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## **New pool of cortical interneuron precursors in the early postnatal dorsal white matter**

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## **Abstract**

The migration of cortical GABAergic interneurons has been extensively studied in rodent embryos, whereas few studies have documented their postnatal migration. Combining *in vivo* analysis together with time-lapse imaging on cortical slices, we explored the origin and migration of cortical interneurons during the first weeks of postnatal life. Strikingly, we observed that a large pool of GAD65-GFP positive cells accumulates in the dorsal white matter region during the first postnatal week. Part of these cells divides and expresses the transcription factor paired box 6 indicating the presence of local transient amplifying precursors. The vast majority of these cells are immature interneurons expressing the neuronal marker doublecortin and partly the calcium binding protein calretinin. Time-lapse imaging reveals that GAD65-GFP positive neurons migrate from the white matter pool into the overlying anterior cingulate cortex (aCC). Some interneurons in the postnatal aCC express the same immature neuronal markers suggesting ongoing migration of calretinin positive interneurons. Finally, BrdU incorporation experiments confirm that a small fraction of interneurons located in the aCC are generated during the early postnatal period. These results altogether reveal that at postnatal ages the dorsal white matter contains a pool of interneuron precursors that divide and migrate into the aCC.

## **Introduction**

In rodents, the vast majority of cortical GABAergic interneurons are generated during the embryonic period in specific subpallial domains (Wonders and Anderson 2006; Gelman and Marin 2010), whereas in humans and non-human primates a large fraction of cortical interneurons originates from proliferative zones of the dorsal pallium (Letinic et al. 2002; Petanjek et al. 2009a). It is generally accepted that the combinatorial expression of a variety of transcription factors control the fate specification of interneurons (Gelman and Marin 2010). In rodents, cortical interneurons arising from the medial ganglionic eminence (MGE) are derived from progenitors specifically expressing the transcription factor Nkx2.1 and express parvalbumin or somatostatin (Xu et al. 2008). Cortical interneurons generated outside of the MGE in other subpallial domains such as the caudal ganglionic eminences (CGE) are thought to represent up to 30% of the total number of cortical interneurons, and express calretinin, reelin, neuropeptide Y (NPY) and the vasoactive intestinal peptide (VIP) (Miyoshi et al. 2010; Vucurovic et al. 2010; Rubin et al. 2010). After being generated in specific subpallial domains, interneurons migrate tangentially to reach the dorsal pallium. The tangential migration of MGE-derived interneurons is well characterized in rodents and occurs almost exclusively prenatally (Marin and Rubenstein 2001; Wonders and Anderson 2006; Gelman and Marin 2010). Few studies have explored the potential for postnatal migration of cortical interneurons generated outside of the MGE. BAC transgenic mice expressing the enhanced green fluorescent protein (GFP) under the control of the serotonin receptor 3A promoter (5-HT<sub>3A</sub>-GFP) label CGE derived cortical interneurons, and data support the possibility that a fraction of 5HT<sub>3A</sub>-GFP<sup>+</sup> cells are generated postnatally and migrate into the cortex during the postnatal period (Inta et al. 2008; Vucurovic et al. 2010). To further examine this possibility, we explored the postnatal generation and migration of cortical interneurons in transgenic mice expressing GFP under the control of the glutamate acid

decarboxylase 65 enzyme (GAD-65-GFP) (Lopez-Bendito et al. 2004). Similarly to 5HT<sub>3A</sub>-GFP+ cells (Vucurovic et al. 2010), GAD65-GFP+ cells mainly populate superficial cortical layers and preferentially label non-MGE derived cortical interneurons (Lopez-Bendito et al. 2004). We found that during the first postnatal week a large pool of GAD65-GFP+ cells accumulates in the dorsal white matter region at the level of prospective aCC. We observed that a fraction of GAD65-GFP+ interneurons continues to be generated postnatally in the dorsal white matter, migrate from this site into the prospective anterior cingulate cortex (aCC) and give rise to calretinin positive cortical interneurons. Fate mapping studies using Nkx2.1-Cre mice or 5-HT3-GFP mice as well as expression of transcription factors favor a LGE/CGE origin of this pool of GAD65-GFP+ cells. These results provide evidence that a pool of dividing interneuron precursors accumulate during the first postnatal week in the medial dorsal white matter of mice and can migrate into the lower cortical layers of the anterior cingulate cortex.

## **Methods**

### ***Animals***

All animal experiments were conducted according to relevant national and international guidelines and in accordance with Swiss laws. The day of the vaginal plug detection was counted as embryonic day (E) E 0.5. We used transgenic mice expressing GFP under the control of the GAD65 promoter (Lopez-Bendito et al. 2004) (referred here as GAD65-GFP mice) and mice were maintained on a C57Bl/6 genetic background. GENSAT BAC transgenic mice expressing enhanced GFP under the control of the 5-HT<sub>3A</sub> receptor (line Tg/Htr3a-GFP) was provided by the GENSAT Consortium to T. Vitalis, and maintained on a mixed Swiss and C57/BL6 background. BAC transgenic Nkx2.1 cre mice were kindly provided by Stewart Anderson (Xu et al. 2008) and were maintained on a C57/BL6 background. The Rosa26R–yellow fluorescent protein (YFP) reporter mice (Srinivas et al. 2001) have been crossed with the Nkx2.1-Cre mice. After Cre-mediated recombination, the mice express YFP under control of the Rosa26 promoter.

### ***Cortical slice preparation and time-lapse imaging***

To monitor the migration of GAD65-GFP<sup>+</sup> cells, timed pregnant embryonic day E17.5 GAD65-GFP<sup>+</sup> dams and GAD65-GFP<sup>+</sup> mice at postnatal day P0.5, P2.5, P4.5, P7 were used. Dams and postnatal pups were deeply anesthetized by intraperitoneal (i.p.) pentobarbital injection (50 mg/kg), all embryos and pups were killed by rapid decapitation, and brains were dissected. Cortical slices (200 μm thick) were cut on a Vibratome 1500 washed in a dissection medium (minimum essential medium (MEM) 1X, 5 mM Tris, 0.5% penicillin and streptomycin) for 5 min; placed on porous nitrocellulose filters (Millicell-CM, Millipore) in 60 mm Falcon Petri dishes and kept in neurobasal medium (Invitrogen) supplemented with 2% B27 (Gibco, Invitrogen), 2 mM glutamine, 1 mM sodium pyruvate, 2 mM N-acetyl-cysteine, 1% penicillin-

streptomycin. Time-lapse imaging was performed 6 hours after slice preparation for over at least 4 hours.

### ***In vivo BrdU incorporation experiments***

For short-term proliferation experiments, BrdU (50 mg/kg, Sigma) was injected i.p. in P0.5 and P7 mice, and animals were sacrificed 2 h after the BrdU injection. For long-term survival experiments, mice were injected with BrdU (3x 20 mg/kg, i.p. at 2 h intervals) at P0.5 and sacrificed at P10 or at P7 and sacrificed at P21.

### ***Tissue processing and immunohistochemistry***

Animals were deeply anesthetized by pentobarbital i.p injection (50 mg/kg), and sacrificed by intracardial perfusion of 0.9 % saline followed by cold 4 % paraformaldehyde (PFA) (pH 7.4). Brains were post-fixed over-night in PFA at 4 °C and coronal sections were cut on a Vibratome (Leica VT100S) (60 µm thick sections) and stored at 4 °C in 0.1 M phosphate buffer saline (PBS). For free-floating immunohistochemistry, sections were washed three times with 0.1 M PBS, incubated overnight at 4°C with a primary antibody diluted in PBS / 0.5 % bovine serum albumin (BSA) / 0.3 % Triton X-100, washed in PBS, incubated with the appropriate secondary antibody for 2 hours at room temperature, counterstained in Hoechst 33258 (1:1000) for 10 minutes, mounted on glass slides and coverslipped with Immu-Mount™ (Thermo Scientific); Primary antibodies were used as indicated in Table I. Secondary Alexa-488, Alexa-568, Alexa-647 antibodies (Molecular Probes, Invitrogen) raised against the appropriate species were used at a dilution of 1:1000. For Ki67 antigen retrieval was performed by treating floating sections in Na-citrate buffer (10mM Trisodium and 10mM citric acid in H<sub>2</sub>O, pH6.0), for 30 min at 80°C. For Ascl1 and Pax6 staining, antigen retrieval was performed by treating floating sections in citric acid (10mM citric acid in H<sub>2</sub>O, pH6.0), for 30 min at 80°C. For BrdU staining, sections were pretreated with 2N HCL for 1 h at 37°C to denature DNA.

### ***Image acquisition and analysis***

Cortical slices were imaged in a thermoregulated chamber maintained at 37°C and CO<sub>2</sub> at 5%. Time-lapse movies were acquired using a fluorescent microscope (Eclipse TE2000, Nikon) equipped with a Nikon Plan 10X / 0.30 objective connected to a digital camera (Retiga EX). Images were acquired using the Open-lab software (version 5.0) every 5 minutes for at least 190 minutes. Time-lapse stacks were generated and analyzed using Metamorph software (version 7.4). All GAD65-GFP+ cells in a region of interest comprising the cortical anlage at the level of the prospective anterior cingulate cortex (aCC) were tracked in at least 3 cortical slices at E17.5 (259 cells), P0.5 (405 cells), P2.5 (548 cells) and P4.5 (571 cells). GAD65-GFP+ cells were tracked in the dorsal white matter pool at P7 (120 cells) in 3 cortical slices. Cells moving less than 5 µm/hr were considered as non-migratory. The directionality of migrating cells in the developing cortex was determined by calculating the percentage of GAD65-GFP+ cells migrating in four quadrants: towards the pial surface, towards the SVZ, and towards the lateral or medial part of the cortex.

A Zeiss LSM 510meta confocal microscope (Zeiss) equipped with a Plan-Neofluar 40X / 0.50 objective was used to obtain confocal images, and the Image-J software was used to quantify the co-localization of GAD65-GFP+ cells with a panel of markers. For the dorsal white matter pool, we determined the percentage of GAD65-GFP+ cells expressing BrdU at P0 (n = 4 brains, 2782 cells) and P7 (n = 3 brains, 3775 cells), Ki67 at P0 (n = 3 brains, 2363 cells) and P7 (n = 4, 5013 cells), Ascl1 at P0 (n = 3, 2006 cells) and P7 (n = 3, 1731 cells), Pax6 at P0 (n = 3, 1758 cells) and P7 (n = 3, 1731 cells), COUP-TFII (n = 3, 3266 cells) and P7 (n = 3, 3327 cells), Olig2 at P0 (n = 3, 3136 cells) and P7 (n = 3, 1788 cells), NG2 at P0 (n = 3, 1024 cells) and P7 (n = 3, 1689 cells), Pdgfra at P0 (n = 3, 799 cells) and P7 (n = 3, 1483 cells). To quantify the percentage of GAD65-GFP+ cells expressing DCX in the aCC, confocal images were taken in the upper

cortical region corresponding to developing layer 2-3, the middle cortical region corresponding to developing layer 5 and a lower cortical region corresponding to layer 6 at P7 (n = 3, 934 cells), P14 (n = 3, 1847 cells), P21 (n = 3, 1326 cells). The percentage of GAD65-GFP+ cells labeled with BrdU were quantified at P10 in lower and upper cortical layers (Layer II-III) (n = 3, 995 cells) and at P21 in lower cortical layers (layer V-VI) (n = 3, 691 cells). Epifluorescent images (Nikon Plan 10 X objective) were taken at the level of the aCC to quantify the percentage of GAD65-GFP+ interneurons expressing VIP (n = 3, x cells), reelin (n = 3, x cells), NPY (n = 3, x cells), calretinin (n = 3, x cells), parvalbumin (n = 3, x cells) and somatostatin (n = 3, x cells). Statistical analysis (GraphPad Prism software, version 4.0) was done using one-way ANOVA with Tukey's multiple comparison tests or using Student's t test. Statistical significance was defined at \*p < 0.05 and \*\* p < 0.01.

## Results

### *A pool of GAD65-GFP+ precursors proliferate and express the transcription factor Pax6 in the dorsal white matter*

Analysis of coronal sections during the first postnatal week revealed that from P0.5 to P7 a pool of densely packed GAD65-GFP+ cells accumulated in the dorsal white matter region located near the cingulum at the level of the prospective anterior cingulate cortex (aCC) (\*, Fig. 1C-D; Supplemental Fig. 1). This postnatal pool of GAD65-GFP+ cells was anatomically distinguishable from the subventricular zone (SVZ) bordering the lateral ventricles, was absent at E17.5 (Fig. 1A) and started to appear at E19 (Fig. 1B). To further characterize GAD65-GFP+ cells of this region that we named the white matter precursor pool (WMPP), we performed immunohistochemistry for a panel of transcription factors that are expressed in different subtypes of neural precursors during development. We observed that a fraction of GAD65-GFP+ cells were immunoreactive for paired box 6 (Pax6) (Fig. E), a homeodomain transcription factor that is strongly expressed during the embryonic period in the pallial ventricular zone (VZ) and to a lesser extent in the subpallial rostral LGE and the caudal LGE corresponding to the CGE (Flames et al. 2007). As the size of the WMPP increased during the first postnatal week, the fraction of GAD65-GFP+ cells expressing Pax6 significantly increased from P0.5 ( $13.0 \pm 0.5$  %) to P7 ( $28.0 \pm 1.9$  %) (Fig. 1E, 1I K). In contrast, WMPP cells expressing COUP-TFII, a transcription factor preferentially expressed in the CGE (Kanatani et al. 2008) significantly decreased from P0 ( $6.9 \pm 0.9$  %) to P7 ( $1.2 \pm 0.1$  %) (Fig. F, K). Immunohistochemistry for Ascl1 (previously Mash1), a basic-helix-loop-helix (bHLH) transcription factor expressed in subpallial progenitors during the embryonic period and in SVZ oligodendrocytes precursors (Kim et al. 2008) revealed that less than 1% of GAD65-GFP+ cells at P0 and P7 expressed Ascl1 in the WMPP (Fig. 1G, 1I, 1K). GAD65-GFP+ cells did not express Tbr2, a transcription factor expressed in pallial basal

precursors of the pyramidal cell lineage (Kowalczyk et al. 2009) (Fig. 1H). Less than 1 % of GAD65-GFP<sup>+</sup> expressed Olig2, a bHLH transcription factor expressed in precursors of the oligodendrocyte lineage in the postnatal SVZ (Marshall et al. 2005) (Fig. 1J). Furthermore less than 1 % of GAD65-GFP<sup>+</sup> cells in the WMPP expressed other markers for oligodendrocyte precursors such as the the platelet-derived growth factor receptor- $\alpha$  (Pdgfra) and the chondroitin sulfate proteoglycan NG2 (Supplemental Fig. 2A-B) (Dayer et al. 2005, Kessaris et al. 2006). To investigate whether some of the GAD65-GFP<sup>+</sup> cells of this pool were able to divide, P0.5 or P7 pups received a single BrdU injection (50 mg/kg i.p.) and were sacrificed 2 h later. At P0.5 and at P7 respectively,  $3.4 \pm 0.5$  % ( $\pm$  SEM) and  $2.2 \pm 0.5$  % of GAD65-GFP<sup>+</sup> cells positive were positive for BrdU, indicating that a small fraction of GAD65-GFP<sup>+</sup> cells of the dorsal white matter proliferate locally (Fig. 1K, 2F). Immunohistochemistry for Ki67, an endogenous cell cycle marker, confirmed the presence of GAD65-GFP<sup>+</sup> proliferating cells positive for Ki67 at P0.5 ( $8.7 \pm 0.5$  %) and P7 ( $5.0 \pm 0.4$  %) (Fig. 1K, 2A-E).

Taken together, these data indicate that a proportion of cells in the WMPP are proliferating, express the transcription factor Pax-6 and to a lesser extent COUP-TFII but not Tbr2 or Ascl1, indicating that they likely originate from the LGE/CGE.

***GAD65-GFP labeled cells in the WMPP are derived originally from the LGE/CGE and not the MGE***

To determine whether WMPP cell could originate from subpallial structures, we used BAC transgenic mice where the second exon of Nkx2.1 was replaced by Cre recombinase (Nkx2.1 Cre) (Xu et al. 2008). Nkx2.1 is a homeodomain transcription factor that has been shown to be required for the normal specification of interneurons derived from the MGE (Sussel et al. 1999). Strikingly fate mapping of Nkx2.1 expressing cells in the Nkx2.1-Cre ROSA-YFP transgenic

mice, revealed that the dorsal white matter at P7 contained nearly no YFP<sup>+</sup> cells, demonstrating that the WMPP is not initially derived from the MGE (Fig. 1L). To further assess the origins of WMPP using a marker for non-MGE derived interneurons, we used the 5HT<sub>3A</sub>-GFP BAC transgenic mice, which label cortical interneurons derived from the CGE (Vucurovic et al. 2010) and the early postnatal subventricular zone bordering the lateral ventricles (Inta et al. 2008) which is likely to be derived from the embryonic LGE (Marshall et al. 2003). In contrast to the fate mapping of Nkx2.1 mice, we observed that the pattern of distribution of 5HT<sub>3A</sub>-GFP<sup>+</sup> cells in the WMPP at P7 was similar to the distribution of GAD65-GFP<sup>+</sup> cells (Fig. 1M). Taken together, these data indicate that GAD65-GFP<sup>+</sup> cells that populate the postnatal WMPP originate from progenitors that are derived from regions outside of the MGE. The fact that WMPP cells express Pax6, the 5-HT<sub>3A</sub> receptor but to a much lesser extent COUP-TFII suggests that they may preferentially derive from the LGE rather than the CGE.

***Part of the GAD65-GFP<sup>+</sup> white matter pool are immature neurons with a migratory profile***

At P7, the vast majority of the GAD65-GFP<sup>+</sup> cells of the WMPP expressed the immature neuronal marker DCX (Fig. 3A). Interestingly, DCX<sup>+</sup> / GAD65-GFP<sup>+</sup> cells harboring a leading process oriented towards the pial surface were also visualized in the overlying prospective aCC, (Fig. 3D3), suggesting that a fraction of DCX<sup>+</sup> / GAD65-GFP<sup>+</sup> cells could exit the dorsal white matter and incorporate into the overlying cortex at P7. DCX<sup>+</sup> / GAD65-GFP<sup>+</sup> cells in the WMPP gradually decreased at further developmental time points but persisted at low levels until postnatal day 21 (Fig. 3B-C). At P14, GAD65-GFP<sup>+</sup> cells in the WMPP were less abundant compared to P7 and tended to form chain-like structures that were strongly DCX immunoreactive (Fig. 3B). At P21, DCX<sup>+</sup> / GAD65-GFP<sup>+</sup> cells displaying a typical migratory interneuron-like morphology could still be observed in the WMPP (Fig. 3E-F). In the vicinity of small GAD65-

GFP+ clusters in the WMPP, individual DCX+ / GAD65-GFP+ with a migratory-like leading process were observed entering the lower layers of the aCC at P21 (Fig. 3E). Taken together these data indicate that a pool of neurogenic DCX+ / GAD65-GFP+ cells accumulate in the WMPP during the early postnatal period, and that a fraction of DCX+ / GAD65-GFP+ cells may exit the WMPP to enter the aCC.

***GAD65-GFP+ immature neurons of the WMPP migrate in the postnatal anterior cingulate cortex***

To monitor the migration of cortical interneurons during the postnatal period, we performed time-lapse imaging on coronal slices of GAD65-GFP+ mice at different developmental time points and focused our analysis on the WMPP and prospective aCC. Time-lapse imaging revealed that at postnatal day 0.5 (P0.5) a large number of GAD65-GFP+ interneurons migrated in the developing aCC whereas a majority of stationary GAD65-GFP+ interneurons were observed at P4.5 (Fig.4A-B). Quantification indicated that the percentage of GAD65-GFP+ interneurons migrating in the aCC significantly decreased from embryonic day 17.5 (E17.5) to postnatal day P4.5 (Fig. 4C). From E17.5 to P4.5 the mean migratory speed of GAD65-GFP+ interneurons significantly decreased (Fig. 4D) and the proportion of GAD65-GFP+ interneurons migrating at low speed intervals significantly increased (Fig 4E), indicating that postnatal maturation renders the developing aCC less permissive for migration. To quantify the directionality of GAD65-GFP+ interneurons, the position of migrating cells was determined in four quadrants after a 190 minutes time-lapse sequence (Fig.4F). GAD65-GFP+ interneurons migrated in all directions with a significantly higher proportion of cells migrating towards the pial surface compared to other quadrants at P0.5 ( $49 \pm 3.7$  % ( $\pm$  s.e.m.)), P2.5 ( $51.5 \pm 5.8$  %) (Fig. 4G). Interestingly, a small fraction of GAD65-GFP+ cells ( $6.4 \pm 1.0$  %) were still migrating within the aCC at P4.5 (Fig.

1C) with a preferential direction towards the pial surface ( $69 \pm 7.6$  %) (Fig 4F, 4G, 4J). Time-lapse imaging of GAD65-GFP+ cells forming the WMPP revealed that during the first postnatal week the vast majority of GAD65-GFP+ cells were motile and displayed a large distribution of migratory speeds (Fig. 3G-H). Furthermore, during the first postnatal week GAD65-GFP+ cells were observed exiting the dorsal white matter region underlying the prospective aCC (Fig. 4I-J), suggesting that this region could contain a reservoir for GAD65-GFP+ cortical interneurons. These results provide direct evidence of GAD65-GFP+ cells exiting the WMPP during the first postnatal week and migrating into the developing aCC.

*A fraction of GAD65-GFP labeled interneurons expressing doublecortin and calretinin integrate the cingulate cortex postnatally*

The presence of a pool of motile GAD65-GFP+ cells immunoreactive for DCX in the postnatal dorsal white matter and the observation during time-lapse imaging that GAD65-GFP+ cells could exit this region and migrate into the overlying aCC indicates that immature DCX positive neurons could populate the overlying aCC during the postnatal period. DCX staining in the aCC revealed that this marker labels a fraction of GAD65-GFP+ cells with a high degree of specificity between P7 and P21 (Fig. 5A). The vast majority (>90 %) of DCX positive cells observed in the aCC at P14 and P21 were GAD65-GFP+. At P7, a large fraction of GAD65-GFP+ cells expressed DCX in layer VI and to a lesser extent in upper cortical layers (Fig. 5A, 5F). Between P7 and P14, the fraction of GAD65-GFP+ neurons expressing DCX significantly decreased in layer VI and layer II-III and very few DCX positive GAD65-GFP+ cells were observed at P14 in layer II-III (Fig. 5F). Between P14 and P21, the percentage of GAD65-GFP+ expressing DCX significantly decreased in layer VI and V, and the remaining DCX+ / GAD65-GFP+ cells were essentially located in deep layer VI bordering the dorsal white matter (Fig. 3C, 5C). DCX+ /

GAD65-GFP+ cells found in the lower cortical layers at P14 and P21 showed various degrees of morphological maturity. At P21, some DCX+ / GAD65-GFP+ cells still displayed a migratory-like morphology (Fig. 5D) whereas others had the morphology of immature neurons in the process of differentiation (Fig. 5E). Immunohistochemistry for a panel of interneuron markers revealed that GAD65-GFP+ cells at P21 expressed markers preferentially expressed in CGE-derived interneurons, such as reelin, calretinin, VIP and NPY (Supplemental Fig. 2C-F, I) (Miyoshi et al. 2010). A very low percentage of GAD65-GFP+ cells expressed MGE-derived markers such as parvalbumin and somatostatin (Supplemental Fig. 2G-H, I). Immunohistochemistry at P21 for interneuron subtype markers further revealed the presence of DCX+ / GAD65-GFP+ interneurons immunoreactive for calretinin in the prospective aCC and in the WMPP (Fig. 5E, Fig. 3F), whereas no DCX+ / GAD65-GFP+ interneurons were immunoreactive for parvalbumin and somatostatin.

***A fraction of GAD65-GFP labeled interneurons in the cingulate cortex are generated postnatally***

To determine whether a fraction of GAD65-GFP+ interneurons could be generated postnatally, P0.5 mice received BrdU injections (3 x 20 mg/kg i.p.) and were sacrificed at P10. Confocal analysis of BrdU cells revealed that  $6.7 \pm 0.4$  % of GAD65-GFP+interneurons in the lower cortical layers (layer V and VI) were BrdU positive, whereas only  $1.8 \pm 0.1$  % of GAD65-GFP+ were BrdU positive in the upper cortical layers (layer II-III). GAD65-GFP+ interneurons labeled with BrdU were also found to express NeuN, DCX and calretinin, further confirming their neuronal identity (Fig. 6A-C). When P7 pups were injected with BrdU (3 x 20 mg/kg i.p.) and sacrificed at P21, we could still detect a small fraction ( $2.8 \pm 0.6$  %) of GAD65-GFP+ interneurons positive for BrdU in the lower cortical layers. No GAD65-GFP+ cells positive for

BrdU were observed in the upper cortical layers II/III. Taken together, these data indicate that a small fraction of GAD65-GFP+ positive interneurons continue to be generated postnatally, belong to the calretinin interneuron subtype and incorporate preferentially into lower cortical layers.

## **Discussion**

In this study we observed that a large pool of interneuron precursors accumulate during the first postnatal week in a dorsal white matter region located ventrally to the prospective anterior cingulate cortex (aCC). Precursors in the WMPP were found to divide postnatally suggesting the presence of local transient amplifying precursors in the WMPP. A substantial fraction of WMPP cells expressed Pax6, to a lesser extent COUP-TFII but not Ascl1, Tbr2 and Olig2. These results as well as fate mapping studies using Nkx2.1-Cre or 5-HT3-GFP transgenic mice strongly suggest that the WMPP pool cells originate from the LGE/CGE but not the MGE. Interneurons immunoreactive for both the immature neuronal marker DCX and the calcium binding protein calretinin were observed in the WMPP and the lower cortical layers of the aCC until postnatal day 21, suggesting an ongoing incorporation of a this interneuron subtype. Time-lapse imaging confirmed that GAD65-GFP+ immature neurons of the WMPP were motile and could migrate postnatally into the overlying prospective aCC. Finally, BrdU incorporation experiments indicated that a small fraction of aCC GAD65-GFP+ positive interneurons expressing calretinin are generated postnatally.

### ***The white matter may serve as a secondary pool of cortical interneuron precursors***

Strikingly, during the first postnatal week a large pool of GAD65-GFP+ cells with an immature-like morphology accumulated in the postnatal dorsal white matter region in the vicinity of the cingulum region and ventrally to the developing aCC. At P7, GAD65-GFP+ cells formed a cell-dense region located dorsally to the SVZ bordering the roof of the ventricles. The size of the pool of GAD65-GFP+ cells was clearly larger at P7 compared to P0 and considerably decreased at P14. DCX has been widely used in studies on adult neurogenesis to characterize newly generated neurons in the dentate gyrus and newborn interneurons migrating towards the olfactory bulb

(Brown et al. 2003). Staining for DCX from P7 to P21 revealed that the vast majority of immature-like GAD65-GFP+ cells in the WMPP expressed DCX, confirming that GAD65-GFP+ cells belong to a neurogenic lineage. Interestingly, short-term BrdU incorporation experiments and labeling with the endogenous cell cycle marker Ki67 revealed that GAD65-GFP+ cells in the dorsal white matter region can divide during the perinatal period. These data suggest that the WMPP contains a population of transient amplifying cells that are likely to contribute to the postnatal expansion of the WMPP from P0 to P7. Time-lapse imaging revealed that GAD65-GFP+ cells constituting the WMPP were motile and could migrate into the overlying aCC. Our findings thus indicate that the WMPP could serve as a reservoir for cortical interneurons incorporating into the developing aCC during the postnatal period. However, given the large amount of GAD65-GFP+ cells accumulating in the dorsal white matter region and the smaller proportion of GAD65-GFP+ cells on time-lapse imaging that were observed entering the aCC it is possible that this pool of GAD65-GFP+ cells could also serve another function during postnatal cortical development. During the embryonic period, it has been shown that transient populations of migrating interneurons could serve as guidepost cells and control thalamocortical pathfinding in the subpallium (Lopez-Bendito et al. 2006) and the navigation of callosal axons (Niquille et al. 2009). It is thus conceivable that the large pool of GAD65-GFP+ cells accumulating in the WMPP during the first postnatal week could play a role in the directional growth of callosal or sub-cerebral projecting axons that exit the cortex and grow through this white matter region from embryonic to postnatal ages.

### ***Origin of GAD65-GFP+ cells in the postnatal white matter precursor pool***

We found that GAD65-GFP+ cells that constitute the WMPP do not express Tbr2, a transcription factor expressed in pallial basal precursors of the pyramidal cell lineage (Kowalczyk et al. 2009).

GAD65-GFP<sup>+</sup> cells in the WMPP only very rarely expressed Olig2, a bHLH transcription factor expressed in precursors of the oligodendrocyte lineage in the postnatal SVZ (Marshall et al. 2005). Furthermore GAD65-GFP<sup>+</sup> cells rarely expressed oligodendrocyte precursor markers such as Pdgfra and NG2 (Kessaris et al. 2006; Dayer et al. 2005). These data strongly suggests that GAD65-GFP<sup>+</sup> cells in the WMPP are thus interneuron precursors and not oligodendrocyte or pyramidal neuron precursors. To assess the origin of the WMPP cells, we used a Nkx2.1-Cre genetic fate-mapping strategy. Strikingly, we observed that the postnatal dorsal white matter region did not contain any Nkx2.1-derived YFP positive cells during the postnatal period, strongly suggesting that the WMPP is not derived from the embryonic MGE. In contrast, we observed that 5HT<sub>3A</sub>-GFP<sup>+</sup> cells formed a pool of cells in the dorsal white matter region that gradually increased from P0 to P7 and that had a similar distribution to GAD65-GFP<sup>+</sup> WMPP cells. These data thus confirm that WMPP cells do not have an MGE origin. Furthermore since 5HT<sub>3A</sub>-GFP<sup>+</sup> transgenic mice labels cortical interneurons derived from the embryonic CGE (Vucurovic et al. 2010) but also olfactory bulb interneurons derived from the LGE (Inta et al. 2008), WMPP cells could originate from either the LGE or the CGE. Interestingly only a small percentage of GAD65-GFP<sup>+</sup> cells expressed COUP-TFII, a transcription factor preferentially expressed in the caudal ganglionic eminence during the embryonic period (Kanatani et al. 2008), suggesting that most WMPP cells are not derived from the CGE. However we cannot exclude that precursor cells originating from the CGE down-regulate COUP-TFII as they reach the dorsal pallium. In contrast, we found that a fraction of GAD65-GFP<sup>+</sup> cells expressed the homeodomain transcription factor Pax6 and that the percentage of Pax6<sup>+</sup> / GAD65-GFP<sup>+</sup> cells significantly increased from P0 to P7. Pax6 is required for the specification of pallial pyramidal neurons (Kroll and O'Leary. 2005), but also of late-born interneurons in the postnatal olfactory bulb (Kohwi et al. 2005). Furthermore Pax6 is expressed in the LGE/CGE, although at lower levels than in the

pallial VZ (Flames et al. 2007). Taken together, we favor the hypothesis that WMPP cells originate from the LGE and not the MGE and that they may share common lineage features with olfactory bulb interneurons such as the requirement of Pax6 expression.

***Immature-like interneurons generated postnatally are found in lower cortical layers of the anterior cingulate cortex***

The presence of a large pool of GAD65-GFP<sup>+</sup> precursor cells in the dorsal white matter during the perinatal period opens the possibility that a fraction of these cells could incorporate the overlying aCC. Several lines of evidence support this hypothesis. First, using time-lapse imaging we observed that GAD65-GFP<sup>+</sup> cells could exit the dorsal white matter and migrate into the overlying aCC. Second, we identified in the developing aCC GAD65-GFP<sup>+</sup> cells that had a migratory morphology and that were positive for the immature neuronal marker DCX. Third, we found that the proportion of GAD65-GFP<sup>+</sup> cells expressing DCX decreased from P7 to P21 following an outside-inside gradient, suggesting that DCX<sup>+</sup> / GAD65<sup>+</sup> preferentially incorporate into the deep cortical layers. Different sets of markers have been used to establish subclasses of interneurons in relationship with their developmental origin (Gelman and Marin. 2010). Here we found that immature DCX<sup>+</sup> / GAD65-GFP<sup>+</sup> interneurons in the aCC at P21 preferentially belonged to the calretinin subfamily of interneurons and not to the parvalbumin or somatostatin MGE-derived subtypes.

The vast majority of cortical interneurons are generated during the embryonic period but a small fraction of cortical interneurons could also be generated during the postnatal period and even in adulthood (Inta et al. 2008, Dayer et al. 2005). In accordance with this latter possibility, we report here further evidence that a small fraction of GAD65-GFP<sup>+</sup> cortical interneurons can be generated postnatally, and that newly generated BrdU<sup>+</sup> / GAD65-GFP<sup>+</sup> cells preferentially incorporate into lower cortical layers. The origin of these newly generated interneurons is still

unclear. A first possibility is that newly generated cortical interneurons could derive from radial glial cells located in the perinatal VZ/SVZ. This appears unlikely since radial glia of either the dorsal or ventral wall of the lateral ventricles were labeled at P0.5 and did not appear to generate a pool of interneurons outside of the rostral migratory stream olfactory bulb system (Merkle et al. 2007). The medial telencephalic wall has been demonstrated to be a source of neurons such as Cajal-retzius cells that migrate tangentially into the developing neocortex (Meyer 2010). Although conceivable that some cortical interneuron could originate from this region, no data presently supports this hypothesis. A more likely possibility is that the small fraction of postnatally generated cortical interneurons detected in the aCC could derive from the transient amplifying cells we observed in the dorsal white matter region.

#### ***Interneuron precursor cells generated in the dorsal pallium of primates***

Although it is difficult to translate cortical development in mice to the much longer time-scale of primate brain development (Rakic 2009), comparative models across species indicate that the first postnatal week in mice corresponds broadly to cortical development in macaques between gestational days 85 to 130 and in humans between gestational days 110 to 170 (Clancy et al. 2001). While the vast majority of cortical interneurons in rodents are generated in subpallial structures (Wonders and Anderson 2006), in humans and in the cynomolgus monkeys a large fraction of cortical interneurons derive from dorsal pallial proliferative zones that appear at later gestational ages, after the early wave of interneurons generated in the ganglionic eminences (Letinic et al. 2002; Petanjek et al. 2009a; Petanjek et al. 2009b). The existence in rodents of a small fraction of proliferating GABAergic progenitors located in the white matter of the early postnatal mouse pallium suggests that this developmental process could have been recruited and expanded during primate evolution. This population of transient amplifying cells located in the

early postnatal white matter region of the rodent dorsal pallium could thus present some analogy to the outer subventricular proliferative zones that contribute to the generation of cortical interneurons at late-phases of cortical development in human and non-human primates (Petanjek et al. 2009b). Support for this hypothesis comes from the fact that late-born cortical interneurons originating from the WMPP in rodents belong to the calretinin subtype. These late-born calretinin positive interneurons do not appear to follow the general rule of inside-outside cortical migration, but are detected in deep cortical layers (Rymar and Sadikot 2007). Interestingly, in primates calretinin positive cortical interneurons are also generated at later developmental time-points, mainly derive from pallial proliferative zones, undergo a significant numerical expansion from rodents to primates and present primate-specific morphological features (Jones 2009, Rakic 2009). The existence of a pool of late-born interneurons belonging to the calretinin interneuron subtype and generated outside of the subpallium suggests that region-specific and cell-specific interneuronopathies could be detected. Interestingly, in brains of human holoprosencephaly with severe striatal hypoplasia and atrophy of the GE calretinin-positive cortical interneurons were not depleted compared to other interneuron subclasses (Fertuzinhos et al. 2009) further suggesting that calretinin positive interneurons have a dorsal pallial origin in primates. As mentioned early, interneuron precursors located in the rodent white matter may not only serve as a reservoir for cortical interneurons but also function as guidepost cells providing directional cues for growing axons (Niquille et al. 2009). In humans, a transient population of poorly differentiated, migratory-like calretinin positive cells were observed at mid-gestation in the developing corpus callosum, suggesting that these cells could be involved in axon guidance (Paul et al. 2007; Jovanovic-Milosevic et al. 2010). It is thus possible that interneuron precursor cells generated in the dorsal pallial could serve a similar function at latter developmental time points and that the recruitment and amplification of these transient migratory populations in primates could provide an

evolutionary mechanism accounting for the significant expansion in size and complexity of the corpus callosum in primates compared to rodents (Molnar et al. 2006, Rakic 2009).

In conclusion, this study describes the existence of a pool of immature and motile GAD65-GFP+ cells in the dorsal white matter region that accumulates during the first postnatal week. Precursor cells in the dorsal white matter region are likely derived from the LGE and not the MGE, express the transcription factor Pax6 and could share common lineage features with calretinin positive interneurons destined to the olfactory bulb. Time-lapse imaging and doublecortin immunohistochemistry indicate that interneuron precursor cells can exit the dorsal white matter region, incorporate into the overlying aCC and preferentially belong to the calretinin interneuron subtype. Finally, this study indicates that a small fraction of GAD65-GFP+ cells incorporating into the aCC can be generated during the early postnatal period, suggesting that the addition of new cortical interneurons could play a role in late-phase maturation of cortical networks.

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## Figure legends

**Figure 1: A large pool of GAD65-GFP labeled cells accumulates in the early postnatal dorsal white matter, expresses Pax6 and COUP-TFII but not Ascl1, Olig2, Tbr2 and is derived from 5-HT3-GFP+ domains.**

(A-D) Epifluorescent images of coronal sections showing that GAD65-GFP+ cells accumulate in the white matter precursor pool (WMPP, \*) between P0.5 (C) and P7 (D). Note that the WMPP is absent at E17.5 (A) and starts to appear at E19 (B). Confocal images of the WMPP at P0.5 showing that a large fraction of GAD65-GFP+ cells expresses the transcription factor Pax6 (E) to a lesser extent the transcription factor COUP-TFII (F) but not the transcription factors Ascl1 (G) and TBR2 (H). (I-J) Confocal images of the WMPP at P7 showing that a fraction of GAD65-GFP+ expresses Pax6 (I3) but not Ascl1 (I1) or the transcription factor Olig2 (J). (K) Graph showing that the percentage of GAD65-GFP+ cells labeled for Ki67 significantly decreases from P0.5 to P7 (\*\*  $p < 0.01$ ). Note also that the percentage of GAD65-GFP labeled cells labeled for Pax6 significantly increases from P0.5 to P7 but significantly decreases for COUP-TFII (\*\*  $p < 0.01$ ). (L) Images showing that the WMPP at P7 contains very few Nkx2.1cre-YFP+ cells. (M) Images showing that 5HT<sub>3A</sub>-GFP+ cells populate the dorsal white matter at P7. White boxed areas depict a higher magnifications image. GFP, GAD65-GFP. Scale bars: 20  $\mu$ m for E-J, L2, M2, 100  $\mu$ m for A-D, L1, M.

**Figure 2: A fraction of GAD65-GFP+ cells proliferate in the postnatal dorsal white matter.**

(A-B) Epifluorescent images showing in the WMPP (\*) a pool of cells labeled with the endogenous cell cycle marker Ki67 at P0.5 (A) and P7 (B). (C) Confocal images of the WMPP at P0.5 showing GAD65-GFP+ cells immunoreactive for Ki67. (D) Confocal image of the WMPP at P0.5 showing a moderately labeled GAD65-GFP+ cell immunoreactive for Ki67 (arrow) and a

strongly labeled GAD65-GFP<sup>+</sup> cell with a migrating neuronal morphology and negative for Ki67. (E-F) Confocal images of the WMPP at P7 showing GAD65-GFP<sup>+</sup> cells immunoreactive for Ki67 (E) and BrdU (F). White boxed areas depict a higher magnifications image. Arrowheads point to double-labeled cells. GFP, GAD65-GFP, SVZ, subventricular zone. Scale bars: 100  $\mu$ m for A, B, 10  $\mu$ m for C, D, E, F

**Figure 3: A large pool of GAD65-GFP<sup>+</sup> cells express doublecortin and migrate in the postnatal dorsal white matter region ventral to the anterior cingulate cortex.**

(A-C) Images showing that at P7 (A), P14 (B) and P21 (C) GAD65-GFP<sup>+</sup> cells persist in the WMPP (\*). At P7 a large number of GAD65-GFP<sup>+</sup> cells in the WMPP express the immature neuronal marker doublecortin (DCX) (A5). Note at P14 the presence of GAD65-GFP<sup>+</sup> cells that form DCX<sup>+</sup> chain-like structures (arrowhead) (B5). (C) At P21 DCX<sup>+</sup>/ GAD65-GFP<sup>+</sup> cells are present in the WMPP but also in deep layer VI of the aCC (arrows) (C5). (D) Confocal images showing at P7 DCX<sup>+</sup> / GAD65-GFP<sup>+</sup> interneurons displaying neuronal migratory morphologies in the aCC (arrowheads) (D3-D4) and a large pool of DCX positive GAD65-GFP<sup>+</sup> cells in the adjacent WMPP (D5-D6). (E) Confocal images showing at P21 in the WMPP a small cluster of DCX<sup>+</sup> cells expressing low levels of GAD65-GFP (arrowhead). In the vicinity of this cluster, a DCX<sup>+</sup> cells with higher expression of GAD65-GFP cell and displaying a migratory-like leading process is observed entering the aCC (arrowhead). (F) Confocal images showing that at P21 DCX<sup>+</sup>/ GAD65-GFP<sup>+</sup> cells with neuronal migratory morphologies and expressing calretinin (arrowhead) are present in the WMPP. (G) Epifluorescent time-lapse sequence of a P7 coronal slice at the level of the dorsal WMPP showing migratory tracks of GAD65-GFP<sup>+</sup> cells (superposed color lines). (H) Graph showing that the majority of GAD65-GFP<sup>+</sup> cells in the WMPP at P7 are motile and display a range of migratory speed. White boxed areas depict higher

a magnification image. GFP, GAD65-GFP. Hst, Hoechst, DCX, doublecortin, SVZ, subventricular zone. Scale bars: 100  $\mu\text{m}$  for A, B1-B4,C, 10  $\mu\text{m}$  for B5,B6, D-G.

**Figure 4: A fraction of GAD65-GFP+ interneurons migrate in the prospective postnatal anterior cingulate cortex.**

(A-B) Epifluorescent images of coronal slices at the level of the aCC at P0.5 (A1) and P4.5 (B1). Time-lapse sequences showing the migration of GAD65-GFP labeled interneurons at P0.5 (A2-A5) and P4.5 (B2-B5). At P0.5 a large fraction of GAD65-GFP labeled interneurons are motile (arrowheads). In contrast, the majority of GAD65-GFP labeled interneurons at P4.5 are stationary (arrows). Superposed color lines represent migratory tracks. (C) Graph showing that the percentage of migrating interneurons in the aCC progressively decreases from P0.5 to P4.5 (\*\*  $p < 0.01$ ). (D) Graph showing a significant decrease in the mean migratory speed of GAD65-GFP+ interneurons in the aCC between E17.5 and P4.5 (\*\*  $p < 0.01$ ). (E) Graph showing a significant shift in the speed distribution of migrating interneurons between E17.5 and P4.5 with a majority of GAD65-GFP+ cells at P4.5 migrating at lower speed intervals (\*\*  $p < 0.01$ ). (F) Scatter graphs showing the final position of GAD65-GFP labeled interneurons in the prospective aCC after a 190 min time-lapse sequence at P0.5 (F1) and P4.5 (F2). The starting point for each cell is the intersection between the x and y axes (0,0) and directionality towards the pial surface is given by positive values in the y axis. Note that interneurons migrate in all directions with a preferential direction towards the pial surface. (G) Graph showing that a significantly higher proportion of GAD65-GFP+ cells at P0.5, P2.5 and P4.5 migrate towards the pial surface compared to other directions. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ). (H) Schematic coronal section depicting during the first postnatal week migration of GABAergic cells from the GAD65-GFP positive WMPP towards cortical regions (I) Epifluorescent images at low (I1) and higher (I2) magnification showing at

P2.5 a pool of GAD65-GFP labeled cells in the WMPP and dorsal SVZ. (J) Epifluorescent time lapse sequence from a 180 min time lapse sequence at P0.5 showing GAD65-GFP+ cells exiting the WMPP and migrating towards the prospective aCC. White boxed areas depict a higher magnification image. GFP, GAD65-GFP, Hst, Hoechst, aCC, anterior cingulate cortex, M, motor cortex, Vent, ventricle, CC, corpus callosum, CPu, caudate putamen SVZ, subventricular zone, Lat, lateral, Med, medial. Scale bars: 100  $\mu\text{m}$  for A1, B1, I, 20  $\mu\text{m}$  for A2-A5, B2-B5, J.

**Figure 5: A fraction of GAD65-GFP+ interneurons expresses the immature neuronal marker DCX in the postnatal aCC.**

(A-C) Confocal images of layer VI of the aCC showing that the fraction of GAD65-GFP+ interneurons expressing DCX gradually decreases between P7 (A), P14 (B) and P21 (C). Note that DCX is preferentially expressed in GAD65-GFP+ interneurons. (D) Confocal images of layer VI of the aCC at P21 showing a GAD65-GFP labeled interneuron with an immature migratory-like morphology. (E) Confocal images of layer VI of the aCC at P21 showing a GAD65-GFP labeled interneuron expressing DCX and the neuronal marker calretinin. (F) Graph showing that the percentage of DCX+ / GAD65-GFP+ significantly decreases from P7 to P21 in all cortical layers (\*\*  $p < 0.01$ ). Note that at P21 DCX+ / GAD65-GFP+ cells are mainly found in layer VI. Arrowheads depict double-labeled cells. GFP, GAD65-GFP. Scale bar: 10  $\mu\text{m}$  for all images.

**Figure 6: A small fraction of GAD65-GFP labeled interneurons in the anterior cingulate cortex are generated postnatally.**

(A-C) Confocal images of layer VI of the aCC at P10 showing GAD65-GFP+ interneurons labeled for BrdU and DCX (A), BrdU and NeuN (B), BrdU and calretinin (C). BrdU was injected

at P0.5 (3 x 20 mg/kg i.p.) and animals were sacrificed at P10. GFP, GAD65-GFP. Scale bar: 10  $\mu$ m for all images.

**Supplemental figure 1: A large pool of GAD65-GFP labeled cells accumulates in the early postnatal dorsal white matter from P0.5 to P7**

(A-E) Epifluorescent images of coronal sections showing that a pool of GAD65-GFP cells accumulates in the WMPP (arrows) between P0.5 (C), P2.5 (D) and P7 (E) at the level of the prospective anterior cingulate cortex. Note that the pool of GAD65-GFP cells is absent at E17.5 (A) and starts to appear at E19 (B). Coronal sections are taken at three rostro-caudal levels encompassing the prospective anterior cingulate cortices. GFP, GAD65-GFP. Scale bar: 100  $\mu$ m for all images.

**Supplemental figure 2: GAD65-GFP+ cells express a variety of interneuron-specific markers but not the Pdgfra and NG2 oligodendrocyte precursor markers.**

(A-B) Confocal images showing that GAD65-GFP+ cells in the early postnatal WMPP at P7 do not express the oligodendrocyte progenitor markers Pdgfra (A) and NG2 (B). (C-H) Confocal images showing that GAD65-GFP+ cells in the anterior cingulate cortex (aCC) express reelin (C), calretinin (D), VIP (E), NPY (F) but not somatostatin (G) and parvalbumin (H). (I) Graph showing that GAD65-GFP+ in upper (I-III) (I1) and lower cortical layers (V-VI) (I2) of the aCC preferentially express markers for CGE-derived interneurons (calretinin, VIP, NPY, reelin) and only rarely markers for MGE-derived interneurons (parvalbumin, somatostatin). CR, calretinin, SST, somatostatin, PV, parvalbumin, RL, reelin, GFP, GAD65-GFP. Scale bar: 10  $\mu$ m for all images.

Figure 1

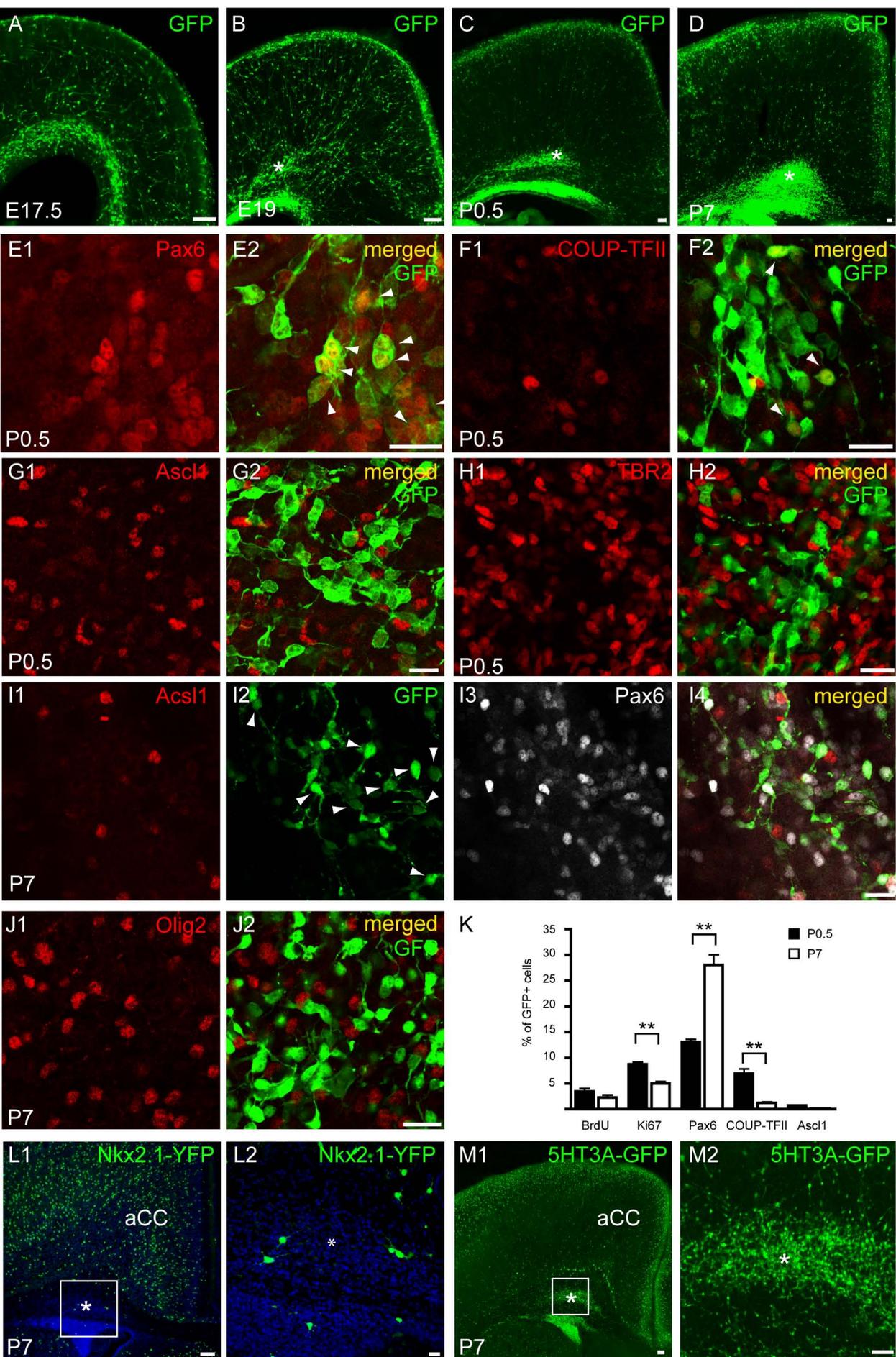


Figure 2

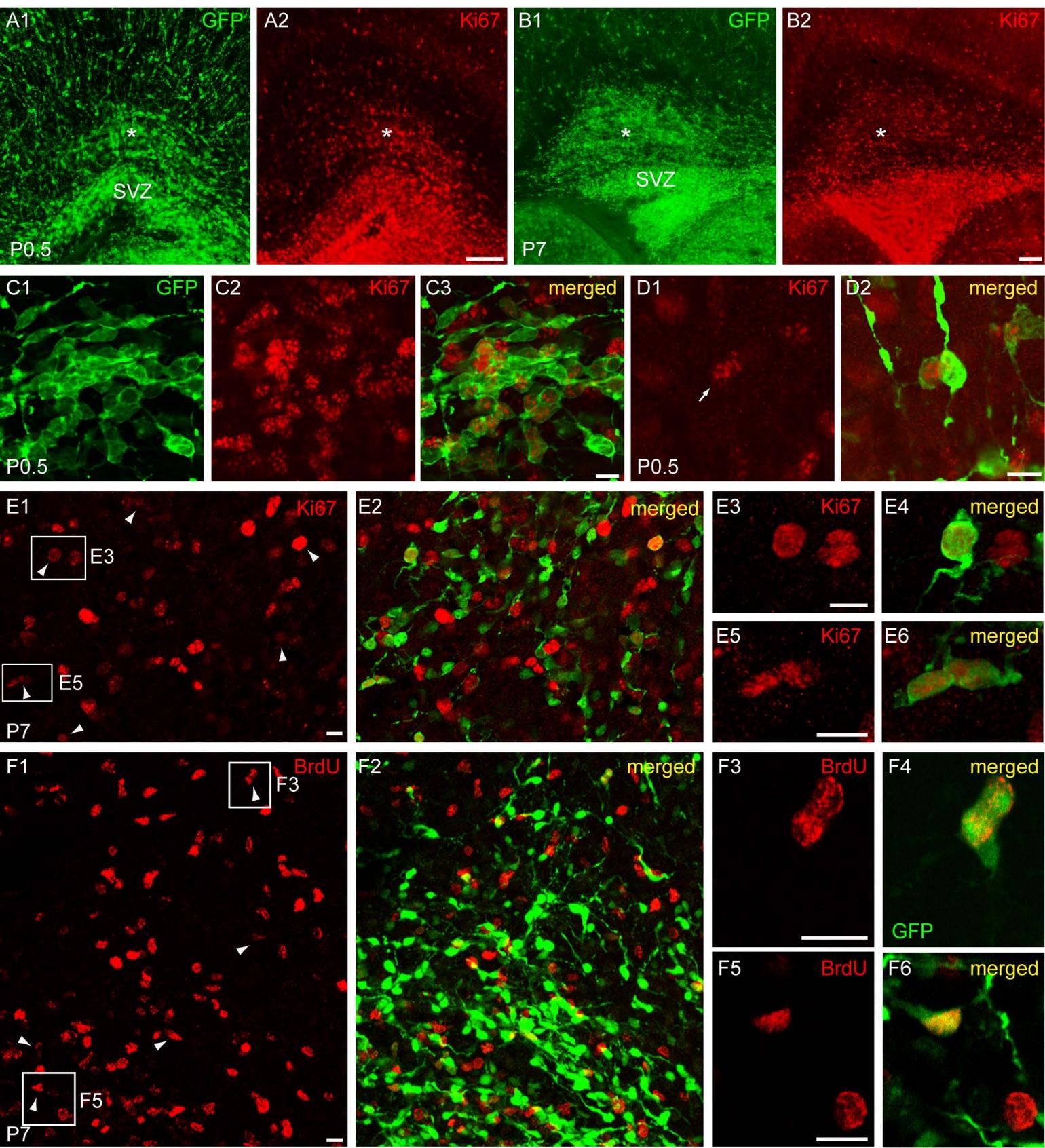
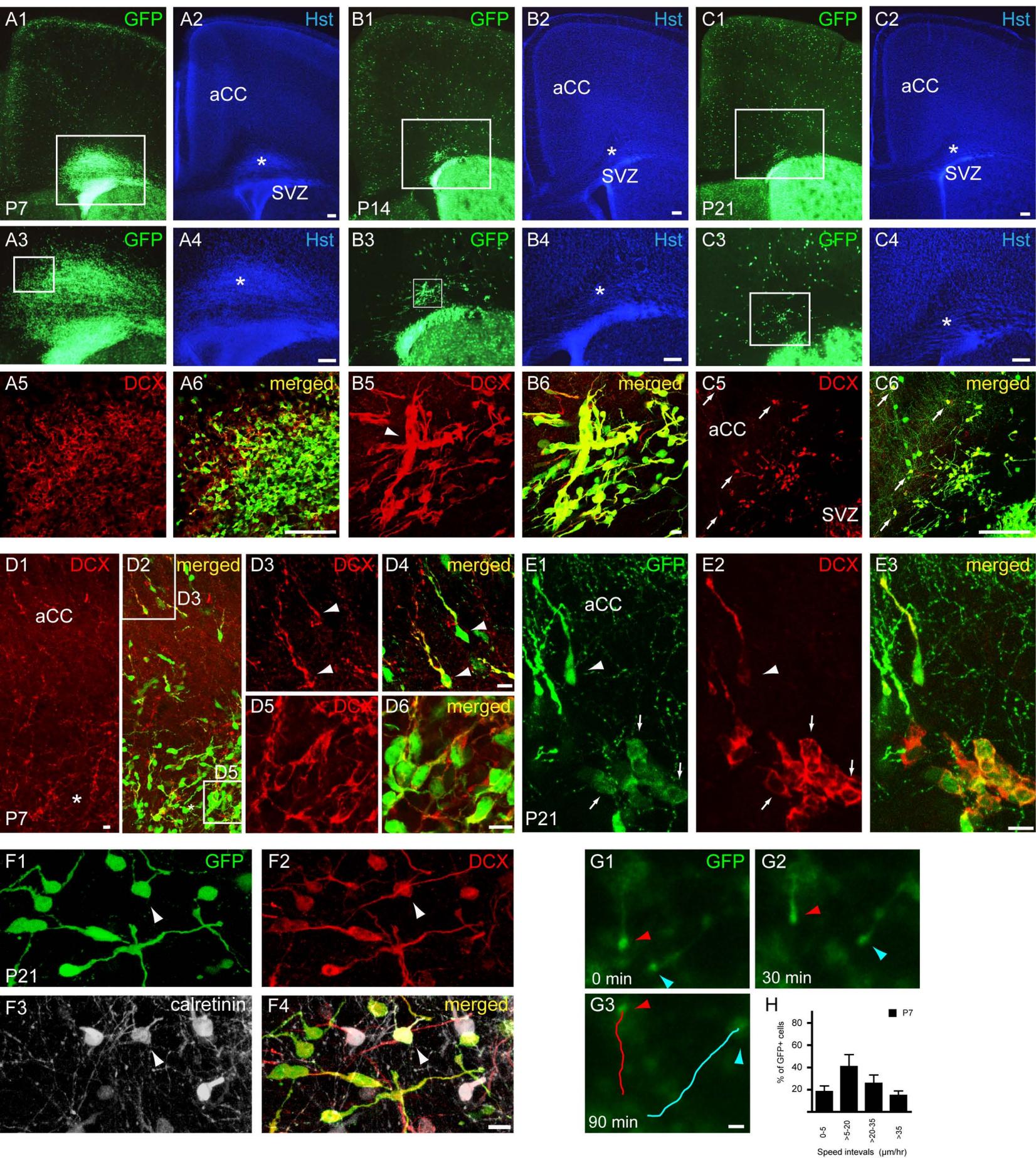


Figure 3



# Figure 4

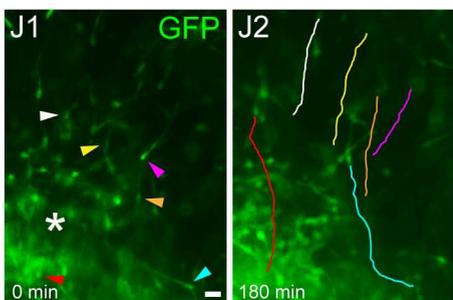
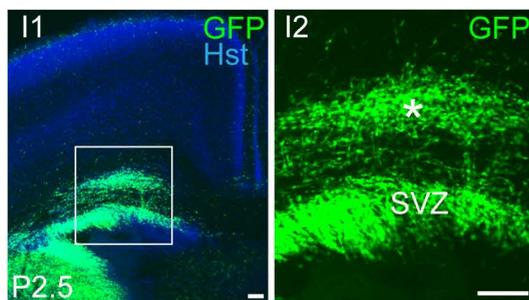
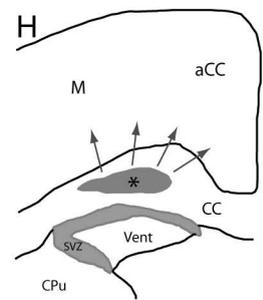
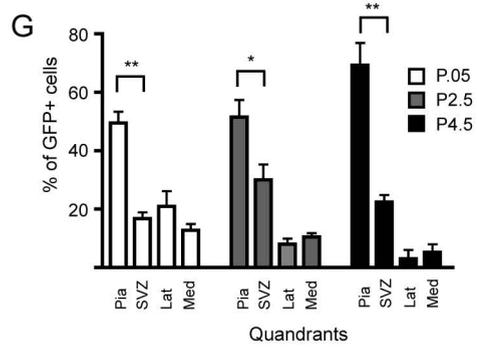
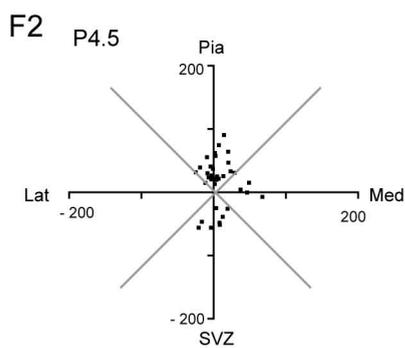
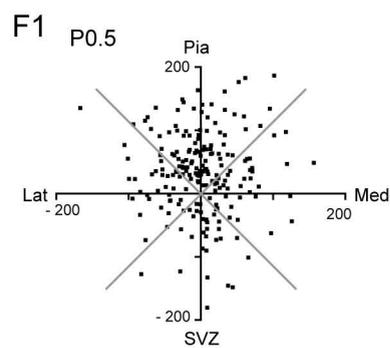
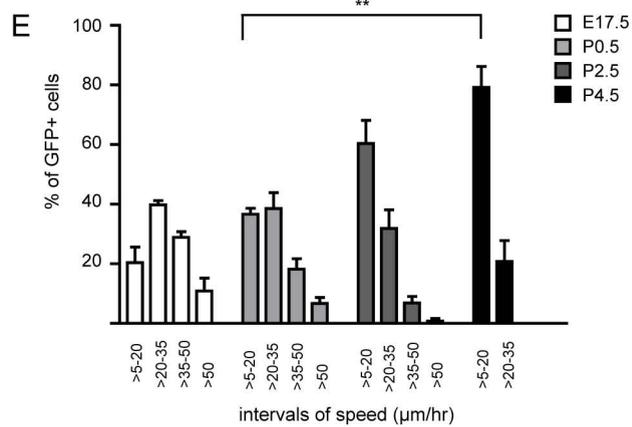
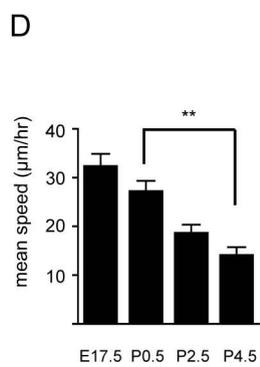
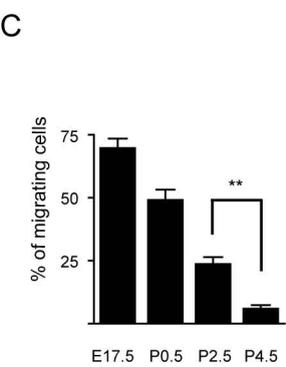
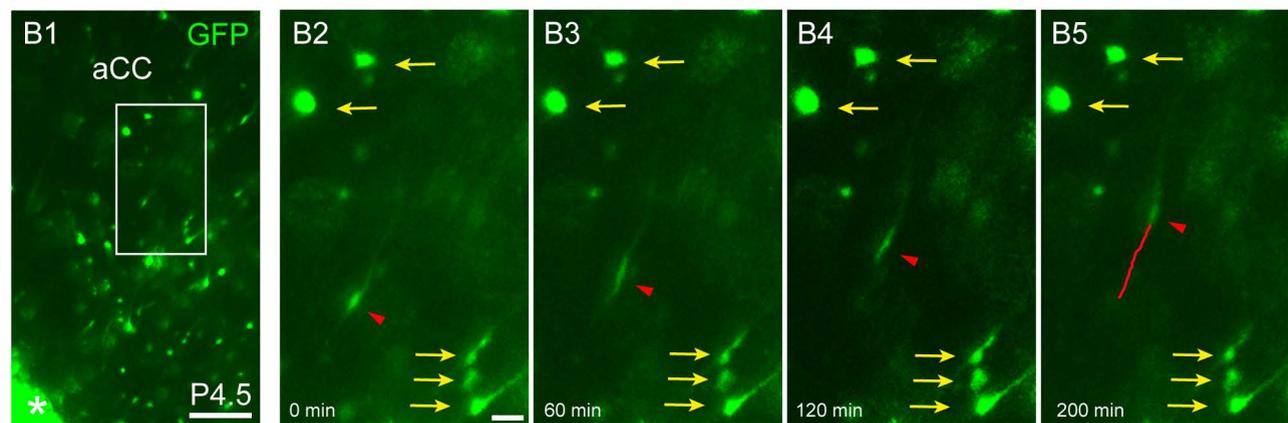
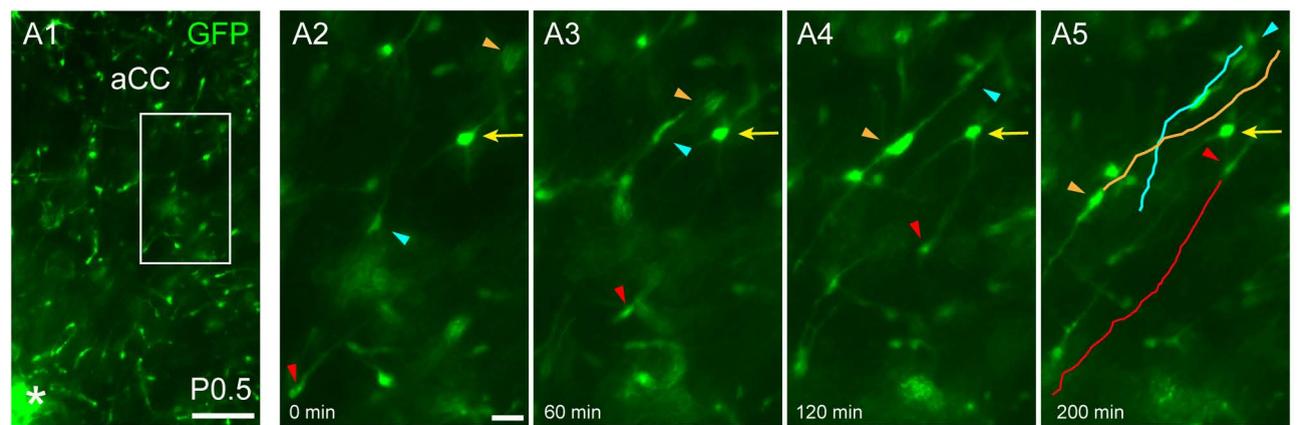
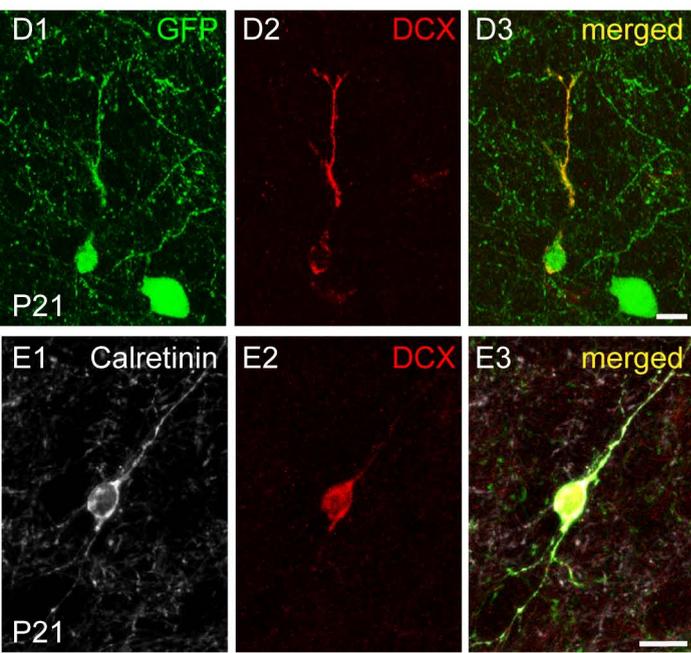
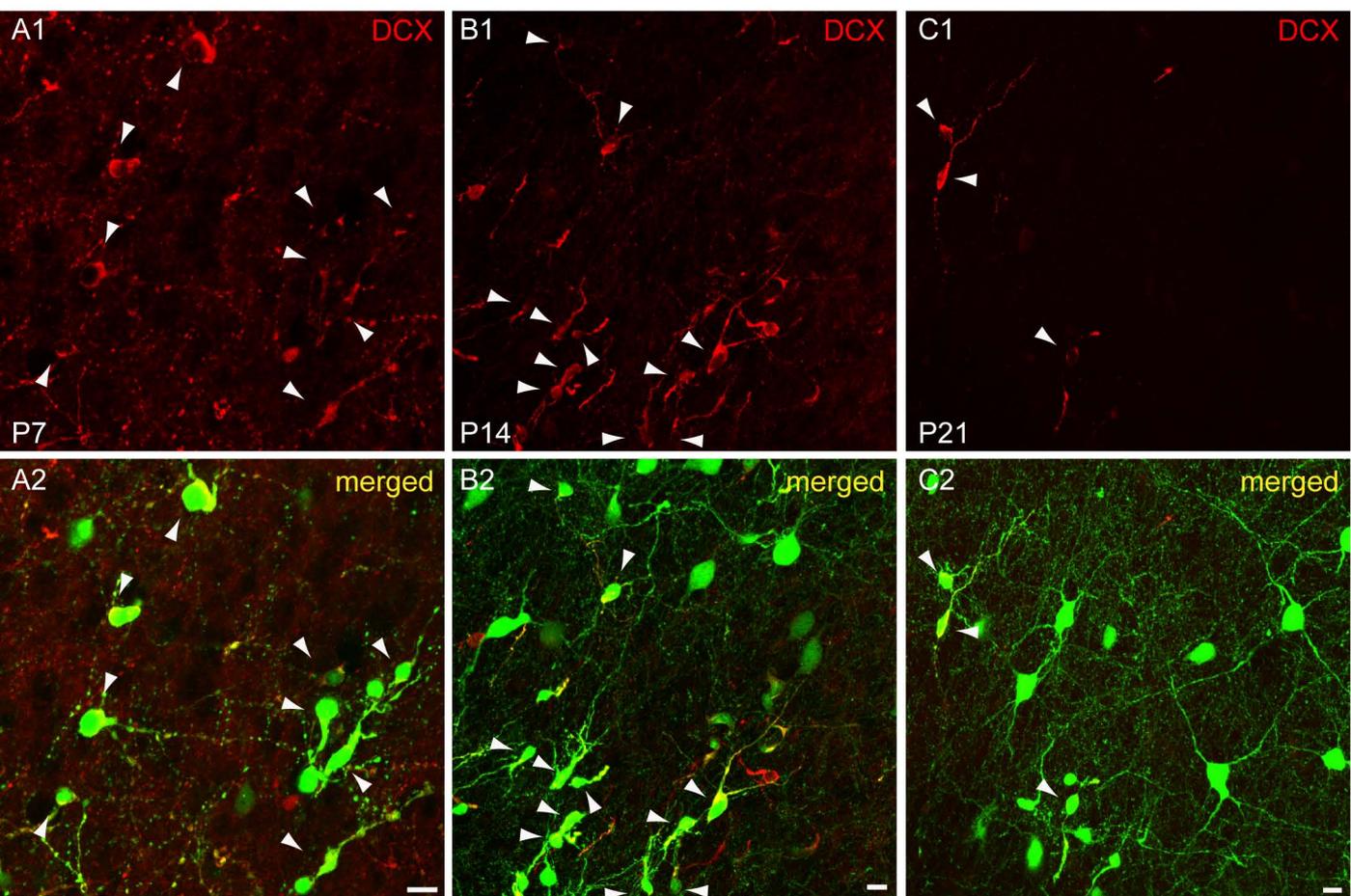


Figure 5



F

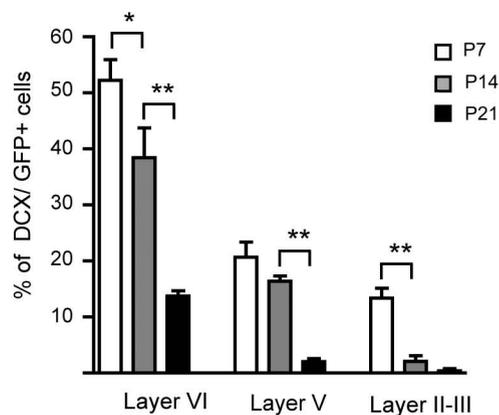
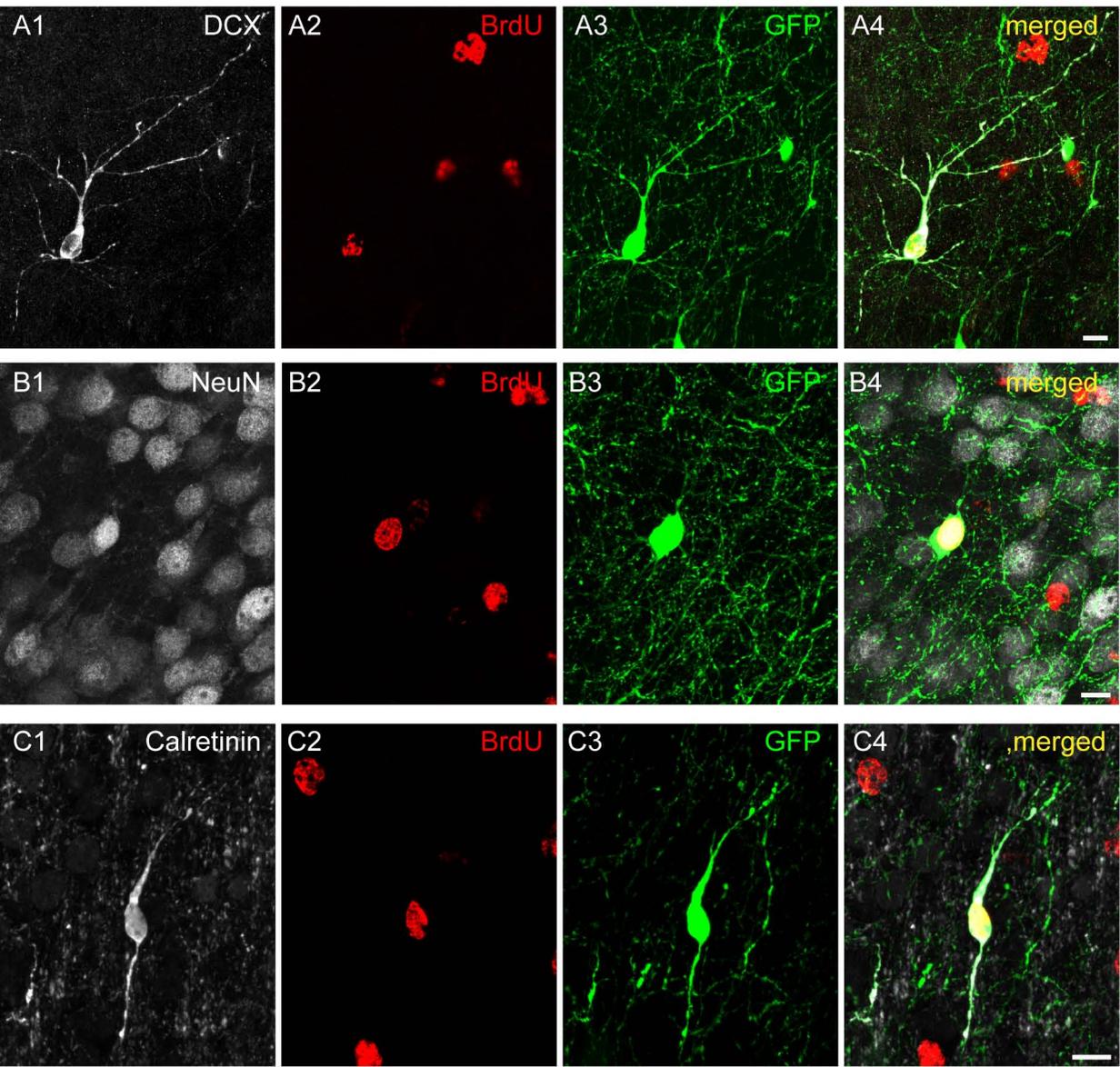


Figure 6



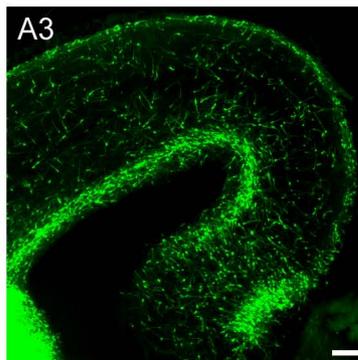
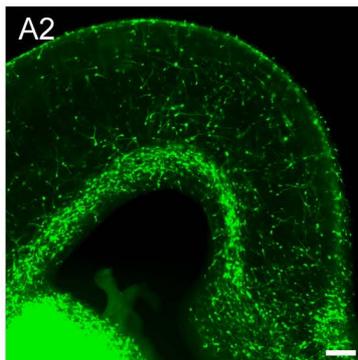
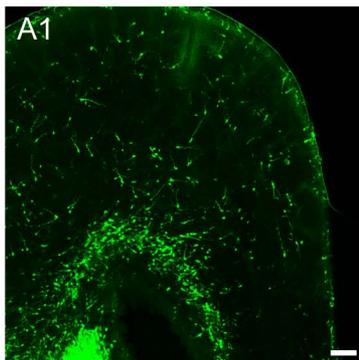
# Supplemental Figure 1

Rostral

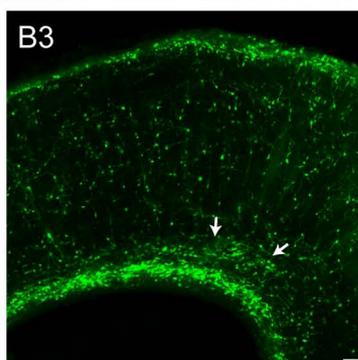
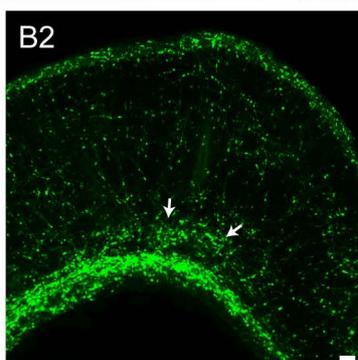
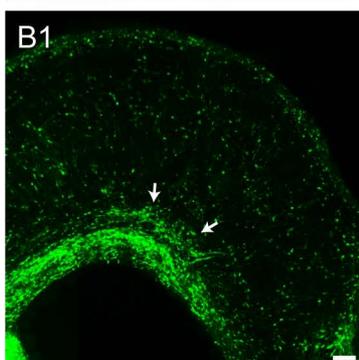
Caudal



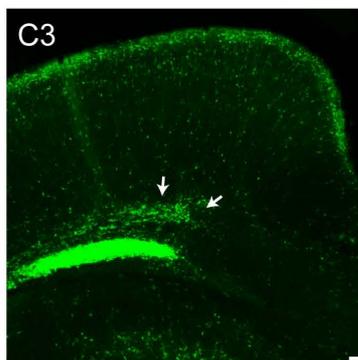
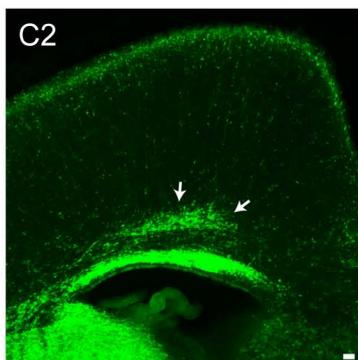
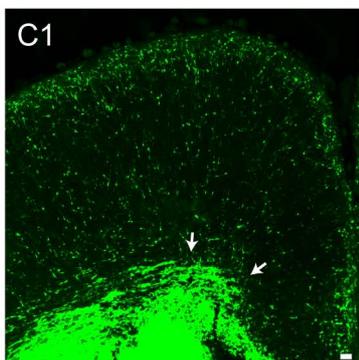
E17.5



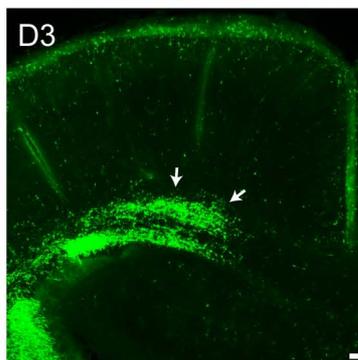
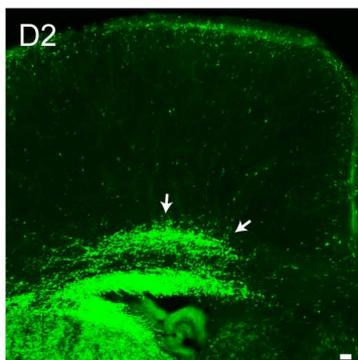
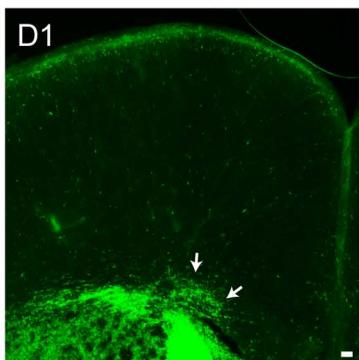
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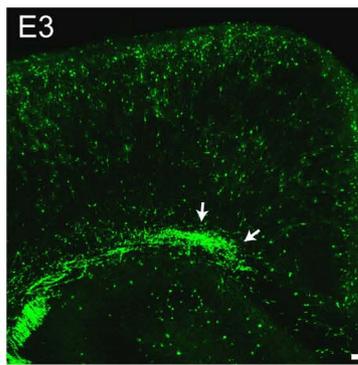
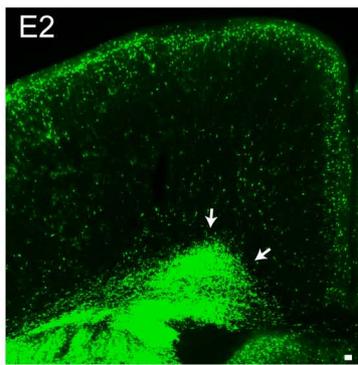
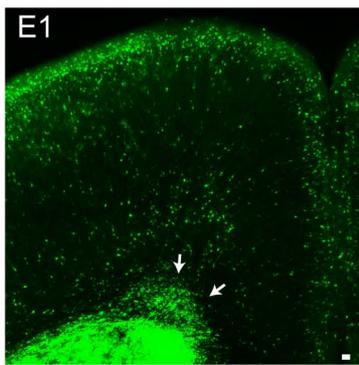
P0.5



P2.5



P7.5



## Supplemental figure 2

