The thalamus regulates retinoic acid signaling and development of parvalbumin interneurons in postnatal mouse prefrontal cortex

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Abstract

GABAergic inhibitory neurons in the prefrontal cortex (PFC) play a crucial role in higher cognitive functions. Although disruption of their normal development is linked to a number of psychiatric disorders, the underlying cellular and molecular mechanisms are poorly understood. Here we found that in postnatal mouse PFC, *Cyp26b1*, a gene encoding a retinoic acid-degrading enzyme, controls the development of medial ganglionic eminence (MGE)-derived interneurons, which we discovered are the main cell population that respond to retinoic acid within this part of the cortex. Mice lacking *Cyp26b1* in layer 6 of the frontal cortex had an increased density of parvalbumin (PV)-expressing, but not somatostatin-expressing, interneurons in medial PFC. Furthermore, initiation of *Cyp26b1* expression in neonatal PFC required the connections between the thalamus and the neocortex. This indicates a novel role of the thalamus to control *Cyp26b1* expression which in turn controls PV interneuron development in the postnatal PFC. We also found that these same connections between the thalamus and the neocortex were required for the proper radial dispersion of MGE derived interneurons in the embryonic cortical plate. Our results show that the thalamus regulates the development of PFC interneurons both by cortex-wide and region-specific mechanisms.

Keywords

prefrontal cortex, interneurons, parvalbumin, retionic acid, CYP26B1, thalamus, thalamocortical axons

Introduction

The prefrontal cortex (PFC) has major connections with several brain other regions including the thalamus, amygdala, hippocampus and striatum, and integrates many modalities of information to execute higher functions including goal-oriented behaviors, social interactions and emotion. Aberrant development of the PFC has been linked to schizophrenia, autism spectrum disorders, attention deficit hyperactivity disorders (ADHD), depression and bipolar disorders(Schubert et al., 2015). More specifically, developmental trajectories of GABAergic interneurons in the PFC, particularly those expressing the calcium-binding protein parvalbumin (PV), are impaired in both human patients and animal models of these disorders(Meechan et al., 2012; Nakazawa et al., 2012; Powell et al., 2012; Gonzalez-Burgos et al., 2015; Caballero and Tseng, 2016; Hashemi et al., 2018). Therefore, determining the normal developmental mechanisms of PFC interneurons has high disease relevance.

Development of cortical interneurons is regulated by both intrinsic and extrinsic mechanisms(Chu and Anderson, 2015; Wamsley and Fishell, 2017). One key extrinsic cue is the input from the thalamus, which regulates migration and maturation of cortical interneurons both prenatally and postnatally in mice(Sugiyama et al., 2008; Marques-Smith et al., 2016; Tuncdemir et al., 2016; Zechel et al., 2016). However, most studies addressing the role of the thalamic input in interneuron development have been performed on primary visual or somatosensory cortex, leaving the mechanisms in PFC understudied. The delayed maturation of PFC interneurons compared with other cortical areas(Gonchar et al., 2007; Nowicka et al., 2009; Ueno et al., 2017) and the distinct set of thalamic nuclei connected with the PFC(Clasca et al., 2012; Nagalski et al., 2016)

suggest the presence of unique extrinsic regulatory mechanisms for interneuron development between the PFC and sensory cortex.

One candidate molecule that may play a role in postnatal development of the PFC is retinoic acid (RA), a small molecule derived from vitamin A. RA is critical for many important aspects of brain development, ranging from rostrocaudal patterning of the hindbrain and spinal cord to synaptic plasticity(Maden et al., 1996; Dupé and Lumsden, 2001; Molotkova et al., 2007; Chen and Napoli, 2008; Chatzi et al., 2011). The RA-degrading enzyme, CYP26B1, is crucial in embryonic development in vertebrates(Yashiro et al., 2004; Hernandez et al., 2007; Gonzalez-Quevedo et al., 2010). In the mouse neocortex, *Cyp26b1* is expressed in the deep layer of the frontal cortex (Allen Brain Atlas). In contrast, *Aldh1a3*, which encodes an RA-synthesizing enzyme, is transiently expressed in the superficial layer of the medial PFC during postnatal development(Wagner et al., 2006). The already established role of RA in development and the unique opposing locations of *Cyp26b1* and *Aldh1a3* expression imply that the balance between production and degradation of RA might play an unexplored role in postnatal development of medial PFC.

In this study, we first demonstrated that in medial PFC, *Cyp26b1* is transiently expressed in layer 6 cells from postnatal day 2 (P2) to P21, a period that overlaps with the early maturation of interneurons in the PFC, and that a subpopulation of PV interneurons are the main cell type that responds to RA. These results led us to hypothesize that RA signaling regulated by *Cyp26b1* plays a role in controlling interneuron development in the PFC. To test this, we generated layer 6- and frontal cortex-specific *Cyp26b1* mutant mice and found that they had an increased density of

PV-expressing neurons in medial PFC at P14 and P21. We further demonstrated that the postnatal expression of *Cyp26b1* in medial PFC is dependent on the connections between the thalamus and PFC. Thus, the thalamus has a postnatal role in regulating the development of PV neurons via inducing the expression of the RA-degrading enzyme CYP26B1 in frontal cortex. Additionally, we found that the thalamus was also required for radial allocation of PFC interneurons during embryogenesis. These results demonstrate that the thalamus plays multiple crucial roles in interneuron development in the PFC first by controlling their laminar positioning during embryonic stages and then by restricting the maturation of PV neurons by inducing a retinoic acid-degrading enzyme.

Results

Cyp26b1, a gene encoding a retinoic acid degrading enzyme, is expressed in a spatially and temporally dynamic pattern in postnatal mouse PFC

In order to identify genes that are enriched in developing PFC, we screened the Anatomic Gene Expression Atlas (AGEA)(Ng et al., 2009). The database showed that *Cyp26b1*, a gene that encodes a retinoic acid (RA)-degrading enzyme that belonged to the cytochrome P450 family 26 (CYP26) proteins, is strongly expressed in the frontal cortex at postnatal day 14 (P14), including the deep layer of medial and ventral PFC as well as the middle layer of lateral cortex extending into the agranular insula. We therefore examined the developmental expression patterns of *Cyp26b1* in more detail by in situ hybridization. Prenatal cortex did not show detectable *Cyp26b1* expression (Fig.1A). At P0, lateral frontal cortex, but not medial PFC, started to showed a robust signal (Fig.1B). A strong expression in medial PFC was first observed at P2 (Fig.1C). Comparison with the established marker of layer 6 neurons, *Synaptotagmin 6 (Syt6)*, showed that *Cyp26b1* is expressed in layer 6 of medial and ventral PFC, as well as in the dorsolateral frontal cortex including the motor area (compare Fig.1D and M). Not only was the expression of *Cyp26b1* spatially restricted, it was also temporally dynamic; in medial PFC, *Cyp26b1* was strong until P14 (Fig.5C), and by P21, it was much weaker compared with ventral and lateral regions (Fig.1E). By P35, there was no detectable expression of *Cyp26b1* in medial PFC (Fig.1F).

The tissue RA level is controlled both by its synthesis from vitamin A and by its degradation by CYP26 enzymes. Members of the aldehyde dehydrogenase 1 family are crucial for synthesizing RA, and two members of this family (ALDH1A2 and ALDH1A3)

are expressed in early postnatal cortex; *Aldh1a2* is broadly expressed in the meninges(Wagner et al., 2002), whereas *Aldh1a3* was specifically expressed in the superficial layer of postnatal medial PFC as well as higher-order visual areas (Wagner et al., 2002, 2006). We found that week expression of *Aldh1a3* is detected in medial PFC at P2 (Fig.1I). By P8, the expression was robust in layer 2 and continued more laterally (Fig.1J). A similar pattern of expression was found at P21 (Fig.1K) and P35 (Fig.1L). There also appears to be *Aldh1a3* expression in medial cortex in the adult (Allen Brain Atlas). Outside of the frontal cortex, we did not detect *Cyp26b1* expression in more caudal parts of the neocortex including sensory areas at any of developmental stages examined (Fig.S1D,E, S3D). *Cyp26b1* was also expressed in the piriform cortex, the amygdala, CA3 and the hilus regions of the hippocampus, and the globus pallidus (Fig.S1C-E). Expression in these regions started during embryogenesis (Fig.S1B,C) and continued into adulthood (Allen Brain Atlas). *Cyp26b1* was not detected in either medial (MGE) or lateral (LGE) ganglionic eminence (Fig.S1A).

In summary, in the medial part of early postnatal PFC, RA is produced by cells in the superficial layer, whereas CYP26B1, a RA degrading enzyme or an “RA sink”, is located in layer 6 (summarized in Fig.1N). These results suggest that RA signaling is spatially and temporally controlled, and that this regulation might play a role in the development of medial PFC.

Parvalbumin-expressing interneurons in medial PFC respond to retinoic acid during early postnatal development

The spatiotemporal expression pattern of *Cyp26b1* prompted us to explore the cellular targets of RA signaling during postnatal PFC development. In order to determine the populations of cells that respond to RA, we analyzed the *RARE-LacZ* transgene, an indicator of the transcriptional activity of the RA receptor, RAR (retinoic acid receptor)/ RXR (retinoid X receptor) heterodimers (Rossant et al., 1991). At P0, expression of β-galactosidase (β-gal) was barely detectable in medial PFC except in radial glial fibers (Fig.2A). At P7, a small number of β-gal-positive cells were detected in medial PFC, mostly in layer 5 (Fig.2B-E). These cells were also positive for SOX6, a marker for GABAergic interneurons derived from MGE(Batista-Brito et al., 2009) (Fig.2B). This was still the case at P14, when the number of β-gal-positive cells greatly increased (Fig.2F-I); analysis of three transgenic brains revealed that 91% of β-gal-positive cells in layer 5 were also SOX6-positive (Fig.2M). Markers of other neuronal types, including SP8 (Fig. 2C,J; caudal ganglionic eminence (CGE)-derived cortical interneurons)(Ma et al., 2012), CTIP2 (Fig.2D,K; layer 5 subcerebral projection neurons as well as some interneurons) (Arlotta et al., 2005; Chen et al., 2005; Nikouei et al., 2016) and TBR1 (Fig.2E,L; layer 6 corticothalamic projection neurons(Hevner et al., 2001)) did not overlap with β-gal, indicating that these types of neurons do not express the molecular machinery necessary to respond to RA via RAR/RXR heterodimers. In summary, within early postnatal medial PFC, the main populations of RA-responding cells were MGE-derived interneurons in layer 5.

Most MGE-derived interneurons in the adult neocortex express either somatostatin (SST) or parvalbumin (PV)(Fogarty et al., 2007; Miyoshi et al., 2007). A majority of these interneurons complete their tangential migration into the neocortex by

birth(Miyoshi and Fishell, 2011; Inamura et al., 2012). However, PV protein or *Pvalb* mRNA is not expressed until much later, indicating that the expression of PV or *Pvalb* is a useful marker of maturation for this lineage of cells. At P8, *Pvalb* mRNA was already abundant in lateral portion of the cortex including motor area (Fig.3A, double arrows), but not in medial PFC (Fig.3A arrow). Thus, maturation of PV neurons is delayed in medial PFC. By P14, cells expressing *Pvalb* mRNA or PV protein were clearly detectable in medial PFC, mainly in layer 5 (Fig.3B, arrow), which further increased by P21 (Fig.3C, arrow).

In order to determine if RA-responding cells are restricted to either PV or SST neurons, we next analyzed *RARE-LacZ* transgenic mice to determine the co-localization of β-gal and PV (Fig.3D-G, I-L) as well as β-gal and SST (Fig.3H,M) at P14 and P21. We found a heavy overlap between PV and β-gal; in three P14 brains (Fig.3D-G), 48% percent of β-gal-positive cells in layer 5 were also PV-positive (Fig.3N). In turn, 45% of PV-positive cells in layer 5 were also β-gal-positive (Fig.3O). Similar patterns were found at P21 (Fig.3I-L). In a sharp contrast, SST show very little overlap with β-gal from P8 through P67 (Fig.3H,M and data not shown); the ratio of SST/β-gal double-positive cells to SST-positive cells was 2.9% (3 out of 103) at P14 and 5.0% (6 out of 121) at P21. Cells expressing SST protein or *Sst* mRNA rapidly appeared by P4; by P7, ~40% of MGE-derived interneurons expressed *Sst*, and this ratio stayed the same until P60 (Fig.S2). This result suggests that by P7, most of the SST neurons already express *Sst* mRNA, and that MGE-derived neurons that are β-gal-positive and SST-negative at P14 or P21 are most likely to be PV neurons. In summary, most of the overlap between

SOX6 and β-gal at P14 and P21 is accounted for by RA-responding neurons of the PV-cell lineage (summarized in Fig.3P).

Cyp26b1 is required for normal development of parvalbumin-expressing interneurons in medial PFC

Because of the strong correlation between the expression of PV and the cell's responsiveness to RA, we hypothesized that development of PV interneurons in medial PFC is regulated by RA signaling and that this regulation depends on CYP26B1. To test this, we generated conditional *Cyp26b1* mutant mice in which *Cyp26b1* is deleted in the PFC. Because the expression of *Cyp26b1* is highly specific to layer 6 in medial PFC, we used *Synaptotagmin-6 Cre* (*Syt6-Cre*) driver mice(Gong et al., 2003; Hsu et al., 2014). Expression *Syt6* is specific to layer 6 in the neocortex (Fig.1M), and *Syt6-Cre* mice allow recombination in layer 6 corticothalamic projection neurons in the frontal cortex including the medial PFC (Allen Brain Atlas; http://connectivity.brain-map.org/). To validate the usefulness of *Syt6-Cre* in knocking out *Cyp26b1* in layer 6 of the postnatal frontal cortex but not in other, potentially relevant *Cyp26b1*-expressing cell populations, we first bred the Cre mice with ZSGreen Cre reporter mice (Ai6)(Madisen et al., 2010). At E12.5, *Sy6-Cre*; *Ai6* brains showed ZSGR expression in the preplate and the meninges but not in other parts of the cortex or in ganglionic eminences (Fig.S3A). Cortical expression of ZSGreen started to be detected at E14.5 (Fig.S3B). At P0, robust signs of recombination were seen in layer 6 of the frontal cortex but not in more caudal cortex (Fig.S3C,D), amygdala (Fig.S3E) or CA3 and hilus regions of the hippocampus (Fig.S3D). Thus, we predicted that the *Syt6-Cre* mice would cause specific deletion of

Cyp26b1 in layer 6 cells of the frontal cortex including medial PFC. In *Syt6-Cre/+*; *Cyp26b1*$^{flox/flox}$ (*Cyp26b1 CKO*) mice, *Cyp26b1* mRNA was not detected in medial PFC (compare Fig.S4B and H as well as C and I), whereas expression in other brain regions including the hippocampus (Fig.S4D,J), agranular insula (Fig.S4C,I), globus pallidus (Fig.S4E,K) or amygdala (Fig.S4F,L) was not affected, confirming an efficient and specific deletion of *Cyp26b1*.

We then counted *Pvalb* mRNA-expressing neurons and compared the numbers between *Cyp26b1* CKO mice and their littermate wild-type controls at P14 and P21. At both stages, the density of *Pvalb*-positive cells was significantly increased in medial PFC of *Cyp26b1* mutant mice (Fig.4C, Fig.S5J). When the PFC was divided into superficial and the deep layers, the difference was seen only in the deeper half of the medial PFC, consistent with the distribution of β-gal expressing cells. In the motor cortex (M), we detected significant reduction in the density of *Pvalb*-expressing cells in deep layers between *Cyp26b1* CKO and control mice, but the difference was not significant with 2-way ANOVA with repeated measures (Fig.4D). More laterally, the putative somatosensory area also did not show significant difference in density (Fig.4E), suggesting that in the CKO mice, the residual *Cyp26b1* expression in layer 5 of lateral frontal cortex (Fig.S4I) might have spared the normal density of *Pvalb*-expressing cells in lateral cortex. In the PFC, normal expression is limited to layer 6, and CKO mice had no residual expression in more superficial layers. In contrast to *Pvalb*-expressing neurons, the number of *Sst*-, *Vip*- (derived from CGE) or *Lhx6*- (derived from MGE) expressing neurons was not significantly different in *Cyp26b1* mutant mice compared with controls (Fig.S5A-I,K). These results indicate that transient expression of *Cyp26b1*

in layer 6 of medial PFC is specifically required for controlling the development of PV interneurons. This is consistent with disproportionately high percentage of PV neurons responding to RA compared with SST neurons (Fig.3). The lack of significant change in the density of *Sst*-, *Vip*- or *Lhx6*-expressing cells suggests that CYP26B1 does not control the fate specification or the number of each interneuron type, but rather the rate of maturation of PV lineage cells specifically.

Cyp26b1 is not expressed in early postnatal medial PFC in the absence of thalamus-PFC connectivity

Because *Cyp26b1* starts to be expressed in medial and ventral PFC when the reciprocal connections between the thalamus and the cortex are being established, we next asked if normal expression of *Cyp26b1* depends on this connectivity. In our previous study, thalamus-specific deletion of the homeobox gene *Gbx2* resulted in severe deficiency of thalamocortical and corticothalamic projections in sensory areas(Vue et al., 2013). Similar to sensory cortex, the PFC also showed a significant reduction in the staining of NetrinG1, a marker of thalamocortical axons (Nakashiba et al., 2002; Vue et al., 2013) in *Gbx2* mutant mice at E16.5 (Fig.S6A,B). Placement of DiI crystals into the medial PFC of *Gbx2* mutants and wild type at P14 revealed that both retrograde labeling of thalamic neurons and anterograde labeling of corticothalamic axons were severely attenuated in *Gbx2* mutants (Fig.S6C-F). These results demonstrate a robust reduction of reciprocal connectivity between the thalamus and PFC in *Gbx2* mutant mice.

We then tested if expression of *Cyp26b1* in the PFC is altered in *Gbx2* mutant mice. Already at P2, the mutant cortex lacked the expression of *Cyp26b1* in medial and ventral PFC (Fig.5A,F), demonstrating that the onset of *Cyp26b1* expression requires thalamocortical interactions. The deficiency of *Cyp26b1* expression continued until P21, when the medial PFC expression of *Cyp26b1* normally started to decline (Fig.5D,I). In contrast, expression of *Cyp26b1* showed no alterations in layer 5 of the lateral frontal cortex including the motor areas, as well as agranular insula (Fig.5A-J). The layer 6 marker, *Syt6* was still highly expressed in the frontal cortex of *Gbx2* mutants (Fig.5K,N), making it unlikely that cell loss in layer 6 caused the reduced expression of *Cyp26b1* in *Gbx2* mutants. Lastly, *Aldh1a3,* which normally shows a similar onset of expression in medial PFC to that of *Cyp26b1*, was qualitatively unaffected in *Gbx2* mutants (Fig. 5L,O). In summary, transient expression of *Cyp26b1* in layer 6 of medial PFC was dependent on the connections between the thalamus and the cortex (summarized in Fig.5M,P). Putting together the role of *Cyp26b1* in development of PV neurons in medial PFC, our results collectively demonstrate a novel, indirect role of the thalamus in regulating the neocortical interneuron development.

Lack of thalamus-PFC connectivity results in early aberrancy of radial positioning of MGE-derived interneurons

In sensory cortex, it is known that thalamocortical afferents affect the development of interneurons by a variety of mechanisms(Sugiyama et al., 2008; Marques-Smith et al., 2016; Tuncdemir et al., 2016; Zechel et al., 2016). At P21, densities of *Pvalb*- and *Sst*- expressing interneurons in medial PFC, specifically in the

middle layers and not in the most superficial and the deepest layers, were significantly reduced in *Gbx2* mutant mice (Fig.6A-C). Already at P0, LHX6-expressing, MGE-derived interneurons showed an altered radial distribution in medial PFC (Fig.6D-G); there was an increase in the density of LHX6-positive cells in layer 6 and below, and a decrease in the middle layers (Fig.6F), while the total density was not significantly different between the two genotypes (Fig.6G). These results on the neonatal PFC are remarkably similar to a recent report on sensory and motor cortex of thalamus-specific *Gbx2* mutant mice(Zechel et al., 2016). Thus, there is an early, cortex-wide role of thalamocortical projections in controlling the radial distribution of MGE-derived interneurons. Because the altered neuronal positioning had already occurred at P0 in *Gbx2* mutant mice, which is before the onset of *Cyp26b1* expression in medial PFC, these early roles are likely to be independent of the later role of the thalamus in regulating the development of PV interneurons via *Cyp26b1*.

Induction of *Cyp26b1* by the thalamus is independent of transmitter release from thalamocortical projection neurons

How does the thalamus control the expression of *Cyp26b1* in layer 6 neurons in medial PFC at early postnatal stages? One likely cue that mediates the role of the thalamus is the transmitter release from thalamocortical axons. To test if the lack of transmitter release phenocopies the lack of the axon projections themselves, we generated mutant mice in which tetanus toxin light chain (TeNT) is ectopically expressed specifically in thalamocortical projection neurons (Fig.7). At E16.5, expression of VAMP2, the cleavage target of TeNT, was dramatically reduced in

thalamocortical axons expressing TeNT, while it was retained in corticofugal axons (Fig. 7A-D, G-J). At P8, expression of *RORβ* in layer 4 of the primary somatosensory area was altered in TeNT-expressing mice, lacking the characteristic barrel-like pattern (Fig. 7E.F). This is consistent with a recent study on *vGluT* mutants(Li et al., 2013) and indicates the role of transmitter release in the formation of normal cytoarchitecture of the primary sensory cortex. In the PFC, however, induction of *Cyp26b1* in the medial and ventral PFC was not qualitatively affected in TeNT-expressing mice (Fig.7K,L), implying a unique cellular mechanism that underlies the induction of *Cyp26b1* expression in early postnatal PFC.

Discussion

In this study, we first demonstrated that *Cyp26b1*, which encodes an RA-degrading enzyme and a critical regulator of retinoid signaling(Duester, 2008; Rhinn and Dolle, 2012), is expressed in developing PFC in a temporally and regionally specific manner. We also showed that PV-expressing interneurons are a major cell population that normally responds to RA via the RAR/RXR receptors. Conditional deletion of *Cyp26b1* in layer 6 of the frontal cortex resulted in an increased density of *Pvalb*-expressing neurons in medial PFC during postnatal development. Expression of *Cyp26b1* in the PFC depended on the connections between the cortex and the thalamus, but not on the transmitter release from thalamocortical axons. These results demonstrate a unique regulatory role of the thalamus in postnatal development of PV interneurons in the PFC (summarized in Fig.8D-G).

Roles of RA signaling in postnatal development of the medial PFC

In early embryonic brain, RA controls rostrocaudal patterning of the hindbrain and spinal cord by regulating the expression of *Hox* genes(Maden et al., 1996; Dupé and Lumsden, 2001). Cells in the subventricular zone of the embryonic LGE express the RA-synthesizing enzyme ALDH1A3, and *Aldh1a3* deficient mice had reduced expression of dopamine receptor D2 (*Drd2*) in nucleus accumbens(Molotkova et al., 2007) and reduced expression of *Gad1* in embryonic GABAergic neurons in the striatum and the cortex(Chatzi et al., 2011), demonstrating a crucial role of RA in early differentiation of embryonic GABAergic neurons. However, much less is known about the roles of RA signaling in postnatal brain development. Systemic administration of RA

into early postnatal mice caused behavioral hyperactivity at the adult stage with an increased number of calbindin-expressing neurons in the cingulate cortex(Luo et al., 2004). Thus, it was expected that RA has a major role in early postnatal brain development. However, the role of RA in PFC development and the underlying cellular and molecular mechanisms had remained unknown.

Our current study revealed that in postnatal PFC, a subpopulation of PV interneurons are responsive to RA via RAR/RXR receptors, and that the lack of the RA-degrading enzyme, CYP26B1, causes an increase in the density of *Pvalb*-expressing cells in medial PFC at P14 and P21. This phenotype should be independent of the proposed, earlier roles of RA in tangential migration of GABA neurons(Crandall et al., 2011), because 1) most MGE-derived interneurons have completed the tangential migration to the cortex by birth, 2) *Cyp26b1* is expressed only postnatally in the medial PFC and is not expressed in embryonic MGE or LGE (Fig.S1A,B), 3) *Syt6-Cre* mice do not cause recombination in MGE-derived interneurons (Fig.S3), and 4) in *Syt6*$^{Cre/+}$*; Cyp26b1*$^{flox/+}$ mice, expression of *Cyp26b1* is not affected in regions outside of the frontal cortex, including the ventral telencephalon (Fig.S4).

In addition, the increased number of *Pvalb*-positive neurons in deep layers of medial PFC was not accompanied by their decrease in upper layers. This suggests that the radial dispersion of PV neurons, which follows their tangential migration, was also unaffected in *Cyp26b1* mutants.

At the adult stage, *Cyp26b1* mutants no longer showed a significant increase in the number *Pvalb*-positive neurons in medial PFC (Fig.S5L-M). In addition, the total number of MGE-derived interneurons marked by LHX6 did not show a significant

change throughout postnatal development. Thus, thee most likely role of *Cyp26b1* in postnatal development of PV neurons in the medial PFC is to slow their rate of maturation by suppressing RA signaling. It is known that neurotrophin (e.g., BDNF) signaling and neuronal activity, likely mediated by NMDA receptors, play a role in postnatal development of PV neurons(Huang et al., 1999; Itami et al., 2000; Patz et al., 2004; Woo and Lu, 2006; Belforte et al., 2010). Further studies are needed to investigate the relationship between these pathways and RA signaling. It will also be informative to analyze various aspects of maturation of PV neurons including electrophysiological properties, formation of perineuronal nets and changes in gene expression(Okaty et al., 2009; Ueno et al., 2017).

Universal and area-specific roles of the thalamus in neocortical development

Previous studies have indicated that thalamocortical afferents have instructive roles in establishing area- and layer-specific gene expression in cortical excitatory neurons as well as in promoting morphological differentiation of excitatory neurons in primary visual and somatosensory cortex(Chou et al., 2013; Li et al., 2013; Vue et al., 2013; Moreno-Juan et al., 2017). Although the underlying cellular and molecular mechanisms have just begun to be elucidated, at least some of the effects appear to be dependent on the release of neurotransmitters from thalamocortical axons(Li et al., 2013). In addition to excitatory neurons, both SST- and PV- interneurons also depend on thalamic afferents for their maturation, likely via glutamatergic synaptic transmission(Sugiyama et al., 2008; Marques-Smith et al., 2016; Tuncdemir et al., 2016). In somatosensory, visual and motor areas, thalamic afferents also control radial

positioning of MGE-derived interneurons before birth, in which the thalamus regulates the expression of the KCC2 co-transporter via the release of glutamate from thalamic axon terminals(Zechel et al., 2016). Are these roles universal throughout the cortex or is there area-specific regulation of cortical development by the thalamus? The thalamus is composed of dozens of nuclei with distinct gene expression profiles(Jones, 2007; Nagalski et al., 2016), and each thalamic nucleus has a unique patterns of axonal projections to different cortical areas(Clasca et al., 2012). The neocortex is also regionally specified before the arrival of the thalamic axons(Rash and Grove, 2006; O'Leary et al., 2007; Hoch et al., 2009), and exhibits a rostral-to-caudal and lateral-to-medial neurogenic gradients(Bayer and Altman, 1991). Thus, it is expected that the nature of interactions with the thalamus varies between different cortical areas.

In fact, expression of ROR*β* and *Lmo4*, which showed abnormal patterns in primary sensory cortex, was not impaired in the medial PFC of *Gbx2* mutant mice (Fig.S7). Instead, our current study has revealed a frontal cortex-specific regulation of *Cyp26b1* by the thalamus at early postnatal stages; loss of axons between the thalamus and the cortex in *Gbx2* mutants prevented *Cyp26B1* from being expression in layer 6 of the frontal cortex. Interestingly, our TeNT model blocking the vesicle release from TCAs was insufficient for recreating this loss of *Cyp26B1.* Therefore, the role of the thalamus in inducing *Cyp26b1* expression in layer 6 PFC neurons is likely independent of transmitter release that involves VAMP2 functions.

The current study also revealed an early role of the thalamus in regulating the radial positioning of MGE-derived interneurons in neonatal PFC. The altered positioning of interneurons was highly reminiscent of what was found in sensory and motor

areas(Zechel et al., 2016). This indicates that the early role of the thalamus in controlling the radial positions of cortical interneurons is shared between many neocortical areas (summarized in Fig.8A-C). Importantly, radial positioning of cortical interneurons is compromised also in *Lhx6* mutant mice, which suggests that the tangential-to-radial switch of interneuron migration in the cortex requires coordination of intrinsic and extrinsic signaling mechanisms during embryonic development.

It is intriguing that in postnatal *Gbx2* mutant mice, even in the absence of normal induction of *Cyp26b1* in medial PFC, the density of *Pvalb*-expressing neurons was decreased, not increased, unlike in *Cyp26b1* mutants. This implies that the thalamus plays additional roles in postnatal development of PV neurons other than by inducing *Cyp26b1*, which include their survival. Thus, it will be important to determine if other known roles of the thalamus in postnatal regulation of the development of PV neurons in sensory cortex (Sugiyama et al., 2008; Marques-Smith et al., 2016; Tuncdemir et al., 2016) also apply to the PFC.

Functional implications of altered PV neuron development in *Cyp26b1* mutant mice

At the systems level, RA regulates cortical synchrony during sleep(Maret et al., 2005), memory and cognitive behaviors(Chiang et al., 1998; Aoto et al., 2008; Nomoto et al., 2012). In addition, aberrant RA signaling is associated with multiple psychiatric disorders including schizophrenia, bipolar disorder and depression in humans(Goodman, 1998; Bremner et al., 2012; Qi et al., 2015; Haybaeck et al., 2015). Because many psychiatric disorders are predisposed by aberrant brain development,

understanding how RA functions in early postnatal brain development is important for determining the long-term consequences of the perturbations of this signaling pathway. We found that in the absence of *Cyp26b1* in frontal cortex during a discrete period of postnatal development, the medial PFC had more *Pvalb*-expressing interneurons than in control mice. It is well established that PV neurons orchestrate activity in local circuits, which leads to oscillatory synchronous network activity in the gamma-band(Cardin et al., 2009; Sohal et al., 2009). Synchronous gamma-band activity in medial PFC is associated with the successful operation of working memory. In a mouse genetic model of schizophrenia that replicates the human the 22q11.2 microdeletion syndrome(Karayiorgou et al., 1995), gamma synchrony and working memory performance were impaired(Sigurdsson et al., 2010), so was the development of PV interneurons(Meechan et al., 2012; Fénelon et al., 2013; Toritsuka et al., 2013; Meechan et al., 2015). Furthermore, mutations in the *Disc1* gene, a significant risk factor for schizophrenia and other disorders including depression and bipolar disorder in humans, cause alterations of PV neurons and reduction of gamma oscillations in mice(Sauer et al., 2015). These findings link PV interneuron abnormalities to changes in prefrontal synchrony and working memory impairment in mouse models of neuropsychiatric disorders. Mutation of CYP26B1 in humans is a risk factor for schizophrenia that reaches genome wide significance(Mistry et al., 2013; Schizophrenia, 2014). Therefore, it will be interesting to test whether aberrant time course of PV neuron development in medial PFC causes altered synchrony and impairment of cognitive behaviors in *Cyp26b1* mutant mice.

Materials and methods

Mice. *RARE-LacZ* transgenic mice(Rossant et al., 1991) were obtained from Jackson Laboratory (stock number: 008477) and were kept in the CD1 background. Frontal cortex-specific *Cyp26b1* mutant mice were generated using BAC (bacterial artificial chromosome) *Syt6-Cre* mice (GENSAT)(Gong et al., 2003; Hsu et al., 2014). Although the endogenous *Syt6* gene is expressed ubiquitously in layer 6 of the neocortex, the BAC Cre line caused recombination specifically in the frontal cortex. We crossed *Syt6*$^{Cre/+}$*; Cyp26b1*$^{flox/+}$ mice and *Cyp26b1*$^{flox/flox}$ mice to generate the conditional mutants. *Cyp26b1*$^{flox/flox}$ mice(Okano et al., 2012) were developed by Dr. Hiroshi Hamada's laboratory (RIKEN CDB, Kobe, Japan) and obtained from Dr. Maria Morasso (NIAMS, Bethesda, MD). *Rosa26*$^{stop\text{-}ZSGreen/+}$ (*Ai6*) mice(Madisen et al., 2010) were obtained from Jackson Laboratory (stock number: 007906). Thalamus-specific *Gbx2* mutant mice were generated by crossing *Olig3*$^{Cre/+}$*; Gbx2*$^{null/+}$ mice and *Gbx2*$^{flox/flox}$ mice as described(Vue et al., 2013). *Gbx2*$^{flox/flox}$ mice were obtained from Jackson Laboratory(Li et al., 2002). *Olig3*$^{Cre/+}$ mice were described previously(Vue et al., 2009; Bluske et al., 2012). The *Gbx2*^{null} allele was generated by crossing *Gbx2*$^{flox/flox}$ mice with the *CMV-Cre* germ-line deleter mice (Jackson Laboratory, stock number: 003465). Mice that express tetanus toxin light chain in thalamic neurons were generated by crossing *Olig3*$^{Cre/+}$ mice and *Rosa26*$^{stop\text{-}TeNT/stop\text{-}TeNT}$ mice(Zhang et al., 2008).

In situ hybridization. cDNAs for the following genes were used: *Cyp26b1*, *Aldh1a3*, *Syt6, Lmo4* (Open Biosystems), *Pvalb*, *Sst*, *Vip* (obtained from Dr. Rob Machold*), Lhx6* (obtained from Dr. John Rubenstein), *RORβ*, (obtained from Dr. Michael Becker-Andre).

Postnatal pups were perfused with 4% paraformaldehyde/0.1M phosphate buffer and the heads were post-fixed until needed. Brains were then taken out of the skull, washed in 0.1M phosphate buffer for 20min and were sunk in 30% sucrose/0.1M phosphate buffer. Coronal sections were cut with a sliding microtome at 50μm-thickness (Leica) or with a cryostat at 20μm- (P2 or younger) or 40μm-thickness (P4 or older) and were mounted on glass slides (Super Frost Plus, Fisher). In situ hybridization was carried out as described(Vue et al., 2007).

Immunohistochemistry. Brains were taken out immediately after perfusion and were postfixed for 1 hour (P0), 1-2 hours (P4-P14) or 1-4 hours (P21). After the post-fixation, the brains were washed in 0.1M phosphate buffer for 20min and were sunk in 30% sucrose/0.1M phosphate buffer. Sections were cut in the same way as those used for in situ hybridization. The following primary antibodies were used: β-galactosidase (Cappel, 55976, goat, 1:100; Abcam, chicken, 1:500), SOX6 (Abcam, rabbit, 1:100), SP8 (Santa Cruz, sc-104661, goat, 1:100), CTIP2 (Abcam ab18465, rat, 1:200), TBR1 (Abcam, ab31940, rabbit, 1:200; Millipore, AB2261, chicken, 1:200), PV (SWANT, rabbit, 1:500), SST (Millipore, MAB354, rat, 1:100), LHX6 (Santa Cruz, sc-271433, mouse, 1:50), VAMP2 (Synaptic Systems, 104 202, rabbit, 1:200), NetrinG1 (R&D Systems, goat, 1:100). Secondary antibodies conjugated with Cy2, Cy3 or Cy5 were obtained from Jackson ImmunoResearch.

Combined in situ hybridization (*Sst*) and immunohistochemistry (SOX6). In situ hybridization was carried out as described above, except that prior to proteinase K

treatment, sections were incubated with 0.3% hydrogen peroxide/PBS for 10 minutes to block endogenous peroxidase activity. After the hybridization, slides were washed as described above, and were incubated for 30 minutes with anti-DIG antibody conjugated with peroxidase, followed by two washes in PBS and one wash with TNT solution (TNT: 0.1M Tris pH7.5, 0.15M NaCl, 0.05% Tween 20). Sections were then incubated for ~30 min in Tyramide Plus fluorescein (ThermoFisher) diluted at 1:250, washed twice in TNT solution and post-fixed. Thereafter, immunostaining with anti-SOX6 antibody was carried out as described above.

Imaging/Binning. For cell counting, images of sections that underwent in situ hybridization or immunostaining were taken using an upright microscope (Nikon E800) with a 2x (in situ hybridization) or a 4x (immunostaining) objective using a digital CCD camera (Retiga EXi, QImaging) and the OpenLab software. The range of coronal sections to be included in the analysis of medial PFC was defined rostrally by the presence of the lateral ventricle and caudally by the presence of the anterior forceps of the corpus callosum. Once the sections were selected, their images were scrambled and each file was given a randomized number so that the person who performed the subsequent analysis was made blind to the genotypes. Cell counting was performed in the putative prelimbic (PL) and infralimbic (IL) areas of the medial PFC (Allen Brain Atlas; (Paxinos et al., 2006; Van De Werd et al., 2010)). Images were stacked using Adobe Photoshop and subsequently binned to distinguish the parts of the medial PFC as shown in Fig.4; each bin is 500μm-high at the medial surface. For sections with in situ hybridization, the most superficial layer (layer 1) was defined as the cell-sparse

layer on DAPI staining. The remaining cortical wall was divided into three bins with equal widths, resulting in 4 layers of bins. Layer 1 and the layer underneath it were named superficial layers and the remaining two layers were named the deep layers. For immunostaining, we used anti-TBR1 antibody for all slides in Cy5 channel and used it as a reference marker of layer 6. Then, the areas excluding layers 1 and 6 were equally divided into three parts, resulting in four layers of bins (shown in Fig.6). For high-magnification images shown in Fig.2 and Fig.3, an Olympus FluoView 1000 confocal microscope was used (40x oil, NA=1.25).

Cell Counting. In imageJ, the Image-based Tool for Counting Nuclei (ITCN) plugin, from the Center for Bio-Image Informatics, UC Santa Barbara, was used to count cells and measure the area of each bin.

Statistical analysis. Paired ratio t-test was used for comparing cell counts between *Gbx2* cko mice and wild-type littermates as well as *Cyp26b1* cko and wild-type littermates. For data shown in Fig.4, Fig.S4 and Fig.6, we performed repeated measures 2-way ANOVA using Prism (versions 6 and 7, GraphPad). Comparison was made between two brains processed and analyzed in the same experiment. Graphs were generated using Prism.

Axon tracing. Small crystals of 1,1′-dioctadecyl-3,3,3′3′-tetramethylindocarbocyanine perchlorate (DiI) were placed on the medial surface of frontal cortex of PFA-fixed P14 *Gbx2* conditional mutant brains and their control littermates. After incubation of the

brains in PFA at 37℃ for 2 weeks, we cut sections at 150μm with a vibrating microtome (Vibratome), counter-stained the sections and mounted them on glass slides for imaging.

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FIGURE LEGENDS

Fig.1 Spatiotemporally regulated expression of *Cyp26b1* and *Aldh1a3* in the PFC

In situ hybridization of frontal sections through PFC at various stages is shown. **A-F.** *Cyp26b1* expression in frontal cortex. At E17.5, *Cyp26b1* is not detected (A). At P0, only a very weak expression is seen in medial PFC (B, arrowhead). At P2, *Cyp26b1* starts to be detected clearly in medial PFC (C, arrowhead). Expression in lateral cortex, especially agranular insula in more superficial layer (B, arrow; C-F, double arrowheads) is strong, which continues into later stages. At P8, expression in medial (arrowhead) and ventral (arrow) PFC is strong (D). At P21, expression of *Cyp26b1* is reduced in medial PFC (E, arrowhead) and is almost undetectable by P35 (F, arrowhead). **G-L.** *Aldh1a3* expression in frontal cortex. At E17.5, expression is detected in LGE (G, arrow) but not in PFC. At P2, clear expression is detected in the superficial layer of medial PFC (I, arrow). The expression continues into P8, P21 and P34 (J-L, arrow). **M.** *Synaptotagmin-6* (*Syt6*), a layer 6 marker, is expressed in the same layer as *Cyp26b1* in medial PFC at P8 (arrowhead). **N.** Schematic summary of the spatial expression patterns of *Cyp26b1* and *Aldh1a3* in medial PFC of early postnatal mouse brains. Scale bar, 1mm.

Fig.2 RA signaling in early postnatal PFC

In all sections, the right hemisphere is shown, and the white dashed line marks the medial surface. Immunostaining for β-gal on frontal sections of P0 (A), P7 (B-E) and P14 (F-K) brains of *RARE-LacZ* transgenic mice. **A.** At P0, β-gal expression is only found in the radial glial fibers (arrow). **B-E.** At P7, a few β-gal-positive cells are found in

the medial PFC, most of which are also SOX6-positive (B; white arrows show double-labeled cells). β-gal does not overlap with SP8 (C), CTIP2 (D) or TBR1 (E). **F-L.** At P14, the number of β-gal-positive cells dramatically increases from P7, and most of these cells are SOX6-positive. F is a 10x image including the medial surface of the brain, and G-I are 40x confocal images of the same region outlined in the square in F. I is the merged image of G and H. Yellow arrow in G,H show SOX6/β-gal double-positive cells, and green arrow in G shows a rare, β-gal-positive, SOX6-negative cells. (J-L) β-gal doses not overlap with SP8, CTIP2 or TBR1. **M.** Average number of β-gal-positive cells per section by layers (mean ± SEM). L1, layer 1 as marked by sparse labeling in DAPI staining. L6, layer 6 as marked by TBR1 staining. The two middle columns represent equal-width bins between layer 1 and layer 6, and roughly correspond to layers 2/3 and layer 4/5, respectively. Because most β-gal-positive cells are immediately above layer 6, and layer 4 is thin in medial PFC (Fig.S6A), the highest peak in the third column likely represents layer 5. **M**. The ratio of SOX6; β-gal-double-positive cells among β-gal-positive cells are shown by layers (mean ± SEM). Scale bars, A: 200μm, B-F:100μm, G-L: 50μm.

Fig.3 Overlapping expression of *RARE-LacZ* transgene and PV in early postnatal PFC

A-C. In situ hybridization of *Pvalb* mRNA on frontal sections of control mice at P8 (A), P14 (B) and P21 (C). In medial PFC, *Pvalb* is un-detectable P8, but robust expression is detected at P14, which further increases by P21 (A-C, single arrow). **D-M.** Double immunostaining for β-gal and PV or β-gal and SST on frontal sections of P14 (D-H) and

P21 (I-M) brains of *RARE-LacZ* transgenic mice. D is a 10x image including the medial surface of the brain, and E-G and J-L are 40x confocal images of the same region outlined in the square in D and I, respectively. G and L are merged images of E and F, and J and K, respectively. H and M are merged 40x confocal images of the medial PFC. Note the heavy overlap between β-gal and PV and little overlap between β-gal and SST (white arrow in M indicates a very rare double-positive cells). **N.** The ratios of PV; β-gal-double-positive cells among β-gal-positive cells in P14 medial PFC are shown by layers (mean ± SEM). **O.** The ratios of PV; β-gal-double-positive cells among PV-positive cells in P14 medial PFC are shown by layers (mean ± SEM). **P.** A schematic summary of the interneuron populations in medial PFC. Based on the results in this study, a subpopulation of PV interneurons respond to RA via RAR/RXR receptor complex. Scale bar, A-C: 1mm, D,I: 100μm, E-H, J-M: 50μm

Fig.4 Increased *Pvalb*-expressing interneurons in medial PFC of *Cyp26b1* knockout mice

A-B,E-F. In situ hybridization of frontal sections of P14 (**A,B**) *Cyp26b1* conditional knockout mice (**B**) and littermate controls (**A**). Expression of *Pvalb* is shown. See Methods on binning the medial PFC. Numbers of *Pvalb*-positive cells in the two superficial bins and two deep bins were added together and compared separately between *Cyp26b1* mutants and littermate controls. **C**-**E** show the result of paired t-tests for cell counts in the medial PFC, motor cortex (D) and somatosensory cortex (E), all on the same frontal sections. Each line connecting red and blue dots represents a pair of brains analyzed in the same experiment (n=5). p-values of ratio paired t-tests for each

layer (superficial, deep, total) are shown. In repeated measures two-way ANOVA, the p-values for layer (superficial vs deep), pair (between control and knockout), and interactions (between layer and pair) are 0.0017, 0.0325 and 0.1416 (P14 in PFC); 0.0441, 0.0961 and 0.7807 (P14 in motor cortex), 0.1771, 0.4751 and 0.5496 (P14 in somatosensory cortex), respectively. Scale bar, 1mm. L1: layer 1

Fig.5 Transient expression of *Cyp26b1* in the PFC does not occur in the absence of thalamus-cortex interactions in *Gbx2* mutant mice.

A-J. in situ hybridization of frontal sections through PFC at various postnatal stages with a *Cyp26b1* probe. In control mice (A-E), *Cyp26b1* expression starts at P2 in medial (A, arrowhead) and ventral (A, single arrow) PFC, and continues until P14 (C). At P21, expression in medial PFC is reduced (D), and is no longer detectable at P35 (E). In addition to medial and ventral PFC, *Cyp26b1* is also expressed in lateral frontal cortex including the motor area (A-E, double arrows) and agranular insula (A-E, double arrowheads). In *Gbx2* mutant mice (F-J), expression of *Cyp26b1* is not induced in medial or ventral PFC at P2 as well as at later stages. Expression in more superficial layer of lateral cortex (G-J, double arrows and double arrowheads) is not affected in *Gbx2* mutant mice. **K,N.** Expression of the layer 6 marker *Syt6* is not affected in *Gbx2* mutant mice. **L,O.** Expression of *Aldh1a3* in layer 2 of medial PFC and anterior cingulate cortex (arrow) is not affected in *Gbx2* mutant mice. scale bar, 1mm. **M,P.** Summary schematic for this figure

Fig.6 Abnormal numbers and radial distribution of MGE-derived interneurons in the medial PFC of P21 and neonatal *Gbx2* mutant mice

A-C. Comparison of *Pvalb*-positive and *Sst*-positive cells in *Gbx2* mutants (red dots) and wild-type littermates (blue dots) in medial PFC at P21. Each line connecting red and blue dots represents a pair of brains analyzed in the same experiment (n=5). A and B show comparison of laminar distribution of *Pvalb*-expressing and *Sst*-expressing neurons, respectively, in *Gbx2* mutant mice and littermate controls. Layer 1 was defined as the cell-sparse layer detected by DAPI staining. The remaining cortical wall was equally divided into three layers. The deepest layer (shown as “L5/6”) contains the entire layer 6 and the deep part of layer 5. C shows the total number of *Pvalb*- and *Sst*-expressing neurons in all layers. P-values of paired t-test for individual layer are shown. In repeated measures two-way ANOVA, the p-values for layer, pair (between control and knockout), and interactions (between layer and pair) are <0.0001, 0.0002 and 0.0001 (*Pvalb*); <0.0001, 0.0247 and 0.0001 (*Sst*), respectively. **D-E.** Representative images of immunostaining for LHX6 in medial PFC of wild-type (D) and *Gbx2* mutant (E) mice at P0. Binning is shown in yellow. Layer 1 (L1) was defined as the cell-sparse layer detected by DAPI staining. Layer 6 (L6) was defined as the layer with TBR1 staining on the same sections (not shown). The intervening region was equally divided into three layers. Sub-layer 6 was defined as the layer below layer 6. Scale bar, 200μm **F-G.** Comparison of LHX6-positive cells in *Gbx2* mutants (red dots) and wild-type littermates (blue dots) in medial PFC at P0. Each line connecting red and blue dots represents a pair of brains analyzed in the same experiment (n=5). **F** shows laminar distribution pattern. P-values of paired t-test for individual layer are shown. In repeated measures

two-way ANOVA, the p-values for layer, pair (between control and knockout), and interactions (between layer and pair) are 0.0001, 0.5950 and 0.0021, respectively. **G** shows the total number of *Pvalb*- and *Sst*-expressing neurons in all layers. P-values of paired t-test are shown. “Sub-L6” was defined as the region below the expression domain of TBR1, which was stained in all immunostaining slides for a reference. *: $p<0.05$, **: $p<0.005$, ***: $p<0.0005$

Fig.7 Normal induction of *Cyp26b1* in PFC in mice expressing tetanus toxin light chain in thalamocortical axons

A-D. Immunostaining for VAMP2 on frontal sections of somatosensory cortex at E16.5 control (A,B) and mutant mice with ectopic expression of tetanus toxin light chain (TeNT) in thalamic neurons (C,D). TeNT expression leads to deletion of VAMP2 specifically in thalamocortical axons at E16.5. Thalamocortical axons are shown by NetrinG1 staining (B,D, green). In control brains, both thalamocortical (shown in asterisk in A-D) and corticofugal (shown in bracket in A-D) axons express VAMP2, whereas in TeNT-expressing mice, VAMP2 staining is specifically diminished in thalamocortical axons (C,D, asterisk). Scale bar, 500μm. **E,F.** Deletion of VAMP2 in thalamocortical axons results in the lack of the characteristic pattern of *RORβ* expression in the barrel field of primary somatosensory cortex at P8 (arrow), similar to the defect found in *Gbx2* mutant mice (Vue et al., 2013). **G-J.** Immunostaining for VAMP2 on frontal sections of prefrontal cortex at P0 control (G,H) and mutant mice with ectopic expression of tetanus toxin light chain (TeNT) in thalamic neurons (I,J). Similar to the somatosensory cortex, VAMP2 staining in thalamocortical axons is diminished in TeNT-expressing mice

(bracket in I, J). Scale bar, 200μm. **K,L.** Expression of *Cyp26b1* in medial (arrowhead) and ventral PFC is intact in TeNT-expressing mice at P8. scale bar, 200μm (G,H,I,J) 1mm (E,F,K,L)

Fig.8 Schematic diagrams of the current finding

A-C. Embryonic roles of thalamocortical axons as observed in neonatal mice. (A) Thalamocortical axons reach the medial PFC (mPFC) by E16.5 and control migration of medial ganglionic eminence (MGE)-derived interneurons. (B) In normal neonatal mice, MGE-derived interneurons have largely completed tangential migration to the mPFC and have taken proper laminar positioning by radial dispersion (arrows). (C) In thalamus-specific *Gbx2* mutant mice, radial positioning of MGE-derived interneurons are aberrant, resulting in their accumulation in layer 6 and below. **D-G.** Postnatal roles of thalamus-PFC interactions and retinoic acid (RA)-degrading enzyme CYP26B1 in the development of PV interneurons in the mPFC. (D) Early postnatal mPFC is positioned between the source of RA synthesis (layer 2, by ALDH1A3) and the RA-degrading “sink” (layer 6, by CYP26B1). Expression of both enzymes is induced early postnatally, but only *Cyp26b1* is dependent on the connections with the thalamus. The main cell population that responds to RA in postnatal mPFC are parvalbumin (PV) interneurons, and their development is controlled by CYP26B1. (E) In normal postnatal mice, PV neurons mature and start to express *Pvalb* mRNA and PV protein mainly in deep layers of mPFC between P7 and P14. (F) In thalamus-specific *Gbx2* mutant mice, *Cyp26b1* is not induced in mPFC. The number of both *Pvalb* and *Somatostatin* (*Sst*)-expressing neurons is reduced in the middle layers due to the earlier defects in radial dispersion

(described in C). (G) In frontal cortex-specific *Cyp26b1* mutant mice, lack of the RA sink in mPFC leads to an increased number of neurons that express *Pvalb* mRNA or PV protein in deep layers.

LEGENDS TO SUPPLEMENTAL FIGURES

Fig.S1 Normal expression pattern of *Cyp26b1* in the mouse forebrain

In situ hybridization on coronal sections is shown. **A,B.** Expression is not detected in LGE or MGE at E14.5, but is already found in the hippocampus (A,B, arrowhead), xyz (A, arrow), globus pallidus ("GP") and amygdala ("Amy") (B). B is more caudal than A. **C.** At E16.5 (C), *Cyp26b1* is detected in hippocampus (arrowhead), piriform cortex ("Pir"), globus pallidus ("GP") and amygdala ("Amy"). **D,E.** This pattern continues into P4 and adulthood (not shown). E is more caudal level than D. Expression in the hippocampus is strongest in CA3 and hilus, whereas multiple nuclei in amygdala show strong expression of *Cyp26b1* (E). Scale bars, A,B: 500μm, C-E:1mm.

Fig.S2 Time course of *Sst* expression in medial PFC of postnatal mice. A. Left is to the midline. *Sst* mRNA was detected by *in situ* hybridization using a Tyramide Signal Amplification (TSA) system, followed by immunostaining with anti-SOX6 antibody. **B.**The ratio of *Sst*+; SOX6+ cells to SOX6+ cells reached a plateau value of ~0.4 by P7. At P0, a much smaller portion of SOX6+ cells expressed *Sst* mRNA. Each dot indicates an average number of cells in medial PFC of at least 3 sections of a wildtype brain.

Fig.S3 Recombination in *Synaptotagmin-Cre* (*Syt6-Cre*) transgene mice

Expression of ZSGreen in *Syt6-Cre/+*; *Ai6* (ZSGreen Cre reporter) mice at E12.5 (A), E14.5 (B) and P0 (C-E). All sections are coronal and the midline is to the left. At E12.5, expression of ZSGreen reporter is found in meninges (A, arrow) and preplate (A, arrowhead), but not in rest of the cortex or medial and lateral ganglionic eminences

(MGE and LGE). At E14.5, a small number of cortical cells (B, double arrows) below the marginal zone (B, arrowhead) start to express ZSGreen. At P0, many layer 6 cells of frontal cortex express ZSGreen (C, arrow), but not in more caudal neocortex (D, “Ncx”), CA1, CA3 and hilus regions of the hippocampus (E, note that strong signal is found in the meninges of the hippocampus) or the amygdala (F, “Amy”). ic, internal capsule. Scale bar, 200μm

Fig.S4 Generation of *Cyp26b1* cko mice

In situ hybridization of frontal sections of P8 (A,B,D-F, G,H, J-L) or P14 (C,I) *Cyp26b1* conditional knockout (G-L) and control littermate (A-F) brains. *Cyp26b1* was conditionally knocked out using the *Syt6-Cre* transgene. *Syt6* is expressed in layer 6 of both control (A) and *Cyp26b1* knockout (G) brain (arrow). Expression of *Cyp26b1* in layer 6 of frontal cortex (arrow) is detected in control, but not in *Cyp26b1* knockout brain at P8 (B,H) and P14 (C,I). Expression of *Cyp26b1* in agranular insula is unchanged in *Cyp26b1* knockouts (arrowhead in B,C,H,I). Expression of *Cyp26b1* in CA3 and hilus region of the hippocampus (D,J, arrowhead), globus pallidus (E,K, arrow) and amygdala (F,L, arrowhead) is unchanged in *Cyp26b1* knockouts. Scale bar, 1mm.

Fig.S5 No significant changes in the number of *Sst-*, *Vip-* and *Lhx6-*expressing interneurons in medial PFC of *Cyp26b1* knockout mice

A-F. In situ hybridization of frontal sections of P14 *Cyp26b1* conditional knockout mice (D-F) and littermate controls (A-C). Expression of *Somatostatin (Sst)* (A,D), *Vip* (B,E) and *Lhx6* (C,F) is shown. Binning and cell counts were done in the same was as shown

in Fig.4 for *Pvalb*-expressing cells. Scale bar, 1mm. **G-I.** Result of statistical analysis. Each line connecting red and blue dots represents a pair of brains analyzed in the same experiment (n=5). P-values of paired t-test for individual layer are shown. **J,K.** At P21, density of *Pvalb*-expressing cells (J), but not *Sst*-expressing cells, is increased in *Cyp26b1* conditional knockout mice. In repeated measures two-way ANOVA, the p-values for layer (superficial vs deep), pair (between control and knockout), and interactions (between layer and pair) are 0.0047, 0.0287 and 0.0637 (*Pvalb*); 0.0065, 0.3621 and 0.7609 (*Sst*), **L-N.** No significant changes in the number of *Pvalb*-, *Sst*- and *Vip*-expressing interneurons in medial PFC of adult (P56-P67) *Cyp26b1* knockout mice. Each line connecting red and blue dots represents a pair of brains analyzed in the same experiment (n=4). P-values of paired t-test for individual layer are shown.

Fig.S6: Thalamus-PFC disconnection in *Gbx2* mutant mice

A, B. NetrinG1 immunostaining at E16.5. In control mice, NetrinG1-labeled thalamocortical axons are visible in coronal sections of frontal cortex. Arrowhead in A shows the medial PFC, where robust labeling is detected. In contrast, NetrinG1-labeling is undetectable in the frontal cortex of *Gbx2* mutant mice, including the medial PFC (arrowhead in B). scale bar, 200μm.

C-F. DiI labeling at P14. DiI placement in medial PFC retrogradely labels medial thalamic nuclei (show more details) in the control brains (**C,D**). In *Gbx2* mutants, the label is severely reduced (**E,F**), indicating the deficiency of both thalamocortical and corticothalamic projections.

Fig.S7 Expression of *RORβ* and *Lmo4* are qualitatively normal in the PFC of *Gbx2* mutant mice at postnatal day 8 (P8).

A,B. Expression of *RORβ* in layer 4 is comparable between control and *Gbx2* mutant (cko) mice (arrows). **C,D.** Laminar expression patterns of *Lmo4* also appear unchanged in *Gbx2* mutants. scale bar=1mm.

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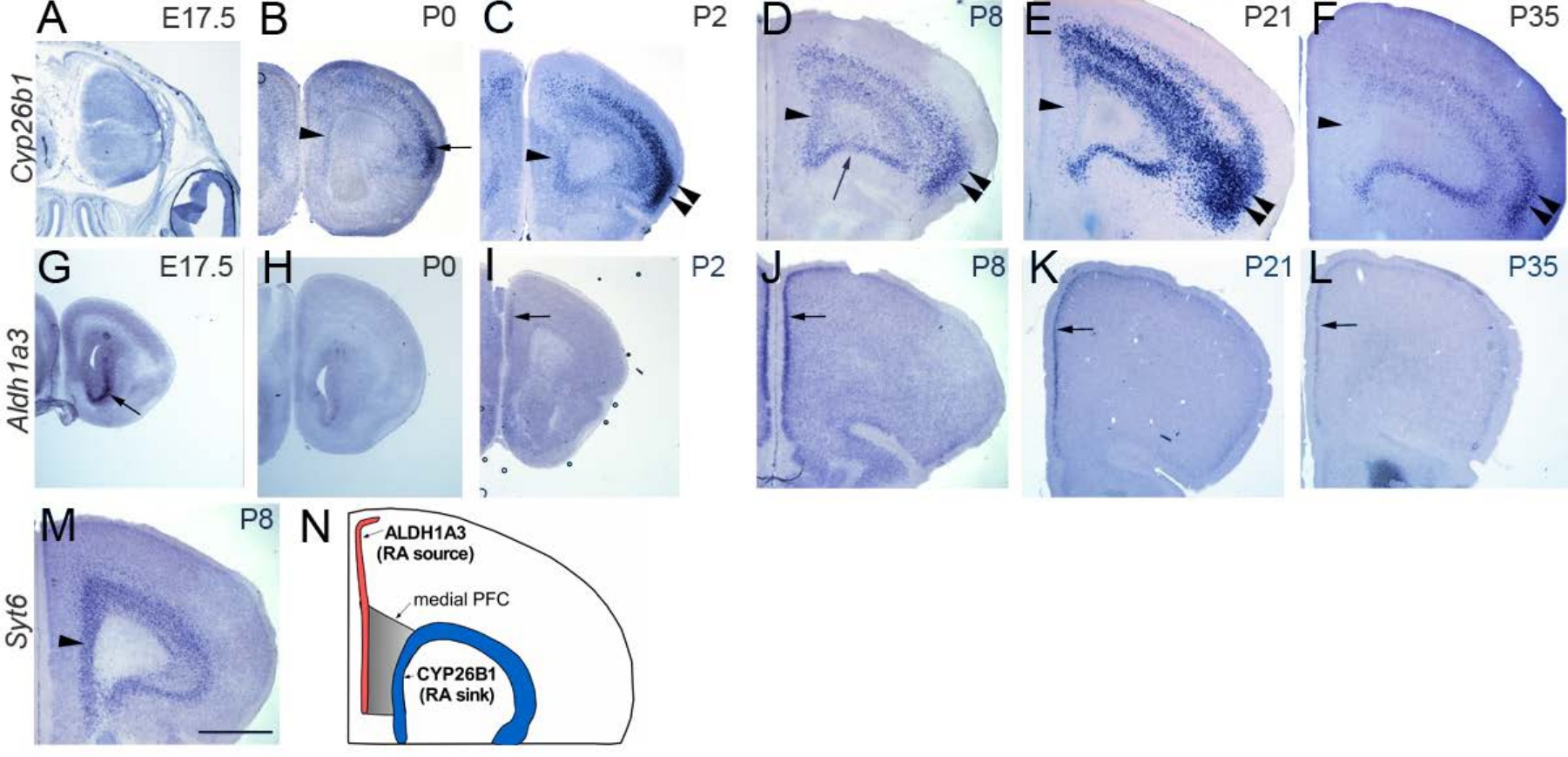

Fig.1 Spatiotemporally regulated expression of *Cyp26b1* and *Aldh1a3* in the PFC

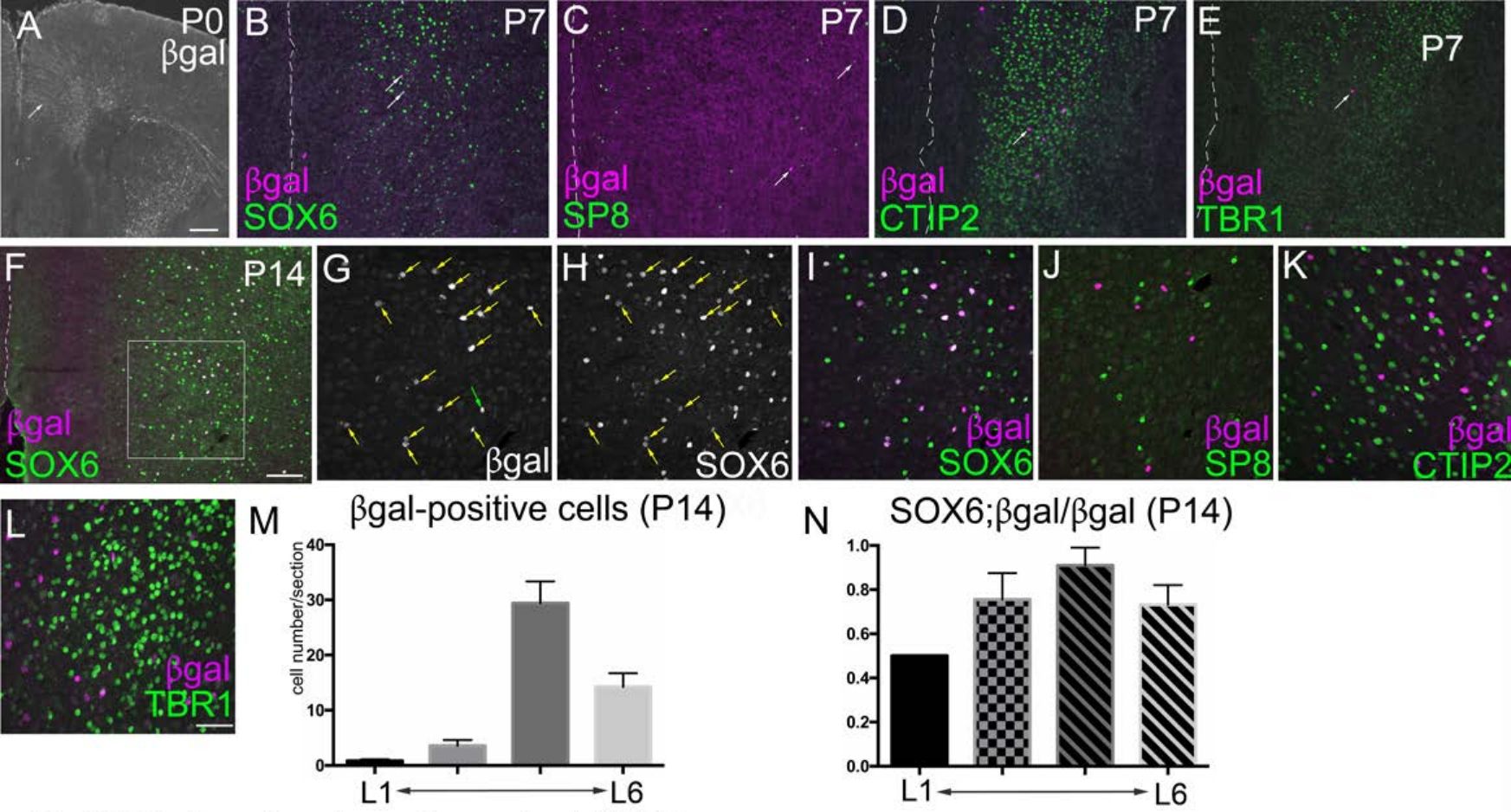

Fig.2 RA signaling in early postnatal PFC

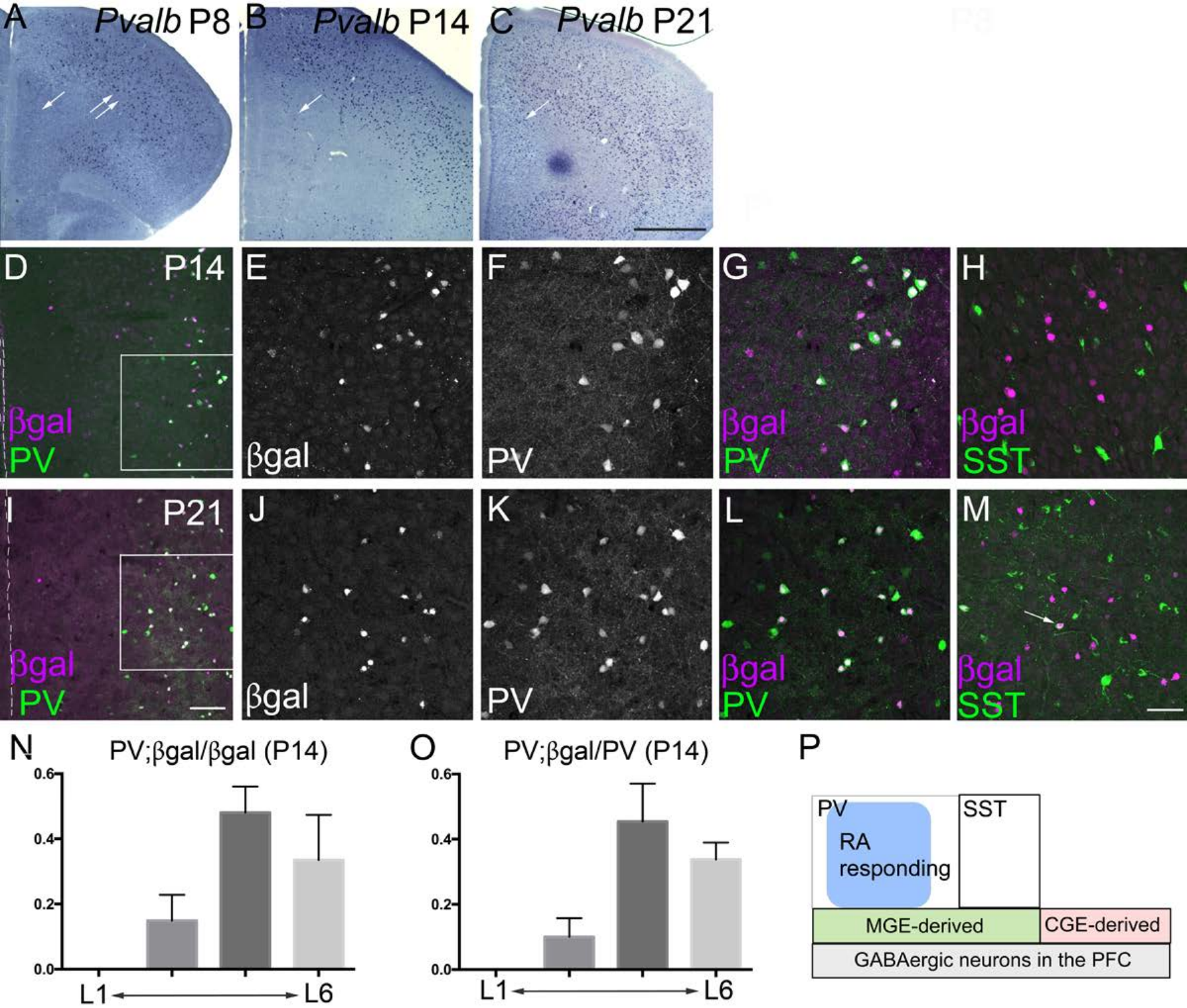

Fig.3 Overlapping expression of RARE-LacZ transgene and PV in early postnatal PFC

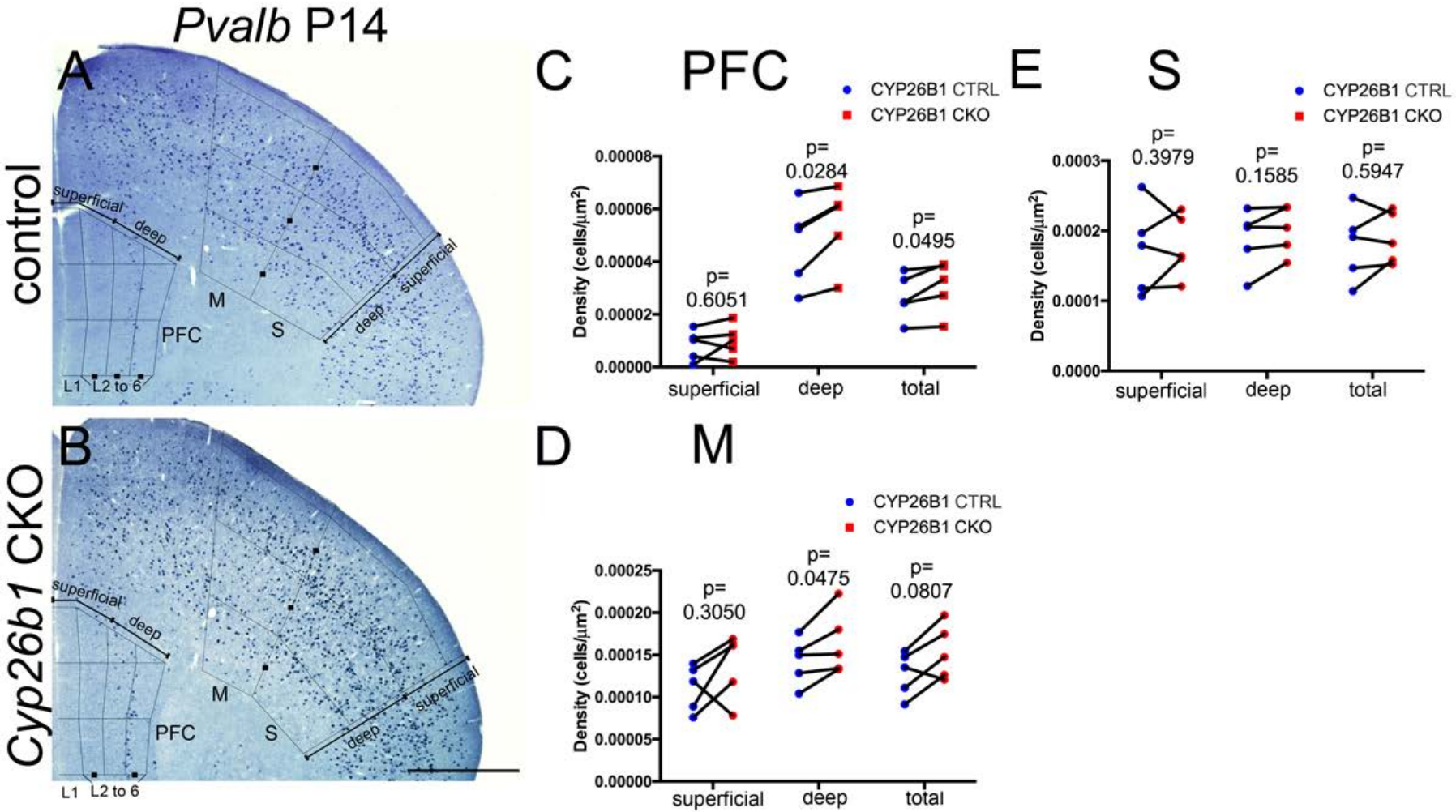

Fig.4 Increased *Pvalb*-expressing interneurons in medial PFC of *Cyp26b1* knockout mice

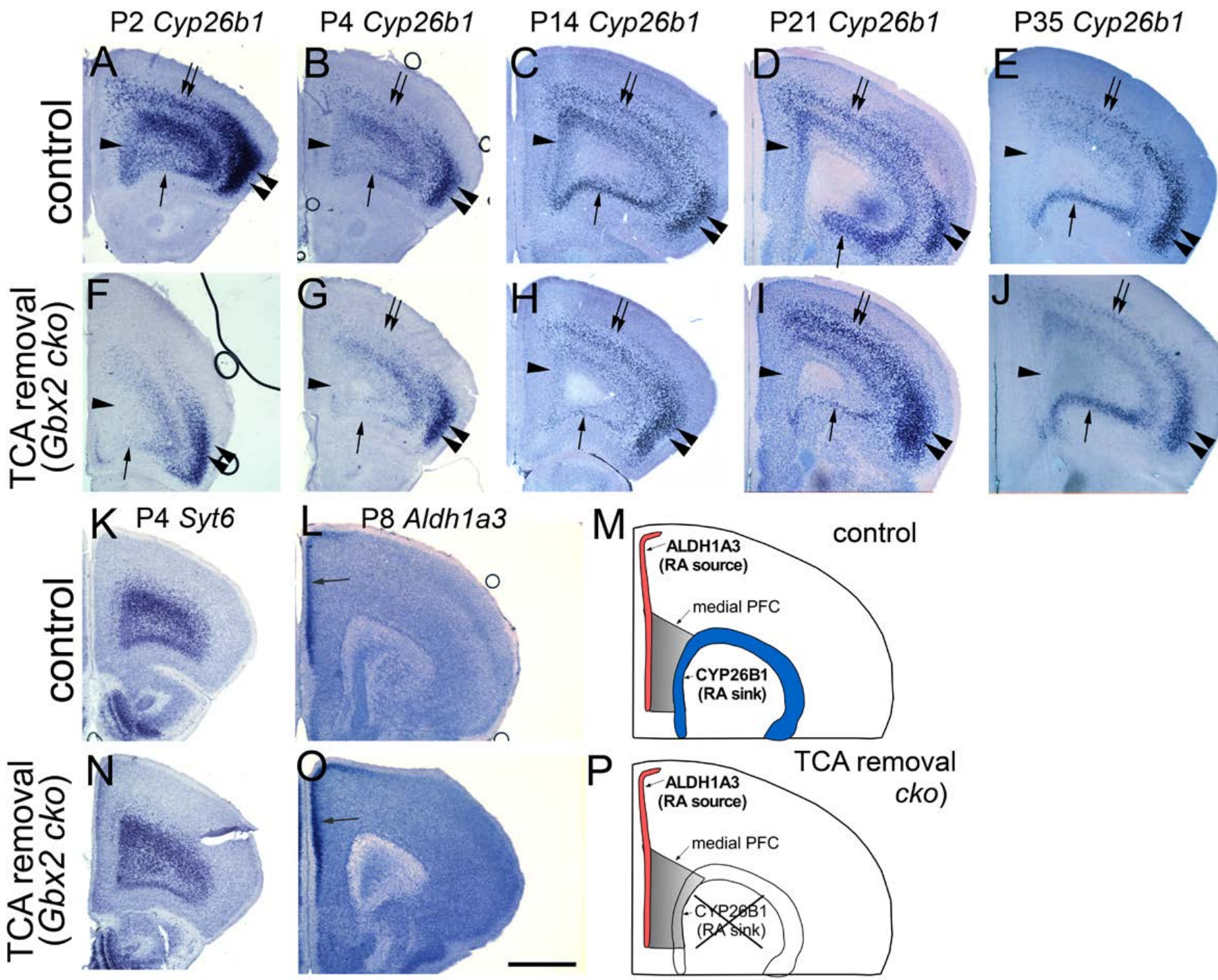

Fig.5 Transient expression of *Cyp26b1* in the PFC does not occur in the absence of thalamus -cortex interactions in *Gbx2* mutant mice.

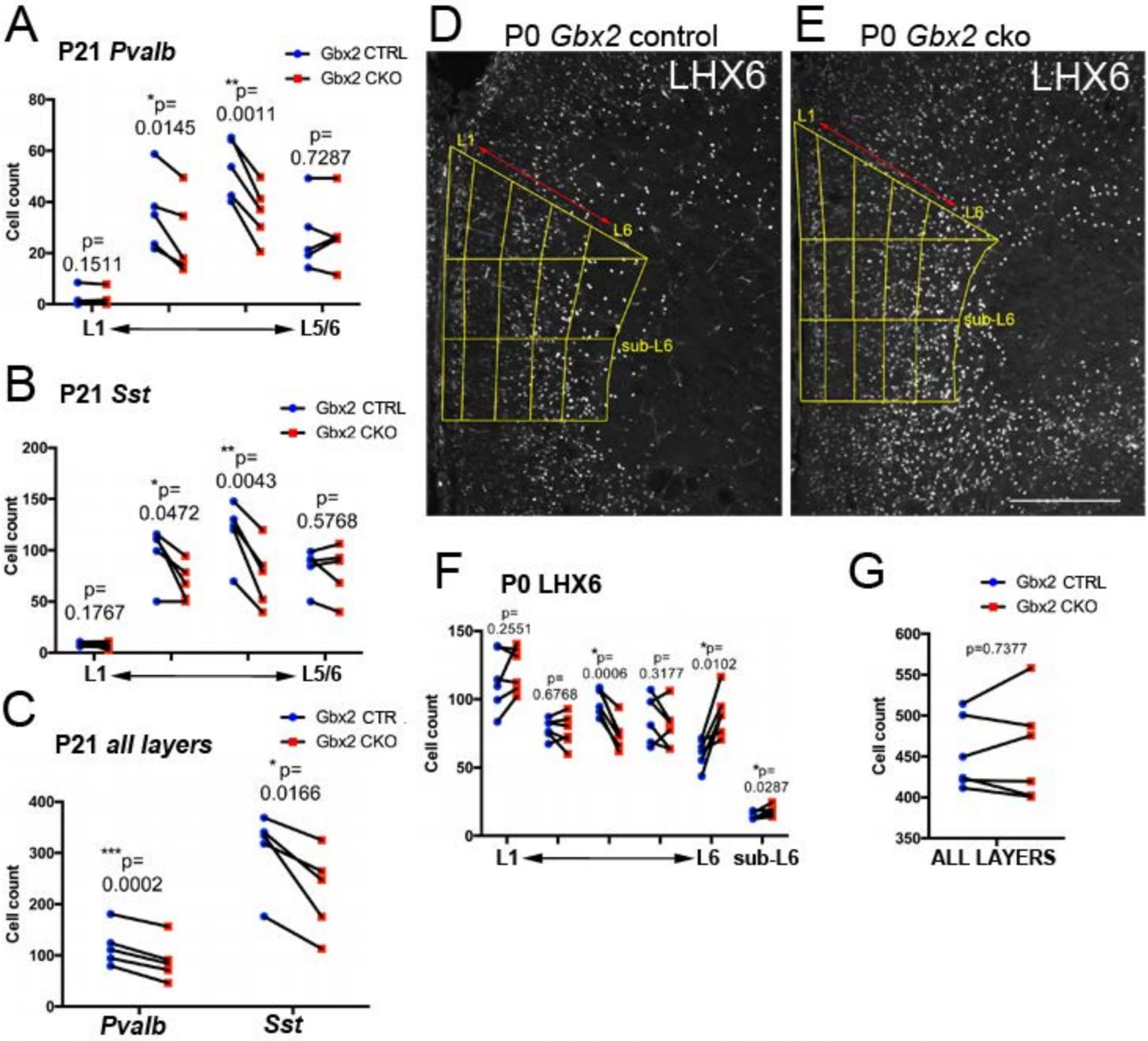

Fig.6 Abnormal numbers and radial distribution of MGE-derived interneurons in the medial PFC of P21 and neonatal *Gbx2* mutant mice

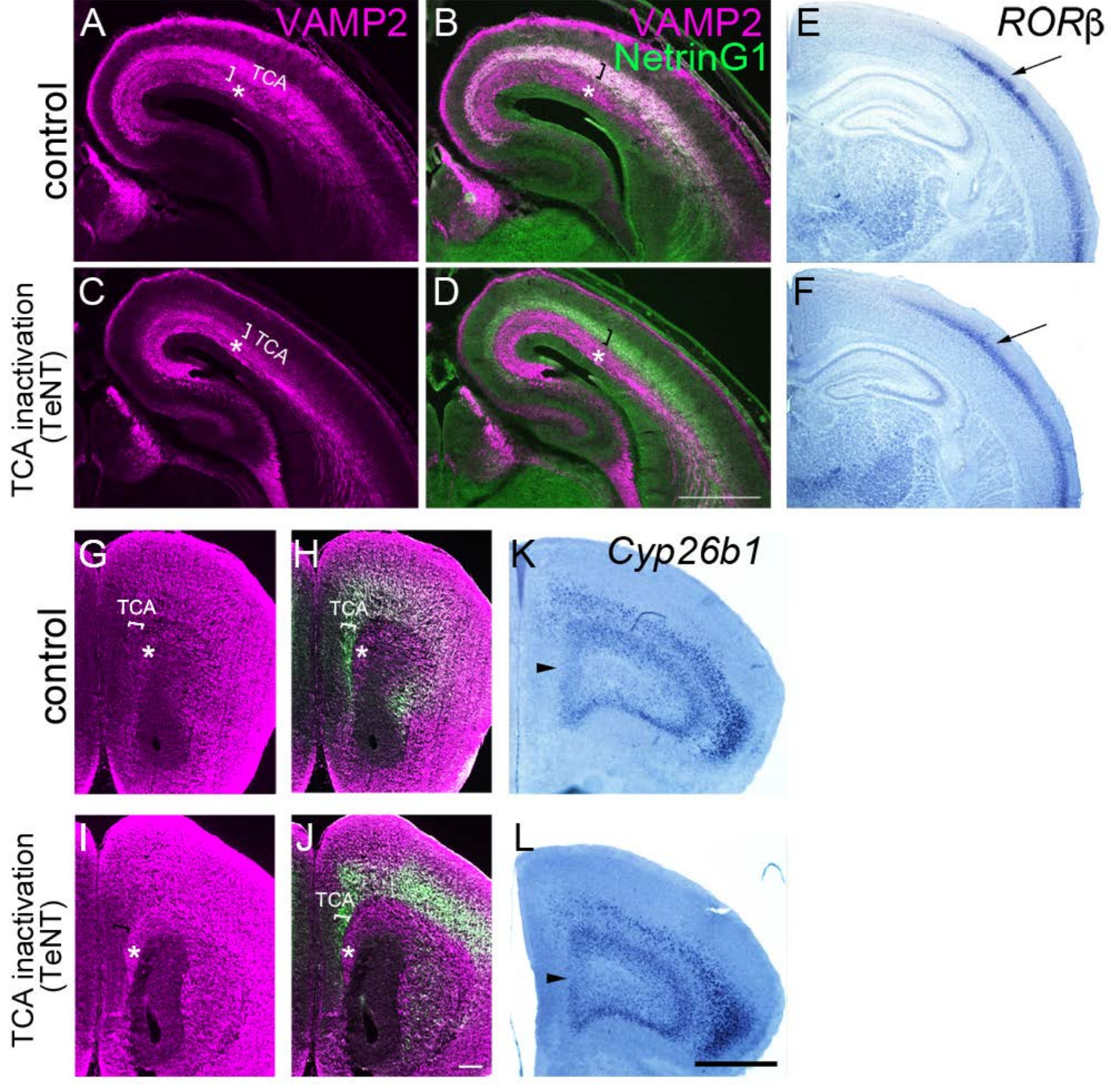

Fig.7 Normal induction of *Cyp26b1* in PFC in mice expressing tetanus toxin light chain in thalamocortical axons

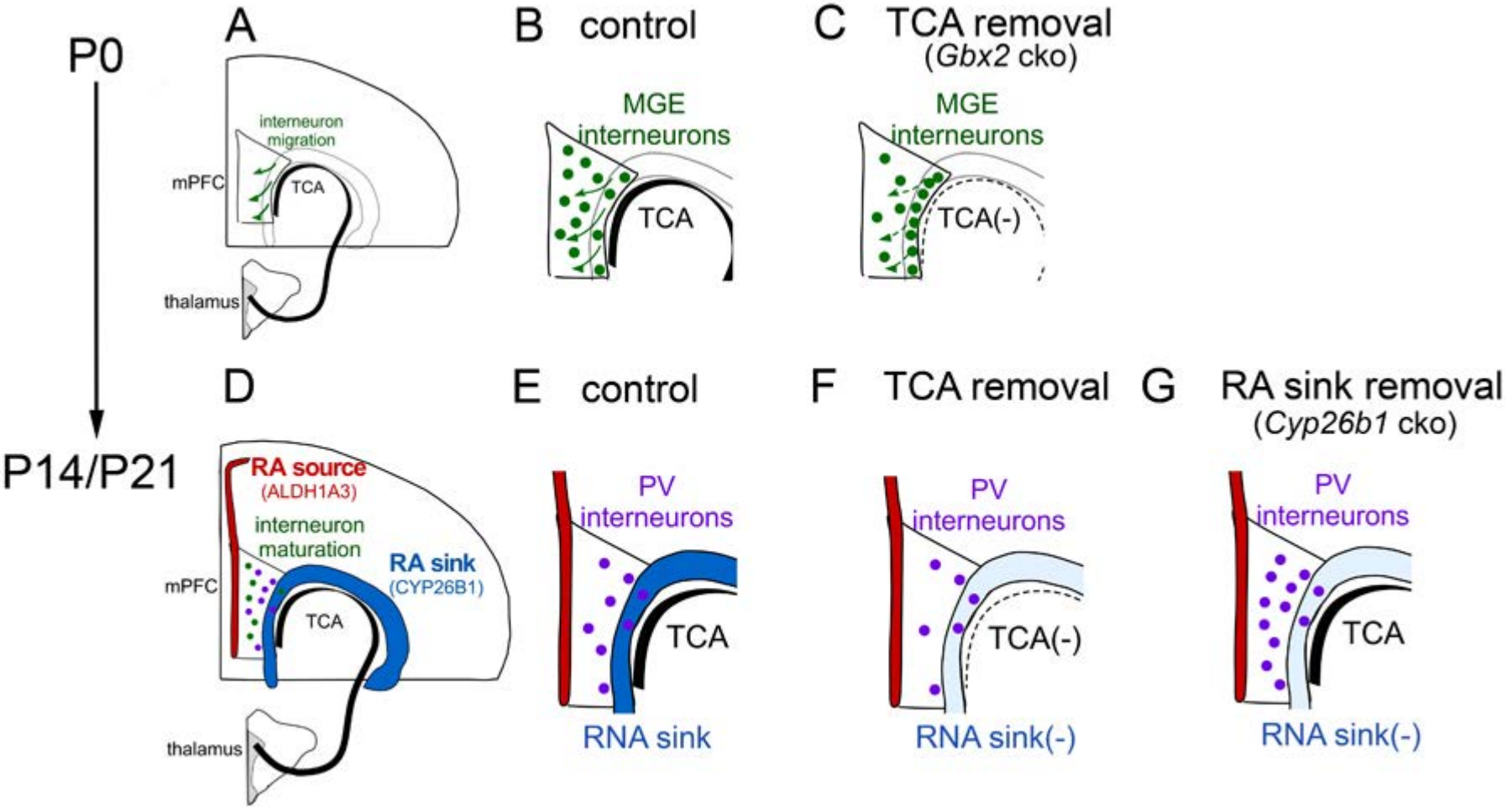

Fig.8 Schematic diagrams of the current finding