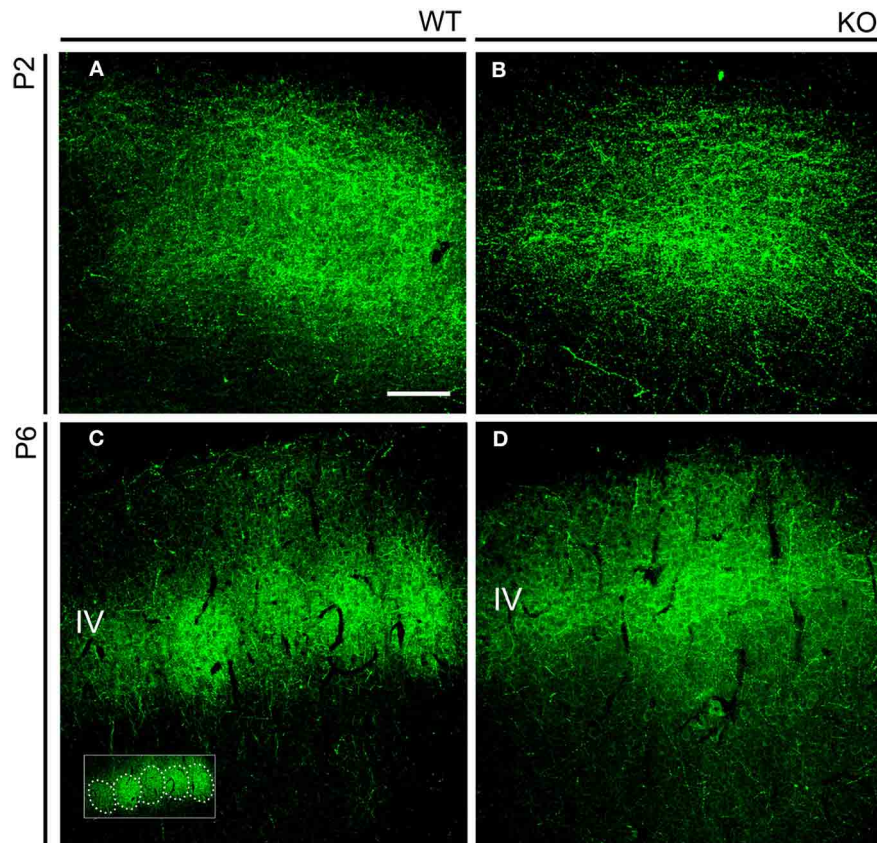
Layer IV of the rodent somatosensory cortex contains multicellular units called barrels so arranged as to form a visible map of the sensory periphery (Woolsey and Van der Loos, 1970; Van der Loos and Woolsey, 1973). This distinctive feature makes the somatosensory system a remarkable model to study molecular mechanisms implicated in the topographic sorting of TC axons to neocortical areas. We focused on the anatomical representation of the sensory map in the somatosensory cortex, whose refinement along development requires the correct targeting of TC axons.

We first analyzed in early postnatal mice the distribution of TC axons within layer IV of the somatosensory cortex (Figure 7). During the first postnatal week, thalamic fibers navigate through layers V and VI and invade layer IV, which is incompletely differentiated. TC axons assume their characteristic periphery-related pattern and impose a barrel arrangement onto layer IV (Agmon et al., 1993; López-Bendito and Molnár, 2003). Developing TC axons ascend to layer IV where they form spatially periodic clusters of terminations of gradually increasing complexity that correspond to the barrels, rather than branching profusely within lower cortical layers with subsequent retraction (Agmon et al., 1993, 1995). To see how TC axons segregate in NCAM null mice,

we analyzed the pattern of distribution of thalamic terminals by means of serotonin (5-HT) immunohistochemistry. Thalamic relay neurons transiently express the plasma 5-HT transporter (5-HTT) and the vesicular monoamine transporter type 2 (VMAT2), allowing 5-HT to concentrate in the TC axons and axon terminals during early postnatal life (Lebrand et al., 1996). 5-HT immunostaining was continuous in the forming layer IV of P2 mice, but at P6 it suggested a discrete pattern in wild type mice, but not NCAM null mice (Figure 7).

Nissl staining of coronal sections of adult brains revealed mild disruptions of the somatosensory map in NCAM null mutant mice (Figures 8A–D). As compared to the well-defined barrels of wild type animals ( $n = 5$ ) due to the presence of distinct barrel septa, null mutant mice ( $n = 5$ ) showed indistinct barrels with no discernible septa between them (Figures 8A,C) and the Nissl stain revealed a microlaminar deposition of cells within layer IV (Figures 8C,D). These changes occurred through all antero-posterior levels of the somatosensory area of the cortex. Layer IV of the somatosensory cortex in null mutant mice remained prominent, suggesting reorganization of TC axons (Figures 8A–B). Cytochrome oxidase histochemistry (Wong-Riley and Welt, 1980) in coronal sections highlighted individual barrels in null mutant mice ( $n = 3$ ), though the staining was





**FIGURE 7 | Serotonin immunohistochemistry in the early postnatal somatosensory cortex. (A,B).** Serotonin immunostaining highlighted the distribution of TC axons in the developing cortex. At P2, at a time barrel units are not yet formed, immunostaining revealed a uniform distribution of axons in both wild type and mutant mice. **(C,D).** At P6,

serotonin immunoreactivity clustered in layer IV in a manner reminiscent of barrels (highlighted in the inset) in wild type animals **(C)**. In P6 NCAM null mutant mice, TC axons also localized to layer IV but showed a continuous distribution **(D)**. Confocal optical sections. IV, cortical layer IV. Bar: 100  $\mu$ m.

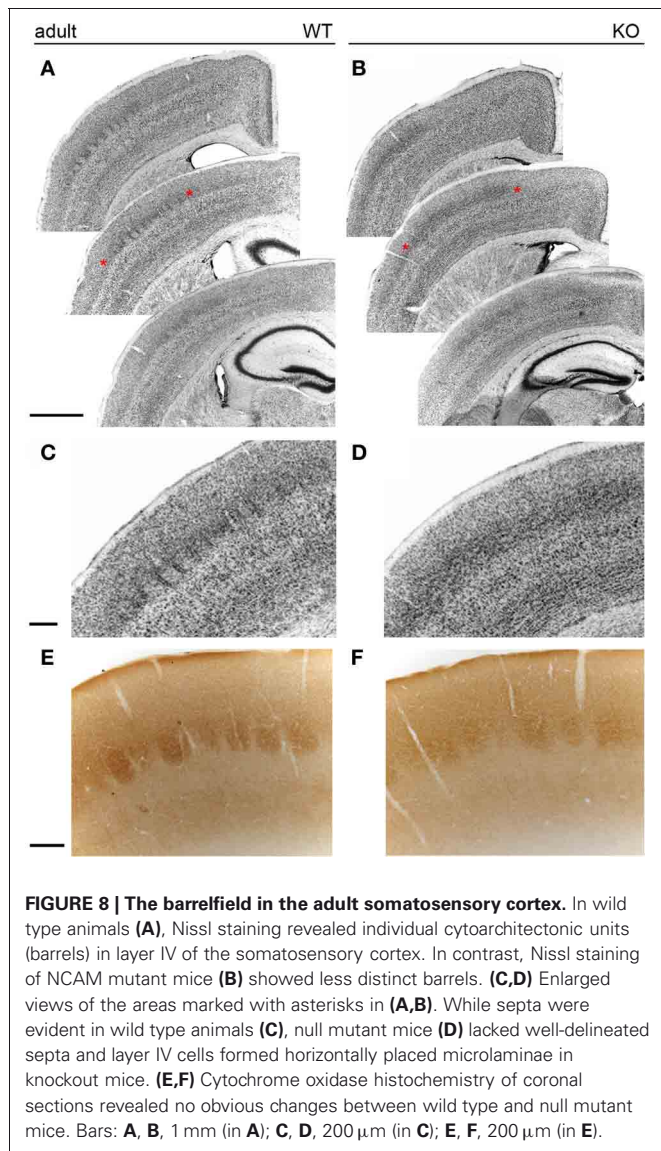
fainter in null mice as compared to their wild type ( $n = 3$ ) littermates, as shown for rostral barrels (**Figures 8E,F**). This was taken as an indication that the overall organization of the somatosensory cortex was preserved in null mutant mice, although the refined cytoarchitectonic organization of the barrels was somewhat altered, indicating that layer IV neurons could be abnormally deployed in the barrels and septa.

Then, we analyzed the somatosensory map in tangential sections of the cortex for the occurrence of topographic changes in adult NCAM null mutants. The posteromedial barrel subfield (PMBSF) contains a group of large, ovoid barrels whose topographic distribution is homeomorphic to that of the largest vibrissa follicles of the animal's snout. The anterolateral barrel subfield (ALBSF) contains similar yet smaller barrels, which correspond to the smallest and less prominent whiskers of the animal's face. As shown in tangential reconstructions of Nissl staining (**Figures 9A,B**), the barrel septa were less prominent in null mutant mice ( $n = 4$ ) than in wild type mice ( $n = 4$ ) and were indiscernible at rostral positions within the PMBSF of null mutant mice (**Figure 9B**). Cytochrome oxidase staining delineated distinct barrels in both genotypes (wild type,  $n = 4$ ; null mutant,  $n = 6$ ). However, in null mutant mice, only the most

caudomedial part of the PMBSF that contains the straddler barrels ( $\alpha$ - $\delta$ ) and the first large barrels of rows A–E, showed such a clear delineation and their overall topography seemed unchanged (**Figures 9C,D**). The other barrels in the PMBSF and in the entire ALBSF were profoundly altered in null mutant mice, and were virtually indistinct. Both Nissl staining and cytochrome oxidase histochemistry unveiled a prominent phenotype trait, the disruption of barrels in the ALBSF and of the most rostral barrels in the PMBSF. These alterations in the barrel field of the somatosensory cortex suggested that the regional alteration of NCAM null TC axons might contribute to disrupted topographic mapping, and that NCAM may be required for the topographical anatomical distribution of TC axons in the somatosensory cortex.

## DISCUSSION

We have shown here that NCAM participates in the guidance of TC axons to the somatosensory cortex, and provide the first evidence for a role for NCAM in TC topography. PSA and NCAM were expressed on developing TC axons from E13.5 to E15.5. In embryonic stages, NCAM null TC axons traversed the ventral telencephalon normally, but a contingent of axons appeared to ectopically branch in the ventral telencephalon and misproject

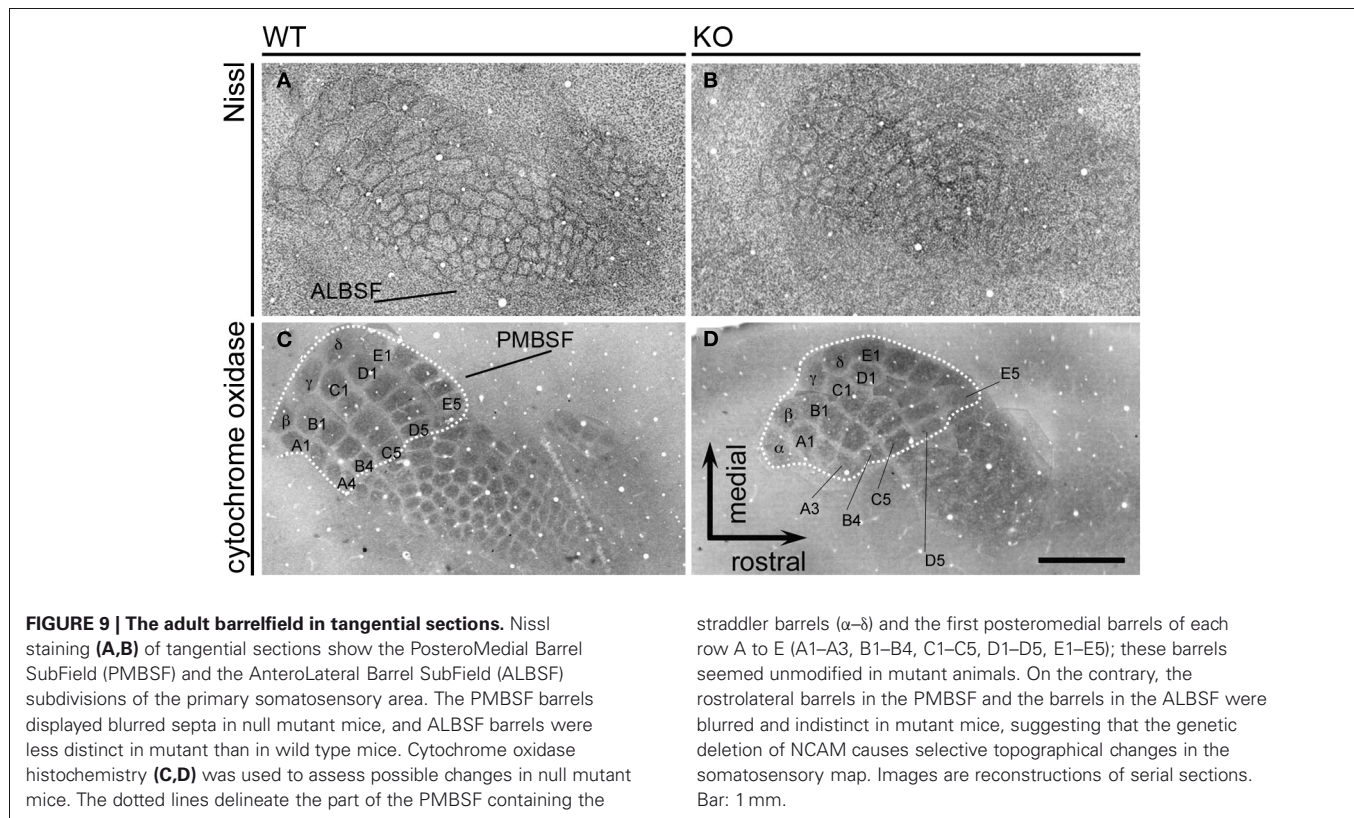


into the dorsal telencephalon in early postnatal stages. In anterograde DiI labeling at E16.5, NCAM null mutant mice appeared to have fewer axons projecting from the middle third of the dorsal thalamus which forms the ventrobasal complex and sends axons to the somatosensory cortex. The rostral third of the thalamus will give rise to ventroanterior and ventrolateral nuclei which project to the motor cortex, while the caudal third of the thalamus will give rise to the dorsal lateral geniculate nucleus that projects to the visual cortex. Accordingly, rostral TC axons in the internal capsule failed to appropriately target the dorsal telencephalon. Together, these results suggest that TC axon guidance to the motor and somatosensory cortices may be altered in NCAM null mice. In accordance with these findings, NCAM null mutant mice displayed aberrant barrel architecture at P6 and in adult somatosensory cortex.

Our findings are distinct from those observed with PSA-deficient mice. TC axons from mice lacking both polysialyltransferases (ST8SiaII and ST8SiaIV) failed to enter the ventral

telencephalon at E15.5, and instead terminated in the reticular thalamic nucleus (Weinhold et al., 2005; Schiff et al., 2011). The consequence of PSA removal is increased and precocious NCAM-dependent adhesion (Hildebrandt et al., 2007). The prominent role of PSA elimination as the origin of this phenotype contrasted with the absence of major phenotype differences in the TC axonal projection in triple knockout animals for ST8SiaII, ST8SiaIV, and NCAM (Schiff et al., 2011), suggesting that additional NCAM removal can restore normal TC axon trajectory in the ventral telencephalon. NCAM null TC axons enter the ventral telencephalon and sort normally at embryonic stages. However, our study also shows that NCAM null TC axons showed pathfinding defects in the ventral telencephalon during the early postnatal life, at the time TC axons establish their connections in the cerebral cortex, and also regional defects in the establishment of the somatosensory map in the cerebral cortex that are described here for the first time. Thus, our new results on a postnatal role for NCAM in TC axon projections complement the role of PSA, by identifying specific roles for NCAM in this trajectory.

The role of other members of the immunoglobulin superfamily of neural cell adhesion molecules in TC axonal pathfinding has been studied intensively in the last few years. L1 family cell adhesion molecules also show shifts of contingents of rostral thalamic axons to caudal regions in the developing and final topographic map. Close homolog of L1, CHL1, directs TC axons to the somatosensory cortex (Wright et al., 2007), and cooperates with L1 to guide axons to the motor cortex (Demyanenko et al., 2011b). Additionally, NrCAM also participates in topographic mapping of TC axons to the motor and somatosensory cortices (Demyanenko et al., 2011a). Together these results suggest that Ig superfamily cell adhesion molecules are critical for proper TC axon guidance to motor and somatosensory cortices. However, this also raises the question as to whether the adhesion molecules cooperate on the same axon or guide subpopulations of axons from the thalamus. Of particular interest in this regard is that L1, CHL1, and NrCAM respond to different guidance cues to promote proper targeting of TC axons. Repellent guidance cues such as ephrins (Dufour et al., 2003) and semaphorins (Wright et al., 2007; Demyanenko et al., 2011a) are expressed in gradients within the ventral telencephalon and allow for preliminary sorting of thalamic axons based on their expression of Eph and Neuropilin receptors. CHL1 interacts with Neuropilin 1 to respond to Sema3A, which is required for proper topographic mapping to the somatosensory cortex (Wright et al., 2007). In mapping motor axons, CHL1, and L1 differentially interact with EphA7 and EphA3, 4 or 7, respectively, to mediate repellent axon guidance in response to ephrinA5 (Demyanenko et al., 2011b). NrCAM associates with Neuropilin 2 and Sema3F to promote proper mapping of motor and somatosensory axons (Demyanenko et al., 2011a). While we did not identify which repellent guidance cue cooperates with NCAM, a recent study has shown that NCAM can associate with EphA3 to promote proper inhibitory synaptic targeting in response to ephrinA5 (Brenneman et al., 2012). The differential responses of these adhesion molecules to repellent guidance cues, suggest that while some axons may express L1, CHL1, and NCAM to respond to ephrinA5, others may only express NrCAM or CHL1.



This combinatorial effect would allow all axons from individual thalamic nuclei to project to their correct targets.

A limitation of this study is that retrograde TC labeling analysis was not performed in P5 mice once the final map is established. While our results suggest that motor and somatosensory axons misproject at early time points, only the somatosensory barrel field was examined in adult animals. TC axons shape the barrel field into the pattern of the whiskers (Erzurumlu and Jhaveri, 1990; Agmon et al., 1993; Schlaggar and O'Leary, 1994; Agmon et al., 1995; Wu et al., 2011), followed by the organization of layer IV neurons into discrete barrel units 1–2 days later (Rice and Van der Loos, 1977; Jhaveri et al., 1991). Therefore, our findings that the formation of barrels in layer IV at P6 were altered and the disorganization of the ALBSF and of the rostralateral tier of the PMBSF in NCAM null mutant mice is likely a result of TC axon misguidance. However, we cannot determine whether these observations are a result of misprojection of axons to the visual cortex or if the axons degenerated.

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The results presented here provide insight into a novel role for NCAM in TC topographic mapping. This may have profound implications in neuropsychiatric disorders where mutations in NCAM or changes in expression are observed, suggesting that TC mapping may be disturbed resulting in impaired sensory function.

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