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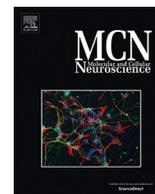
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PlexinD1 and Sema3E determine laminar positioning of heterotopically projecting callosal neurons



Theodora Velona^a, Mike Altounian^a, Micaela Roque^a, Mélanie Hocine^a, Anaïs Bellon^{a,b}, Carlos Garcia Briz^c, Pascal Salin^a, Marta Nieto^c, Sophie Chauvet^{a,1}, Fanny Mann^{a,*,1}

^a Aix-Marseille Université, CNRS, IBDM, 13288 Marseille, France

^b Aix-Marseille Université, INSERM, INMED, 13273 Marseille, France

^c Department of Molecular and Cellular Biology, Centro Nacional de Biotecnología (CNB-CSIC), Campus de Cantoblanco, Darwin 3, 28049 Madrid, Spain

ABSTRACT

The corpus callosum is the largest bundle of commissural fibres that transfer information between the two cerebral hemispheres. Callosal projection neurons (CPNs) are a diverse population of pyramidal neurons within the neocortex that mainly interconnect homotopic regions of the opposite cortices. Nevertheless, some CPNs are involved in heterotopic projections between distinct cortical areas or to subcortical regions such as the striatum. In this study, we showed that the axon guidance receptor PlexinD1 is expressed by a large proportion of heterotopically projecting CPNs in layer 5A of the primary somatosensory (S1) and motor (M1) areas. Retrograde tracing of M1 CPNs projecting to the contralateral striatum revealed the presence of ectopic neurons aberrantly located in layers 2/3 of *PlexinD1* and *Sema3e* mutant cortices. These results showed that Sema3E/PlexinD1 signalling controls the laminar distribution of heterotopically projecting CPNs.

1. Introduction

The 6-layered neocortex of mammals comprises a wide variety of neuron subtypes, including excitatory pyramidal neurons and inhibitory interneurons. Pyramidal neurons are subdivided into three subclasses according to the laminar position of their cell bodies and axonal targets. Corticothalamic projection neurons (CTPNs) are located in layer 6. Subcerebral projection neurons (SCPNs), which project to the brainstem and spinal cord with collaterals to the striatum and thalamus, reside in the deep part of layer 5 (layer 5B). Corticocortical projection neurons including callosal projection neurons (CPNs) that send long-range horizontal projections between hemispheres *via* the corpus callosum are found across multiple cortical layers, with CPNs particularly concentrated in cortical layers 2/3 and layer 5 and to a lesser extent in layer 6.

Previous studies have characterized the transcriptional diversity of CPNs using mouse callosal neurons identified by *in vivo* retrograde labelling or on the basis of the expression of the fate regulator of callosal neurons, special AT-rich sequence-binding protein-2 (Satb2) (Fame et al., 2011; Molyneaux et al., 2015, 2009). These studies identified genes whose expression defines subpopulations of CPNs with a specific laminar or sublaminar distribution. Among these genes, *PlexinD1*, which encodes the PlexinD1 cell surface receptor for semaphorins, is

expressed at high levels in CPNs in the superficial part of layer 5 (layer 5A) and at lower levels within CPNs of other cortical layers (Molyneaux et al., 2009). Based on this result, it has been proposed that cells expressing PlexinD1 probably represent a particular population of callosal neurons, which unlike the majority of CPNs that interconnect symmetric (homotopic) regions of the cerebral cortex, make heterotopic projections to the contralateral striatum.

The existence of crossed (transcallosal) corticostriatal projections is well documented in rodents as well as in monkeys and humans (Carman et al., 1965; Innocenti et al., 2017; Lieu and Subramanian, 2012; Morishima and Kawaguchi, 2006; Shepherd, 2013; Wilson, 1987). These projections originate from pyramidal neurons (called intratelencephalic (IT)-type neurons) located in layer 5A of the motor and premotor cortex, which often project bilaterally into the ipsilateral and contralateral striatum. These neurons are clearly distinct from another type of corticostriatal neuron, the SCPNs of layer 5B (also called pyramidal tract (PT)-type neurons) whose descending axons emit collaterals only in the ipsilateral striatum. Injections of adeno-associated virus (AAV)-Cre-dependent expression vectors into the primary motor cortex of *PlexinD1* bacterial artificial chromosome (BAC)-Cre transgenic mice labelled axons projecting bilaterally into the striatum, with only sparse labelling of the fibres extending through the internal capsule (IC) and cerebral peduncle (CP) *via* the PT (Gerfen et al., 2013). These

* Corresponding author at: Aix Marseille Université, CNRS, UMR 7288, Institut de Biologie du Développement de Marseille (IBDM), Case 907 – Parc Scientifique de Luminy, 163 avenue de Luminy, 13288 Marseille Cedex 9, France.

E-mail address: fanny.mann@univ-amu.fr (F. Mann).

¹ Equal contribution.

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Table 1

Experimental details for stereotaxic injections.

Regions of tracer injections (M1, primary motor cortex; S1, primary somatosensory cortex; PMC, premotor cortex). The stereotaxic coordinates are given in mm from Bregma with respect to the antero-posterior (AP) and medio-lateral (ML) axes and from the brain surface for the dorso-ventral (DV) axis.

Region	Stereotaxic coordinates (mm)	Tracer	Volume (nl)	Number of animals injected
M1	AP: +0.91 ML: +1.34 DV: -0.75	Alexa 647-CTB	100	<i>Plxnd1</i> -eGFP: n = 3 <i>Plxnd1^{lox/-};Emx1^{Cre}</i> : n = 3 <i>Sema3e^{-/-}</i> : n = 3 WT and controls: n = 6
Striatum	AP: +0.01 ML: +2.28 DV: -2.6	Alexa 555-CTB	100	
S1	AP: -0.23, ML: +3, DV: -0.75	Alexa 647-CTB	400	<i>Plxnd1</i> -eGFP: n = 3
PMC	AP: +2.77, ML: +1.87, DV: 1.28	Alexa 555-CTB	400	

Table 2

List of antibodies used in this study.

Antibody name	Host animal	Dilution	Source	Identifier
Primary antibodies				
anti-GFP	Chicken	1:500	Aves	Cat#GFP-1020 RRID: AB_10000240
anti-Satb2	Mouse	1:80	Abcam	Cat#ab51502 RRID: AB_882455
anti-Ctip2	Rat	1:500	Abcam	Cat#ab18465 RRID: AB_10015215
Anti-Cux1 CDP (M-222)	Rabbit	1:200	Santa Cruz Biotechnology	Cat#sc-13024 RRID: AB_2261231
Anti-Cux1 CDP (C-20)	Goat	1:200	Santa Cruz Biotechnology	Cat# sc-6327 RRID: AB_2087003
Anti-PlexinD1	Goat	1:150	R&D Systems	Cat# AF4160 RRID: AB_2237261
Secondary antibodies				
Alexa Fluor 488 anti-Chicken IgY	Donkey	1:500	Jackson ImmunoResearch Labs	Cat#703-545-155 RRID: AB_2340375
Alexa Fluor 568 anti-Mouse IgG	Donkey	1:500	Thermo Fisher Scientific	Cat# A10037 RRID: AB_2534013
Alexa Fluor 647 anti-Rat IgG	Donkey	1:500	Jackson ImmunoResearch Labs	Cat#712-605-153 RRID: AB_2340694
Alexa Fluor568 anti-Rabbit IgG	Donkey	1:500	Thermo Fisher Scientific	Cat#A10042 RRID: AB_2534017
Alexa 568 anti-Goat IgG	Donkey	1:500	Thermo Fisher Scientific	Cat#A11057 RRID: AB_2534104

results support the idea that PlexinD1 is mainly expressed by corticostriatal neurons of the IT subtype (Gerfen et al., 2013).

CPNs of cortical layer 5 are also involved in other types of long-range heterotopic projections. For example, in the mouse sensorimotor cortex, a small population of layer 5 neurons extends transcallosal projections that end in the contralateral premotor cortex (PMC) (Mitchell and Macklis, 2005). Whether PlexinD1 is also expressed in this subtype of heterotopically projecting CPNs has not been investigated.

The function of PlexinD1 in CPNs also remains unexplored. PlexinD1 is a receptor for different members of the semaphorin (Sema) family. This receptor can directly bind Sema3E and Sema4A and associates with neuropilins to form a receptor complex for Sema3C and Sema3G (Gu et al., 2005; Liu et al., 2016; Toyofuku et al., 2007; Yang et al., 2015). In the nervous system, Sema3E is the main ligand of PlexinD1 whose activation regulates various developmental processes, including neuronal migration, axon guidance and synapse formation (Chauvet et al., 2017; Oh and Gu, 2013). Since the temporal profile of *Plxnd1* gene expression in CPNs reaches a maximum at early postnatal stages, it may participate in late stages of CPN development, such as final maturation and refinement of adult connectivity (Molyneaux et al., 2009). However, a possible role of PlexinD1 in the formation of heterotopic transcallosal projections remains unknown.

The work presented in this paper used a *Plxnd1*-enhanced green fluorescent protein (eGFP) reporter mouse line to characterize the axonal projection patterns of PlexinD1-expressing CPNs. Our results showed that PlexinD1 is expressed by at least two major types of CPNs

with long-range heterotopic projections residing in layer 5A of the motor and somatosensory cortex, respectively. We further demonstrated ectopic misprojections to heterotopic contralateral targets formed by upper layer CPNs in the motor cortex of mice lacking *Plxnd1* or *Sema3e* genes. Together, our results showed that PlexinD1 contributes to defining the laminar distribution of heterotopically projecting CPNs in cortical layer 5A.

2. Materials and methods

2.1. Generation, maintenance and genotyping of mutant mice

All animal procedures were conducted in accordance with the guidelines from the European Community (EU Directive 2010/63/EU for animal experiments) and from the French Ministry of Agriculture (agreement number F1305521) and were approved by the local ethics committee (C2EA-14 agreement 2015060510102024- V7 #1186).

Transgenic mice expressing eGFP under the control of the *Plxnd1* promoter [Tg(Plxnd1-EGFP)HF78Gsat/Mmucd] were purchased from the Mutant Mouse Resource Research Centers (MMRRC). *Plxnd1*; *Emx1^{cre}* and *Sema3e* null mice have been previously reported (Burk et al., 2017; Chauvet et al., 2007). The genotypes of the mice were determined by PCR (Burk et al., 2017; Chauvet et al., 2007). Both males and females were used throughout the study.

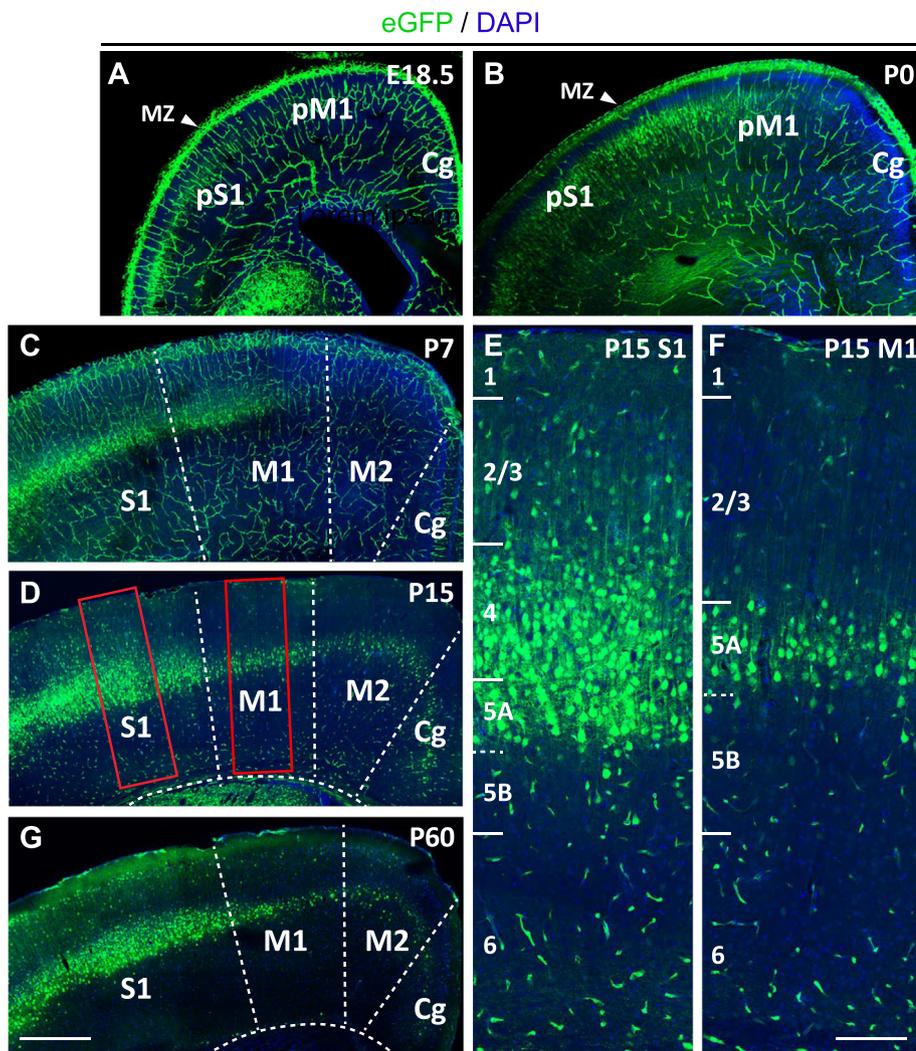


Fig. 1. Distribution of eGFP in the *Plxnd1*-eGFP mouse neocortex.

(A–D) Distribution of eGFP fluorescence in coronal sections of E18.5.

(A), P0 (B), P7 (C) and P15 (D) *Plxnd1*-eGFP brains. (E–F) Enlargement of boxed areas in (D) showing the laminar distribution of eGFP+ neurons in somatosensory (E) and motor (F) areas at P15. Cortical layers were identified using DAPI staining. (G) Distribution of eGFP fluorescence in coronal sections of adult P60 *Plxnd1*-eGFP brain.

Cg: cingulate cortex, M1: primary motor cortex; M2: secondary motor cortex, MZ: marginal zone, pM1: presumptive motor cortex, pS1: presumptive somatosensory cortex, S1: primary somatosensory cortex. Scale bars: 500 μm (A–D, G), 150 μm (E, F).

2.2. Retrograde labelling with cholera toxin B subunit

Adult (8–10 weeks) mice were anaesthetised by intra-peritoneal injection of 100 mg/kg ketamine (Imalgene, Merial) and 10 mg/kg xylazine (Rompun, Bayer) and placed in a stereotaxic apparatus. Small craniotomies were made on the skull. Solutions of cholera toxin subunit B (CTB) conjugated to Alexa Fluor 555 (Life technologies, Cat# C34776) or Alexa Fluor 647 (Life technologies, Cat# C34778) (1 μg/μl in phosphate buffered saline (PBS)) were injected at a flow rate of 10 nl/s using a programmable nanolitre injector (Nanoject III, Drummond, Cat# 3-000-207). Table 1 contains the relevant experimental details, including the areas injected, tracer type and tracer volume. After surgery, mice were injected with 5 mg/kg carprofen (Rimadyl, Pfizer) to reduce postoperative inflammation and pain. Seven days after CTB injection, mice were anaesthetised with ketamine and xylazine, perfused intracardially with 10 ml PBS followed by 20 ml PFA (4% in PBS). Dissected brains were post-fixed in PFA (4% in PBS) overnight at 4 °C and kept in PBS at 4 °C for further processing.

2.3. Immunohistochemistry

Fixed brains were embedded in 4% agar (Invitrogen, Cat# 30391-023) in PBS and sectioned into 50–200 μm-thick sections using a Leica Vibratome (Cat#VT1000S). Immunostaining was performed according to standard procedures. Sections were incubated in blocking buffer containing 0.3% Triton X-100 (Acros Organics, Cat#AC215682500)

and 10% goat serum (VWR, Cat#S2000–100) in PBS for 1 h at room temperature (RT). For staining with anti-PlexinD1 antibody, sections were incubated for 2 h at RT in blocking buffer containing 0.25% Triton X-100 and 0.02% gelatine (Sigma, Cat# G9382-500G) in PBS. Sections were then incubated with primary antibodies diluted in blocking buffer overnight at 4 °C. The following day, sections were washed and incubated with secondary antibodies for 2 h at RT. Table 2 lists the antibodies used in this study.

2.4. Whole-mount immunostaining and tissue clearing

Whole-mount immunostaining with anti-eGFP was performed following the 3DISCO protocol (Belle et al., 2014), and staining with anti-PlexinD1 was performed using the iDISCO+ protocol (Renier et al., 2014).

2.5. Imaging and analysis

Brain sections were imaged using a confocal microscope (Zeiss LSM 780 and LSM 880). Image processing was performed in Photoshop CS6 (Version 13.0.1) and ImageJ. To quantify the layer position of labelled cells, we divided the cortical thickness into 10 bins. The correspondence between bins and cortical layers was determined by overlaying with staining against *Cux1* (layers 2/3), *Ctip2* (layers 5B, 6) and *Tbr1* (layer 6). In S1, bins 2–3 represent layers 2/3, bin 4–5 represent layer 4, bin 6 represents layer 5A, bin 7 represents layer 5B, and bins 8–10

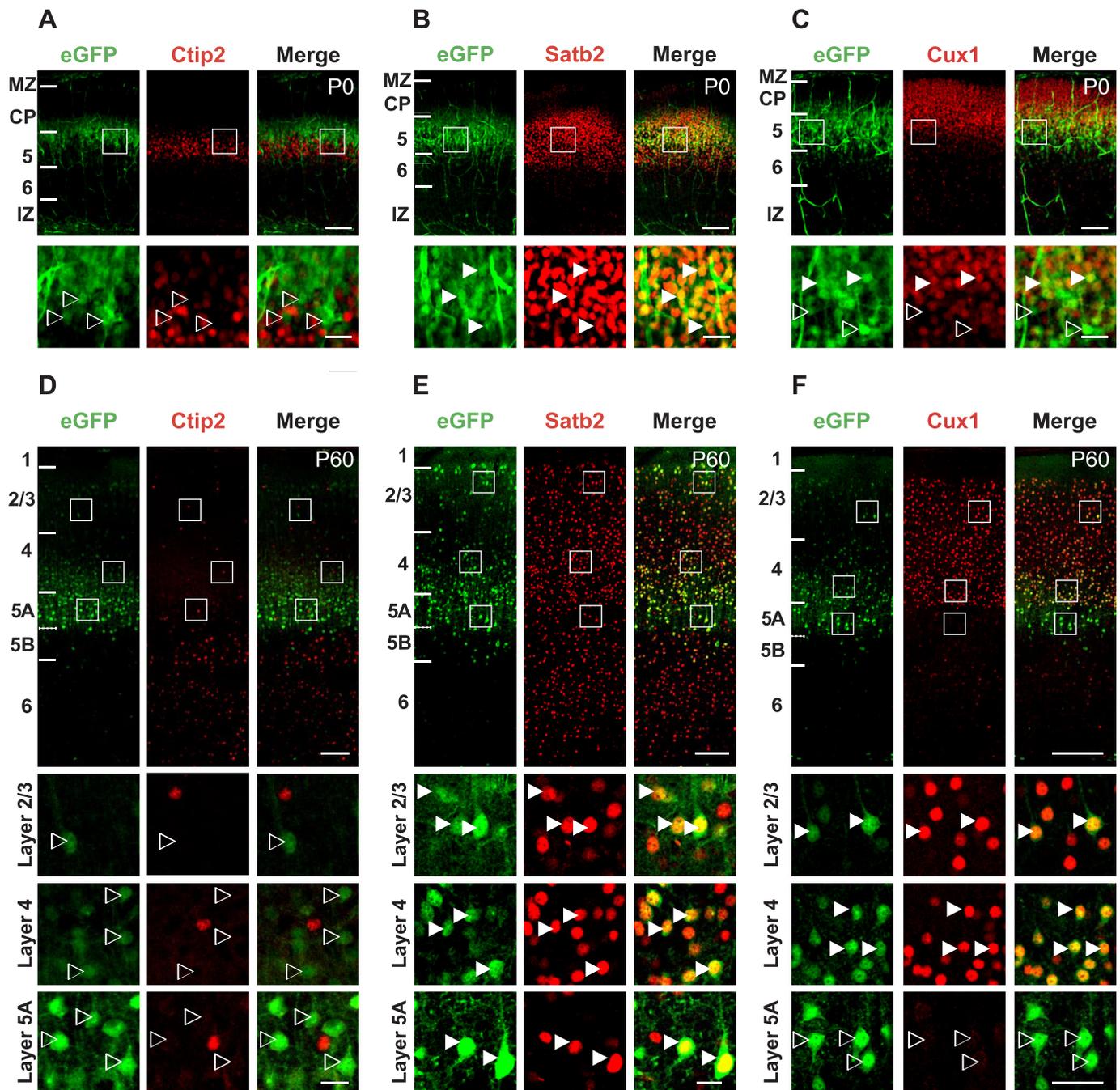


Fig. 2. Molecular signature of eGFP⁺ neurons.

Double labelling of eGFP with Ctip2, Satb2 or Cux1 on sections through the S1 area of P0.

(A–C) and P60 (D–F) *Plxnd1*-eGFP mice. The boxed areas are enlarged to show colocalization of eGFP with Satb2 or Cux1 (white arrowheads) but not with Ctip2 (empty arrowheads). CP: cortical plate, IZ: intermediate zone, MZ: marginal zone.

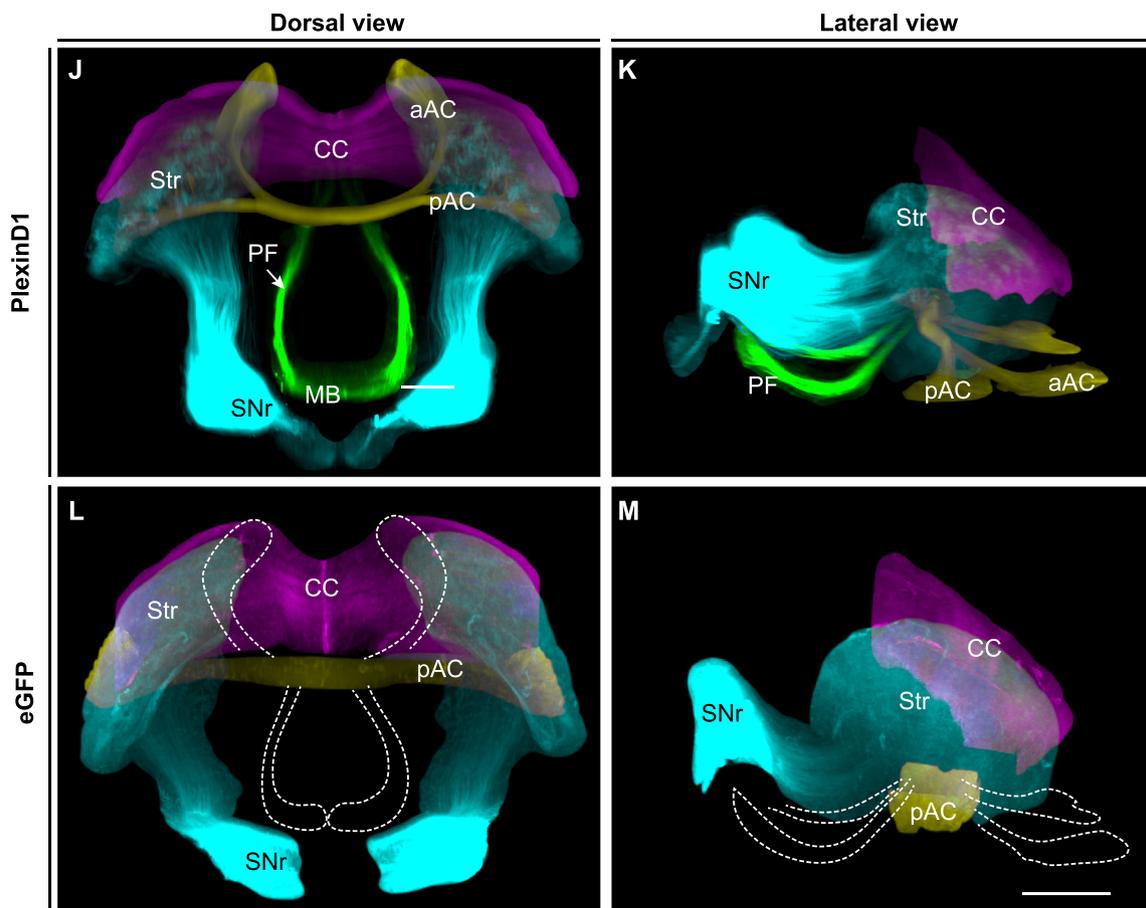
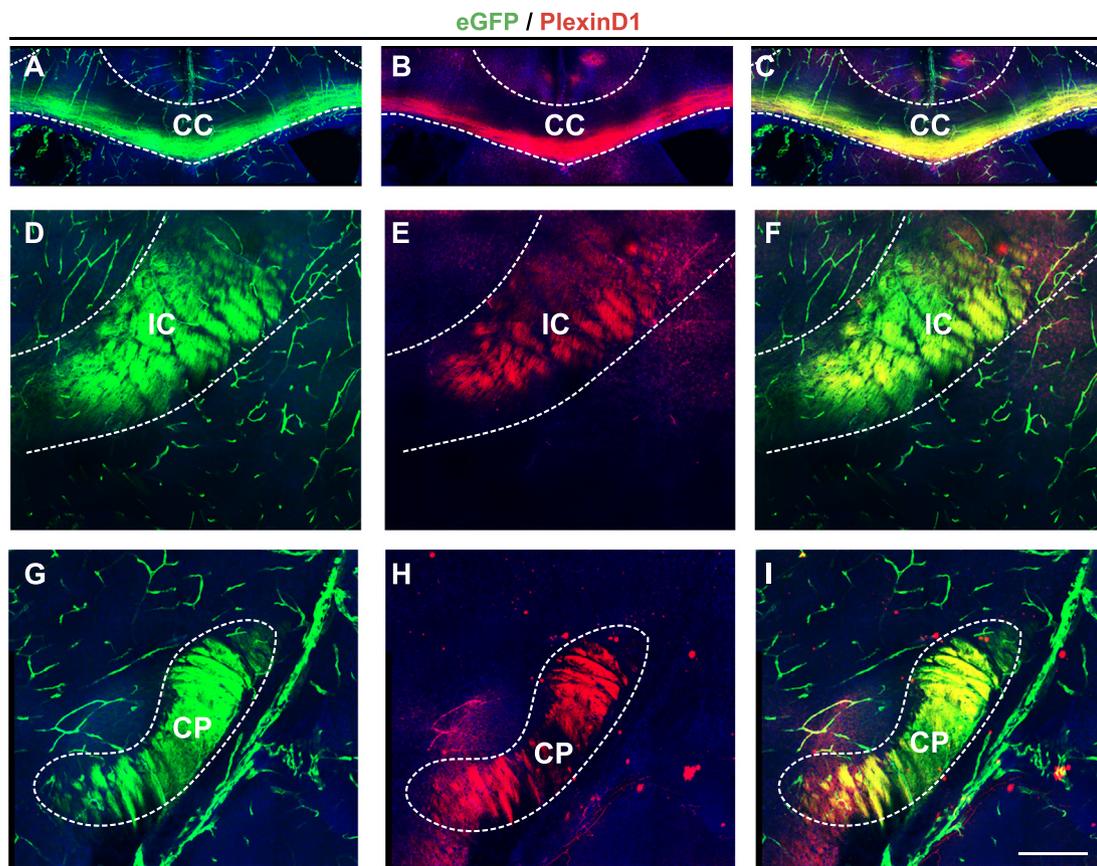
Scale bars: 80 μ m (A–C), 20 μ m (enlarged panels in A–C), 40 μ m (D–F), 10 μ m (enlarged panels in D–F).

represent layer 6. In M1, bins 2–4 represent layers 2/3, bin 5 is layer 5A, bins 6–7 are layer 5B, and bins 8–10 are layer 6. Cell counting was performed using the Photoshop counting tool.

3D imaging of cleared brains was performed on a light-sheet fluorescence microscope (LaVision BioTec Ultramicroscope II) using InspectorPro software (LaVision BioTec). 3D volume images were generated using Imaris $\times 64$ software (version 8.4.1, Bitplane). Segmentation of distinct axonal tracts was performed manually using ‘Isosurface’ (Imaris) by creating a mask around each volume, followed by ‘Isosurface’ performed automatically on the first segmentation. 3D pictures were generated using the ‘snapshot’ tool.

2.6. Statistics

Statistical analyses were performed using GraphPad Prism Version 6.05 (GraphPad Software, San Diego, CA, USA). For each experiment, the normal distribution of the data was examined using a D’Agostino–Pearson omnibus test. The estimate of variance was determined by the standard deviation of each group. Since data were nonparametric, the Mann–Whitney test was used to compare the means of two groups of data, and the Kruskal–Wallis test or one-way ANOVA followed by the Sidak multiple comparisons test was used to compare differences between more than two groups. Statistical significance was



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Fig. 3. Axonal projections of eGFP⁺ neurons

(A–I) Double immunofluorescence staining with anti-eGFP and anti-PlexinD1 antibodies in P3.

Plxnd1-eGFP brains revealed co-labelled axons in the ventral part of the corpus callosum. (A–C), internal capsule (D–F) and cerebral peduncles (G–I).

(J–M) Three-dimensional reconstructions of light-sheet microscopy images from neonatal (P3) *Plxnd1*-eGFP mouse brains immunolabelled for PlexinD1 (J, K) and eGFP (L, M). (J, L) Dorsal views and (K, M) lateral views of the immunolabelled brains. PlexinD1 labels axons in the corpus callosum (purple), striatonigral tract (blue), anterior commissure (yellow), and postcommissural fornix (green) (J, K). eGFP partially recapitulates PlexinD1 expression and labels axons of the corpus callosum, striatonigral tract and posterior limb of the anterior commissure (L, M). The dotted lines delineate the PlexinD1-expressing tracts that do not express eGFP (anterior limb of the anterior commissure and postcommissural fornix). eGFP expression pattern has been confirmed on sections and whole brains of 13 *Plxnd1*-eGFP mice from P0–P3.

aAC: anterior limb of the anterior commissure, CC: corpus callosum, CP: cerebral peduncle, IC: internal capsule, MB: mammillary bodies, pAC: posterior limb of the anterior commissure.

PF: post-commissural fornix, SNr: substantia nigra pars reticulata, Str: striatum.

Scale bars: 100 μ m (A–I), 1000 μ m (J–M).

set at $p < 0.05$. No statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally employed in the field. Statistical details (n , p value, statistical test used) can be found in the Results section and figure legends.

3. Results

3.1. *PlexinD1* expression in the developing and adult brain

To study the expression of PlexinD1 in the mouse neocortex, we used BAC transgenic mice in which expression of eGFP is under control of the *Plxnd1* promoter (hereafter referred to as *Plxnd1*-eGFP mice) (Bribián et al., 2014; Burk et al., 2017). Fluorescent *in situ* hybridization of *Plxnd1* mRNA on the cortex of adult *Plxnd1*-eGFP mice showed that eGFP transgene expression faithfully recapitulates the endogenous expression of PlexinD1 in all cortical layers of both S1 and M1 areas (Fig. S1). The embryonic expression pattern of PlexinD1 (E12.5–E16.5) has been previously described (Bribián et al., 2014; Deck et al., 2013). Here, we focused our analysis on later developmental stages (E18.5–P15) and adulthood. From E18.5 to P7, brain blood vessels were eGFP-positive, consistent with the known expression of PlexinD1 by endothelial cells (Gitler et al., 2004; Gu et al., 2005). eGFP was also expressed from E18.5 to P7 in the marginal zone (MZ) of the cortex where hem-derived Cajal-Retzius (CR) neurons reportedly express PlexinD1 (Fig. 1A–C). Consistent with the transient nature of CR cells, this expression disappeared from the MZ at P15 (Fig. 1D). Neuronal expression of eGFP was also observed from E18.5 to P7 in the deep cortical plate. This expression followed lateral-to-medial and anterior-to-posterior gradients, which reflect the gradients of neurogenesis (Fig. 1A–C). At P15, when laminar differentiation is completed, eGFP-positive neurons were found in layer 5A across all neocortical areas, including S1 and M1 areas (Fig. 1D–F). In addition, eGFP-positive cells were found in layer 4 of S1, and in layers 2/3 of S1 and M1 where expression levels were lower (Fig. 1E–F). This layer-specific expression pattern was maintained in the brains of adult mice (Fig. 1G).

3.2. *PlexinD1* is expressed by CPNs

PlexinD1 is reportedly expressed by CPNs (Molyneaux et al., 2009). We confirmed this finding using the following markers for CPNs subtypes: *Ctip2* defines subcortical projection neurons (Arlotta et al., 2005), *Satb2* defines callosal neurons (Alcamo et al., 2008; Britanova et al., 2008; Leone et al., 2015), and *Cux1* defines layer 2/3 callosal neurons (Rodríguez-Tornos et al., 2016). In the presumptive somatosensory cortex of *Plxnd1*-eGFP neonates, eGFP⁺ cells co-expressed the transcription factor *Satb2* but not *Ctip2* (Fig. 2A–B). In addition, the upper layer eGFP⁺ cells co-expressed *Cux1* (Fig. 2C). The co-expression pattern of eGFP with *Satb2* or *Cux1* was maintained in the adult S1 cortex (Fig. 2D–F). A similar colocalization pattern was observed for eGFP and *Satb2* in the M1 area, with the eGFP-positive cells in layer 2/3 also expressing *Cux1* (Fig. S2).

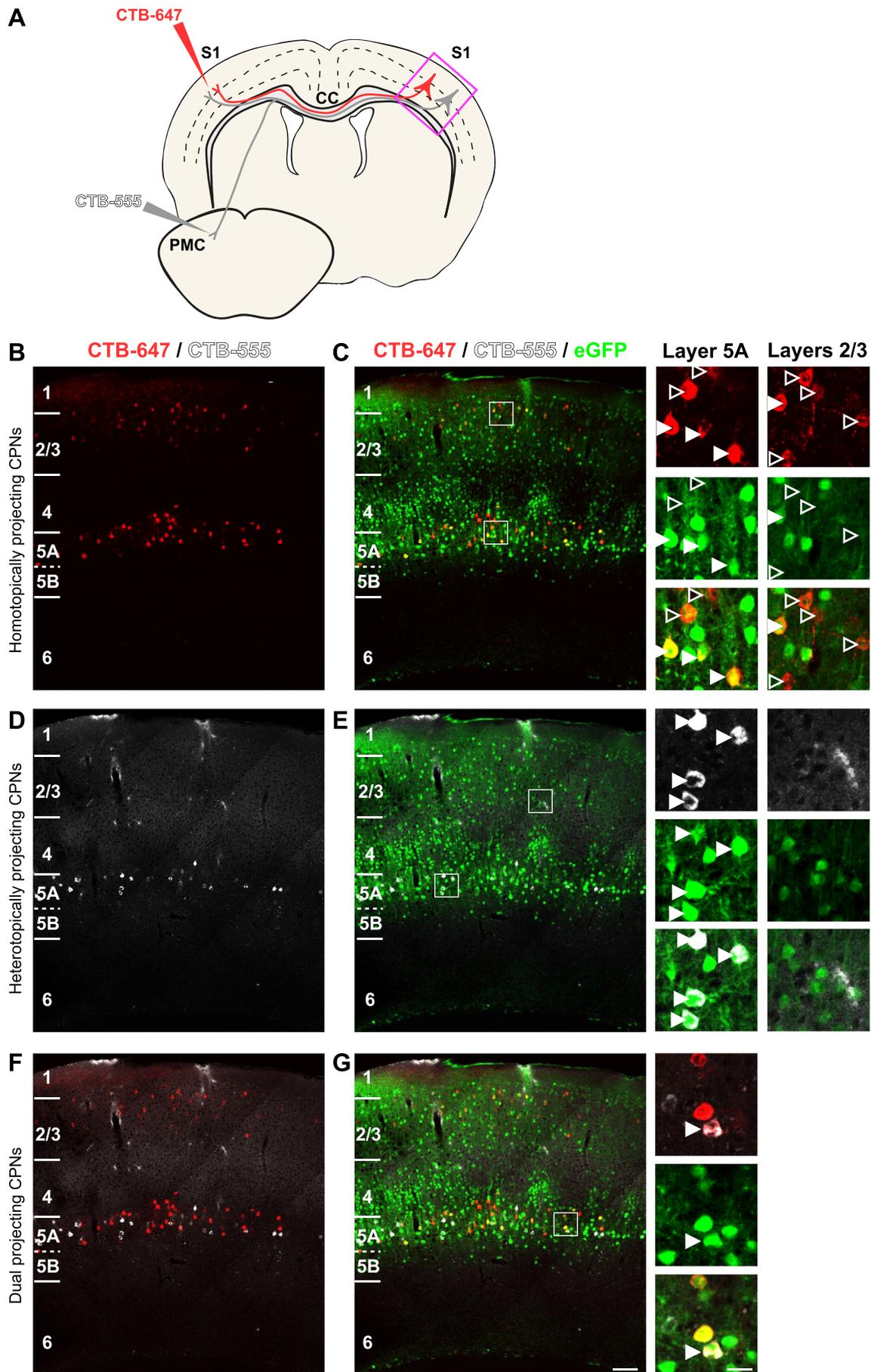
Callosal identity was further confirmed by analysing the main

output projection paths of the cortex. As expected, in P3 *Plxnd1*-eGFP brains, immunostaining for eGFP and PlexinD1 was observed in the corpus callosum where expression was restricted to the ventral portion of the tract (Fig. 3A–C). We also found co-labelling of eGFP and PlexinD1 in the IC and midbrain CPs, the pathway for SCPNs of layer 5 (Fig. 3D–I). Indeed, retrograde labelling by injection of DiI in the CP labelled neurons in layer 5 of the cortex. However, these neurons were located in layer 5B just beneath eGFP-expressing layer 5A neurons (Fig. S3A–B). Retrograde-labelled eGFP⁺ neurons were instead found in the striatum (Fig. S3C–F), indicating that the eGFP⁺/PlexinD1⁺ fibres observed in the IC and CP are part of the striatonigral tract (Burk et al., 2017). 3D imaging of eGFP⁺ and PlexinD1⁺ tracts in cleared brains further confirmed expression in the corpus callosum and striatonigral tracts (Fig. 3J–M). Together, these data indicated that PlexinD1 is specifically expressed in a subset of callosal neurons during cortical development and in the adult brain.

3.3. *PlexinD1* is expressed by heterotopically projecting CPNs

A large majority of CPNs interconnect symmetrical (homotopic) regions of both cortical hemispheres, but connections between non-homologous (heterotopic) regions have also been demonstrated. The following types of long-range heterotopic callosal projections originating from layer 5 CPNs have been described in the mouse brain: a small proportion of layer 5 CPNs in the sensory-motor cortex project to the contralateral PMC (Mitchell and Macklis, 2005), and some layer 5 CPNs in motor and premotor cortices project to the contralateral cortex and striatum (Sohur et al., 2014). The expression of PlexinD1 in layer 5A CPNs has suggested that they might represent heterotopically projecting neurons (Molyneaux et al., 2009).

To address this idea, we used a double retrograde labelling technique in adult *Plxnd1*-eGFP mice. In the first setting, we injected Alexa Fluor 647-conjugated CTB (CTB-647) in the S1 cortex and Alexa Fluor 555-conjugated CTB (CTB-555) in the PMC of the same hemisphere. This injection allowed us to simultaneously retrogradely label CPNs with homotopic S1 > S1 and heterotopic S1 > PMC projections residing in the contralateral S1 cortex (Figs. 4A and S4A). Consistent with the known distribution of CPNs, homotopically projecting S1 > S1 callosal neurons (CTB-647⁺) were distributed across cortical layers 2/3 and 5 (Fig. 4B). In layer 5A, 61% of CTB-647⁺ neurons were eGFP⁺ ($n = 631$ CTB-647⁺/eGFP⁺ neurons, 433 CTB-647⁺/eGFP⁻ neurons, 15 sections, 3 mice; Fig. 4C). In contrast, heterotopically projecting S1 > PMC neurons (CTB-555⁺) were largely restricted to layer 5A, and most of these neurons were eGFP⁺ (73%, $n = 200$ CTB-555⁺/eGFP⁺ neurons, 80 CTB-555⁺/eGFP⁻ neurons, 15 sections, 3 mice; Fig. 4D–E). These results suggest that layer 5A eGFP⁺ neurons might represent callosal neurons that send long-range dual projections to the homotopic region of the contralateral cortex and rostrally to the contralateral PMC (dual-projecting S1 > S1/PMC neurons). Consistent with this idea, we found that the large majority of neurons double labelled for CTB-647 and CTB-555 were indeed eGFP⁺ neurons (96%, $n = 23$ CTB-647⁺/CTB-555⁺/eGFP⁺ neurons, 2 CTB-647⁺/CTB-



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Fig. 4. eGFP⁺ CPNs in the S1 area send heterotopic projections to the contralateral PMC.

(A) Schematic representation of dual retrograde labelling of homotopic (S1 > S1) and heterotopic (S1 > PMC) transcallosal projections with two CTB-conjugated fluorophores (CTB-647 and CTB-555) in the brains of adult *Plxnd1*-eGFP mice.

(B–G) Visualization of eGFP⁺ CPNs in the S1 cortex with retrograde labelling from contralateral S1 (CTB-647⁺) (B–C), from contralateral PMC (CTB-555⁺) (D–E) or from both regions (CTB-647⁺/CTB-555⁺).

(F–G). The boxed areas are enlarged to show colocalization of CTB-conjugated fluorophores with eGFP (white arrowhead) or absence of colocalization (empty arrowhead).

Scale bars: 40 μm (B–G), 10 μm (enlarged panels).

555⁺/eGFP⁻ neurons, 15 sections, 3 mice; Fig. 4F–G). Together, these data indicated that PlexinD1 is expressed by a large proportion of layer 5A CPNs in S1 that send long-range projections to heterotopic targets and/or send dual projections to homotopic and heterotopic areas in the contralateral cortex.

In a second setting, CTB-647 and CTB-555 were injected into the M1 cortex and dorsolateral sector of the striatum, respectively. The distribution of homotopically M1 > M1 and heterotopically M1 > Str projecting neurons was assessed in the contralateral M1 cortex (Figs. 5A and S4B). Similar to the results in the somatosensory cortex, homotopically projecting M1 > M1 neurons were distributed in cortical layers 2/3 to 5, whereas heterotopically projecting M1 > Str neurons were principally located in layer 5A. Within layer 5A, 59% of (homotopic) CTB-647⁺ ($n = 277$ CTB-647⁺/eGFP⁺ neurons, 221 CTB-647⁺/eGFP⁻ neurons, 22 sections, 3 mice; Fig. 5B–C) and 57% of heterotopic CTB-555⁺ ($n = 351$ CTB-655⁺/eGFP⁺ neurons, 211 CTB-555⁺/eGFP⁻ neurons, 22 sections, 3 mice; Fig. 5D–E) neurons were eGFP⁺ (Fig. 5B–E). In addition, eGFP⁺ cells in layer 5A represented 69% of CTB-647/CTB-555 double-labelled neurons (dual-projecting M1 > M1/Str neurons) ($n = 16$ CTB-647⁺/CTB-655⁺/eGFP⁺ neurons, 10 CTB-647⁺/CTB-555⁺/eGFP⁻ neurons, 22 sections, 3 mice; Fig. 5F–G). These data indicated that PlexinD1 is expressed in M1 by CPN subpopulations that send transcallosal projections to the contralateral striatum and/or send dual projections to the contralateral cortex and striatum.

3.4. Abnormal laminar distribution of heterotopically projecting CPNs in mice lacking *Plxnd1* and *Sema3e* genes

PlexinD1 exerts a diverse range of physiological activities, including regulation of cell survival and migration, axon growth and guidance, and synapse formation (Oh and Gu, 2013). To evaluate the role of PlexinD1 in CPNs, we used conditional knockout mice that lack PlexinD1 in cortical glutamatergic neurons (*Plxnd1*^{lox/-}; *Emx1*^{cre} mice). In adult mutant brains, the expression patterns of the cortical layer markers *Cux1* (layers 2–4), *Ctip2* (layers 5B and 6) and *Er81* (layer 5) were indistinguishable from those in control mice (Fig. S5A–C). In addition, labelling of early-born neurons by injection of EdU at E13.5 showed no difference in the distribution of EdU-labelled neurons in the mature motor cortex (P16) of control and conditional *Plxnd1* mutants (Fig. S5D–E). Overall, these results suggested normal cortical lamination in *Plxnd1* conditional mutants.

Homotopically and heterotopically projecting CPNs in the M1 of *Plxnd1*^{lox/-}; *Emx1*^{cre} mice were labelled selectively by contralateral injections of CTB-647 and CTB-555 in the motor cortex and dorsolateral striatum, respectively (Fig. S6). The distribution of homotopic M1 > M1 CTB-647⁺ cells was similar to that in control mice (control wild-type and *Plxnd1*^{lox/+}; *Emx1*^{cre} mice were pooled together as there were no differences between these two groups) (Fig. 6A and D). Numerous cells were retrogradely labelled by CTB-555 in *Plxnd1*^{lox/-}; *Emx1*^{cre} mice, indicating that heterotopic M1 > Str transcallosal projections are made independently of PlexinD1 expression. However, we noticed that CTB-555⁺ and double-labelled CTB-647⁺/CTB-555⁺ neurons were distributed differently across cortical layers in *Plxnd1*^{lox/-}; *Emx1*^{cre} mice, with a significantly higher proportion of heterotopically M1 > Str and/or dual-projecting M1 > M1/Str neurons in

upper layer 2/3 compared to that in control mice (Fig. 6B–C and E–F). Since *Sema3E* is the main ligand for PlexinD1, we investigated the distribution of homotopically and heterotopically projecting CPNs in the motor cortex of *Sema3e*^{-/-} mice (Fig. S6), which exhibit a normal distribution of the layer-specific markers *Cux1* and *Er81* (Fig. S7A–B). The expression of *Plxnd1* was also unaffected in the mutant cortex (Fig. S7C) and PlexinD1⁺ callosal fibres extended normally to both hemispheres (Fig. S7D). A significant difference was observed in the distribution of heterotopically projecting M1 > Str CTB-555⁺ neurons between mutant and control brains, with ectopic cells in cortical layers 2/3 of *Sema3e*^{-/-} mice (Fig. 7). These results suggested that PlexinD1 and *Sema3E* are required for the correct laminar distribution of heterotopically projecting M1 > Str CPNs in the motor cortex (Fig. 8).

4. Discussion

Comparative transcriptome analyses previously identified *Plxnd1* as a gene expressed in restricted subpopulations of CPNs and excluded from other projection neurons in the developing neocortex (Molyneux et al., 2009). Here, we showed that *Plxnd1* is expressed by some CPNs distributed in layer 5A, which is reminiscent of the location of CPNs making heterotopic long-range axonal projections. Retrograde labelling confirmed that *Plxnd1* is expressed by virtually all (96%) dual-projecting S1 > S1/PMC CPNs located in layer 5A of the somatosensory cortex that extend axonal connections to both homotypic and heterotopic locations in the contralateral cortex. In addition, in the motor cortex, 60% of dual-projecting M1 > M1/Str CPNs extending collaterals to the contralateral striatum appeared to express *Plxnd1*. Therefore, we propose that *Plxnd1* is a common identifying marker for two types of regionally restricted dual-projecting CPNs.

Although the impressive molecular diversity of CPN subtypes is emerging, markers to further subdivide the different CPN subpopulations that project to multiple targets remain limited. In the adult motor cortex, *Fezf2* labels the majority of layer 5A CPNs with contralateral striatal projections (Tantirigama et al., 2016). The expression pattern of *Fezf2* may overlap with that of PlexinD1. More recently, the lipid-bound scaffolding domain protein Caveolin-1 (*Cav1*) was found to be expressed during development by a restricted population of layer 5A CPNs in S1 but was excluded from M1. *Cav1*⁺ cortical neurons represent CPNs with simultaneous callosal projection to contralateral S1 and ipsilateral projection to PMC (MacDonald et al., 2018). Given that over two-thirds of the dual-projecting S1 > S1/PMC CPNs also send a projection to the ipsilateral PMC (Mitchell and Macklis, 2005), *Cav1* is most likely co-expressed with PlexinD1 in these neurons. Rapid advances in single cell analysis will certainly aid future studies to identify other genes that work in combination with *Plxnd1* to organize the diversity and area-specific features of dual-projecting CPNs.

Since PlexinD1 is enriched on callosal axons at P3, we investigated whether it is involved in axon guidance and targeting. Our results revealed that PlexinD1 expression in layer 5A CPNs of the motor cortex is not necessary for the development and maintenance of long-range heterotopic projections to the contralateral striatum. Alternatively, we found that loss of PlexinD1 function leads to the appearance of ectopic dual-projecting M1 > M1/Str neurons in layers 2/3 of the motor cortex, while in the control condition, all CPNs in upper neocortical layers projected to the contralateral mirror cortex. PlexinD1 signalling

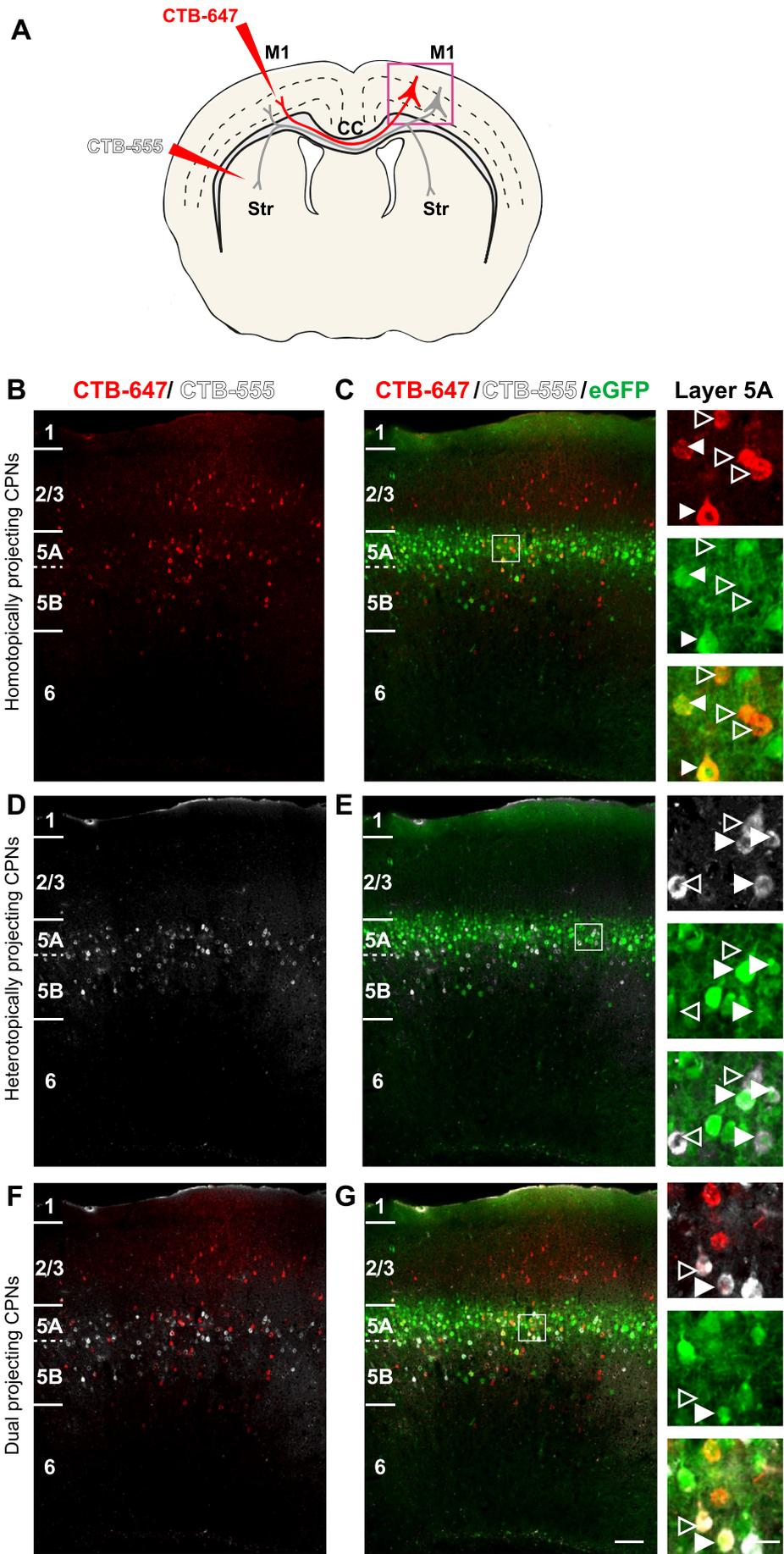


Fig. 5. eGFP⁺ CPNs in the M1 area send heterotopic projections to the contralateral striatum. (A) Schematic representation of dual retrograde labelling of homotopic (M1 > M1) and heterotopic (M1 > Str) transcallosal projections with two injections of CTB-conjugated fluorophores (CTB-647 and CTB-555) in the brain of adult *Plxnd1*-eGFP mice. (B–G) Visualization of eGFP⁺ CPNs in M1 cortex with retrograde labelling from contralateral M1 (CTB-647⁺) (B–C), from contralateral striatum (CTB-555⁺) (D–E) or from both regions (CTB-647⁺/CTB-555⁺) (F–G). The boxed areas are enlarged to show colocalization of CTB-conjugated fluorophores with eGFP (white arrowhead) or absence of colocalization (empty arrowhead).

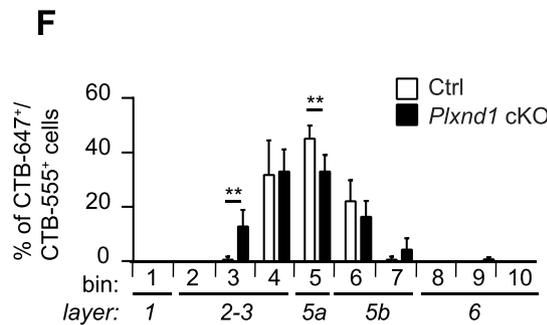
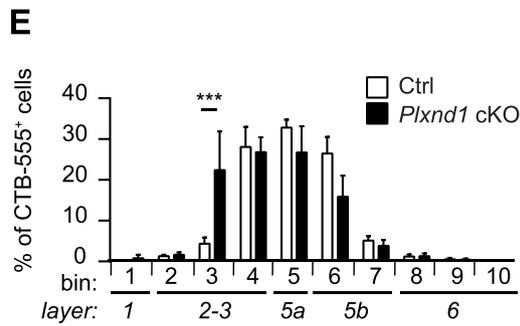
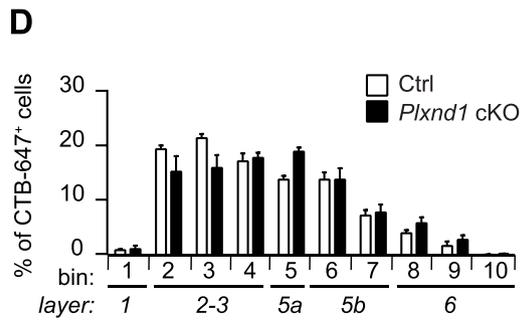
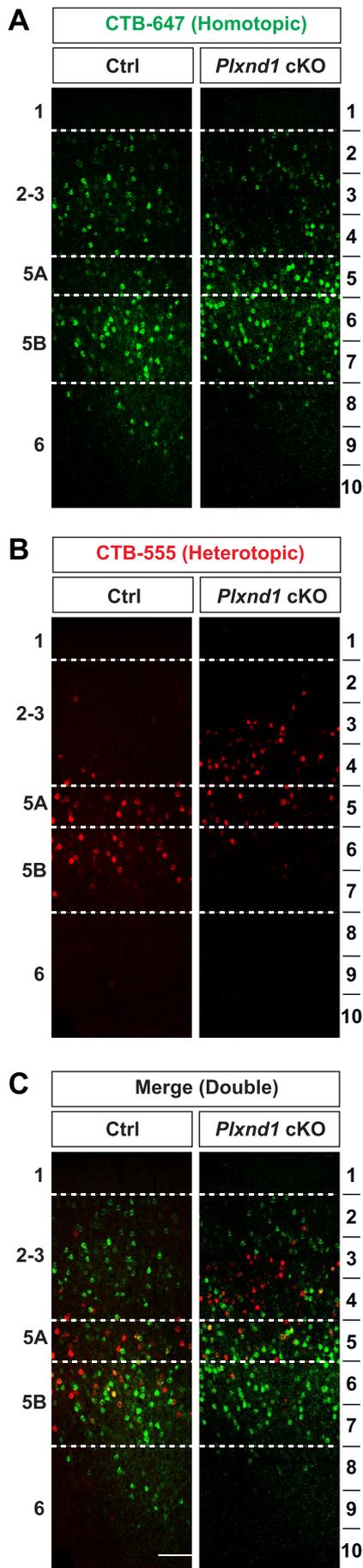
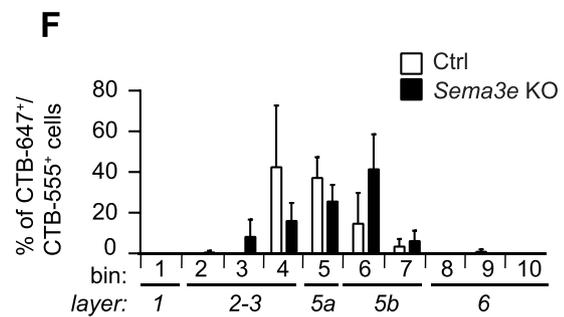
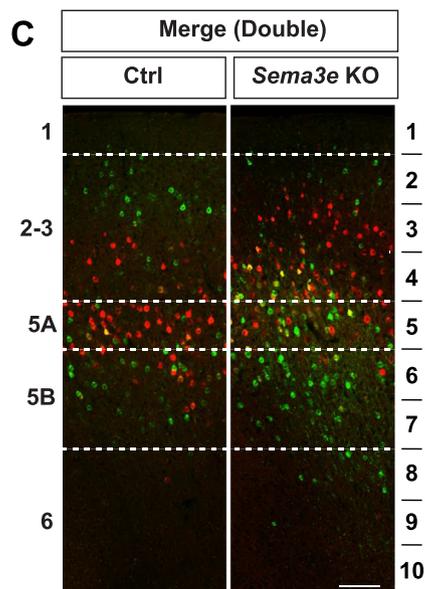
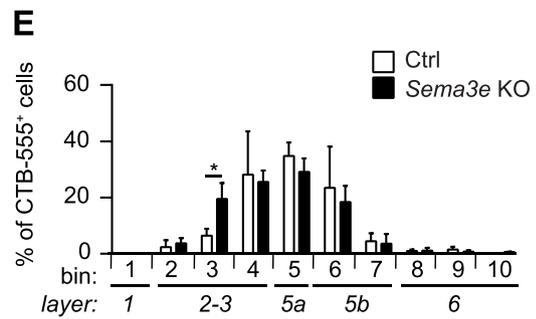
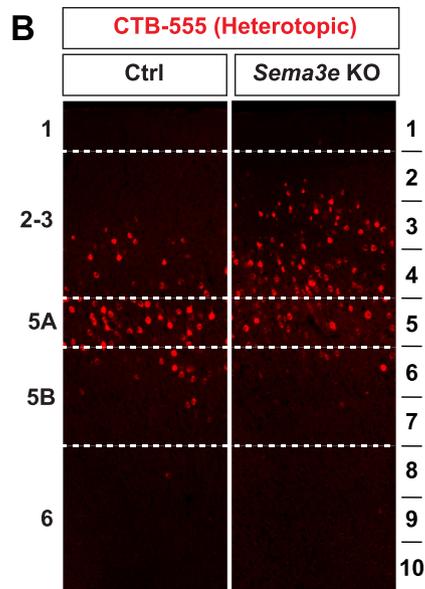
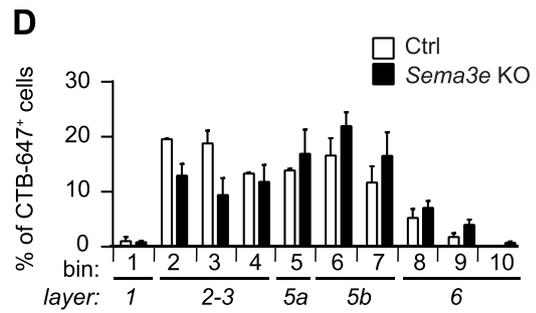
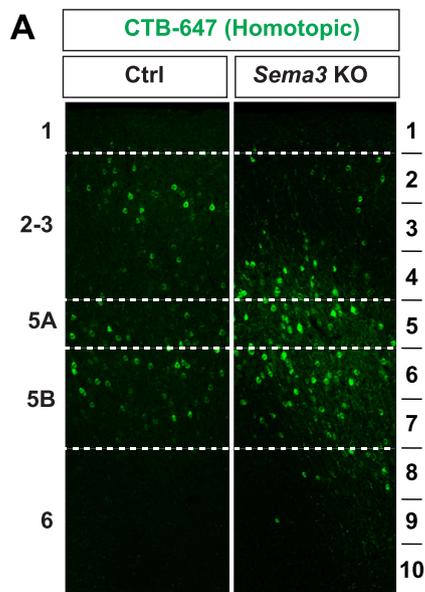


Fig. 6. Mispositioning of heterotopically projecting CPNs in the cortex of *Plxnd1* conditional mutant mice.

Adult Ctrl (control) and *Plxnd1* cKO (*Plxnd1*^{lox/-}; *Emx1*^{cre}) mice were subjected to dual injections of CTB-647 and CTB-555 in M1 and dorso-lateral striatum, respectively. (A-C) Distribution of CPNs in M1 cortex with retrograde labelling from contralateral M1 (CTB-647⁺) (A), from contralateral striatum (CTB-555⁺) (B) or from both regions (CTB-647⁺/CTB-555⁺) (C).

(D-F) Quantification of the laminar distribution of CTB-647⁺ (D), CTB-555⁺ (E) and double-labelled CTB-647⁺/CTB-555⁺ (F) neurons. Ten equal-sized bins were drawn over each image. Data represent the mean \pm SEM. Ctrl: *n* = 6 mice, 23 sections; *Plxnd1* cKO: *n* = 4 mice, 16 sections. **, *p* < 0.01; ***, *p* < 0.001 with two-way ANOVA followed by the Sidak multiple comparisons test.

Scale bar: 100 μ m.



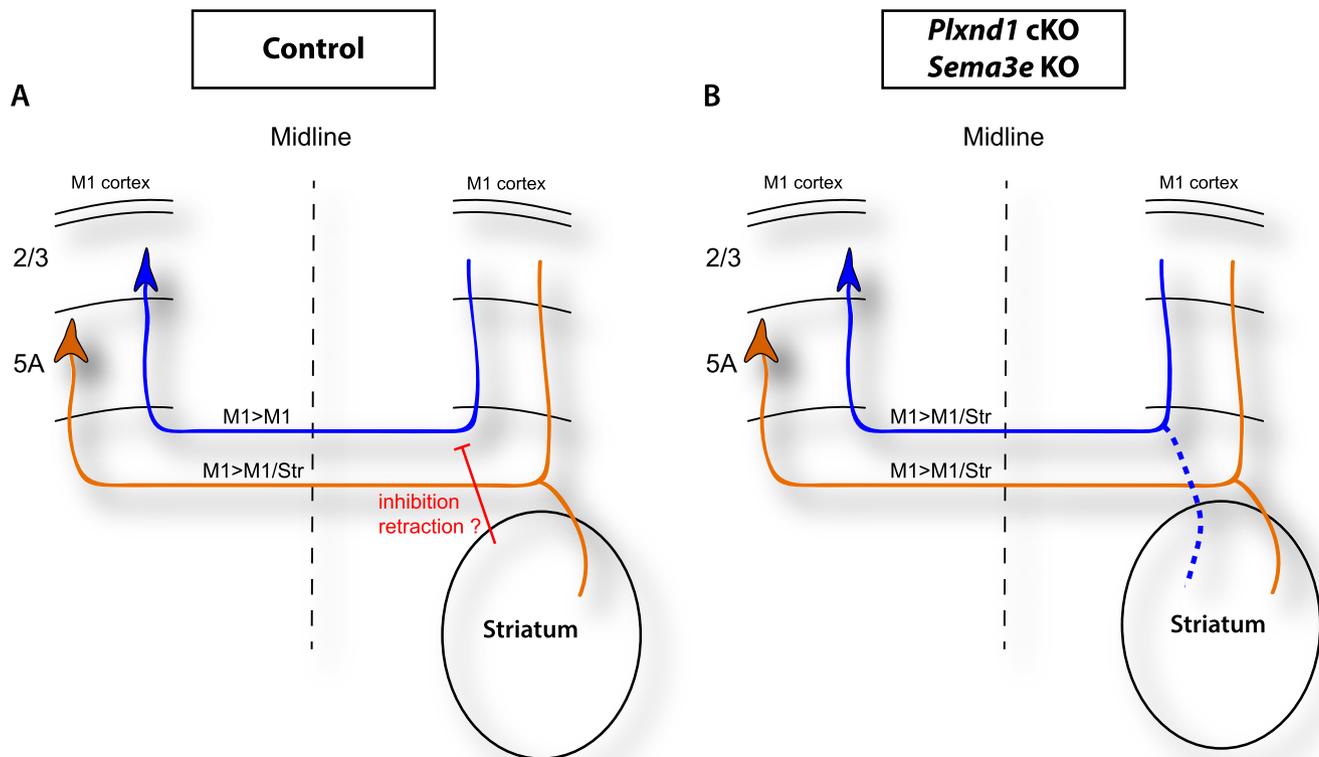
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Fig. 7. Mispositioning of heterotopically projecting CPNs in the cortex of *Sema3e* null mice

Adult control (Ctrl) and *Sema3e* KO (*Sema3e*^{-/-}) mice were subjected to dual injections of CTB-647 and CTB-555 in M1 and dorsolateral striatum, respectively. (A-C) Distribution of CPNs in M1 cortex with retrograde labelling from contralateral M1 (CTB-647⁺) (A), from contralateral striatum (CTB-555⁺) (B) or from both regions (CTB-647⁺/CTB-555⁺) (C).

(D-F) Quantification of the laminar distribution of CTB-647⁺ (D), CTB-555⁺ (E) and double-labelled CTB-647⁺/CTB-555⁺ (F) neurons. Ten equal-sized bins were drawn over each image. Data represent the mean \pm SEM. Ctrl: 3 mice, 12 sections; *Sema3e* KO: *n* = 3 mice, 12 sections. *, *p* \leq 0.05 with two-way ANOVA followed by the Sidak multiple comparisons test.

Scale bar: 100 μ m.

**Fig. 8.** PlexinD1 and Sema3E regulate the laminar distribution of CPNs with heterotopic corticostriatal projections.

(A) Schematic summary showing the laminar distribution of CPNs with heterotopic M1 > M1/Str (orange) in layer 5A. All CPNs in layers 2/3 establish homotopic M1 > M1 projections (blue).

Sema3E/PlexinD1 signalling may prevent them from projecting to the striatum. (B) In *Plxnd1* cKO (*Plxnd1*^{lox/-}; *Emx1*^{cre}) and *Sema3e* KO (*Sema3e*^{-/-}) mice, some CPNs in superficial layers send aberrant heterotopic projections to the contralateral striatum (dotted line).

regulates radial migration of newborn neurons in the rat neocortex (Chen et al., 2008), suggesting that layer 5A dual-projecting CPNs may have migrated into upper layers of the *Plxnd1* mutant cortex. However, our data do not support this view. Indeed, the expression of cortical layer markers in the mutant cortex was undistinguishable from that in the control cortex, and the laminar distribution of EdU-labelled neurons was unaffected. Therefore, it is more likely that the ectopic projections to the contralateral striatum observed in the mutant mice arise from the small subset of layer 2/3 neurons that normally express PlexinD1 (shown by *in situ* hybridization in Fig. S1C and D).

How does PlexinD1 regulate the formation of corticostriatal projections by layer 2/3 neurons? *In situ* hybridization data, including those reported in the Allen Brain Atlas (<http://www.brain-map.org>), indicate that *Sema3e* is expressed in the striatum at P4 and is maintained at P14, a time window that covers critical events in the initial development and refinement of crossed corticostriatal projections (Sohur et al., 2014). There are at least two possible scenarios for ectopic crossed corticostriatal projections. First, the repellent activity of Sema3E could prevent PlexinD1-positive CPNs of layers 2/3 from extending axon collaterals into the striatum that begins to be innervated by other cortical fibres around P3-P4. At this stage of development, a surplus of cortico-striated connections is created, which is then refined

through regulated pruning mechanisms. In a second scenario, Sema3E/PlexinD1 could initiate the retraction of transient projections established by layers 2/3 CPNs to the striatum.

Both hypotheses would explain contralateral misprojections of layer 2/3 neurons in the mature cortex of *Plxnd1* and *Sema3e* mutant mice. However, these hypotheses do not explain how layer 5A CPNs, which express high levels of PlexinD1, can form and maintain dual projections to the contralateral striatum. Therefore, PlexinD1-expressing neurons residing in cortical layers 2/3 and 5A may possibly respond differentially to Sema3E. According previous research, the effect of Sema3E/PlexinD1 signalling on axon guidance depends on neuron-type specific combinations of receptor molecules. More specifically, the expression of the neuropilin-1 (Nrp1) co-receptor inhibits the response of PlexinD1-positive axons to Sema3E, while axons that concomitantly express Nrp1 and VEGFR2 switch responses from repulsion to attraction (Bellon et al., 2010; Chauvet et al., 2007). Interestingly, Nrp1 shows increasing expression from P4 to P14 in layer 5 of the motor cortex (data from the Allen Brain Atlas). In contrast, cortical neurons in layers 2/3 do not express Nrp1 during these stages of development. Therefore, these two types of neurons are likely to respond differently to Sema3E, with only layer 2/3 neurons being sensitive to the repellent activity of Sema3E. This model would be consistent with the observed lack of effect of

Sema3E/PlexinD1 signalling on the development of dual-projecting M1 > M1/Str layer 5A CPNs.

5. Conclusion

The data presented in this study characterized the long-range connectivity of CPN subpopulations defined by the expression of the axon guidance receptor PlexinD1 and identified a role of PlexinD1 in the regulation of the laminar organization of dual-projecting M1 > M1/Str CPNs. Our results also revealed a class of PlexinD1⁺ neurons in layer 4 of the somatosensory cortex that are not CPNs. Layer 4 neurons comprise the following morphological subclasses: spiny stellate cells, star pyramidal neurons and a few pyramidal cells (Harris and Shepherd, 2015). It will be interesting to determine whether PlexinD1 is a marker for one or more of these neuron subclasses. Our data also showed that PlexinD1 continues to be expressed in the adult brain where its function remains unknown. In the adult *Drosophila* nervous system, Semaphorin/Plexin signalling is implicated in homeostatic plasticity through regulation of presynaptic neurotransmitter release (Orr et al., 2017). Such a function of PlexinD1 signalling in the stabilization of cortical circuits remains to be explored.

Author contributions

Study concept and supervision: F.M., and S.C. Data acquisition: T.V., M.A., M.R., M.H., A.B., C.G. and P.S. Data analysis and interpretation: T.V., S.C., M.N. and F.M. Drafting of the manuscript: T.V., S.C. and F.M.

Declaration of competing interest

The authors declare they have no competing financial interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.mcn.2019.103397>.

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