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# The Wnt receptor Ryk controls specification of GABAergic neurons versus oligodendrocytes during telencephalon development

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## SUMMARY

GABAergic neurons and oligodendrocytes originate from progenitors within the ventral telencephalon. However, the molecular mechanisms that control neuron-glial cell-fate segregation, especially how extrinsic factors regulate cell-fate changes, are poorly understood. We have discovered that the Wnt receptor Ryk promotes GABAergic neuron production while repressing oligodendrocyte formation in the ventral telencephalon. We demonstrate that Ryk controls the cell-fate switch by negatively regulating expression of the intrinsic oligodendrogenic factor Olig2 while inducing expression of the interneuron fate determinant Dlx2. In addition, we demonstrate that Ryk is required for GABAergic neuron induction and oligodendrogenesis inhibition caused by Wnt3a stimulation. Furthermore, we showed that the cleaved intracellular domain of Ryk is sufficient to regulate the cell-fate switch by regulating the expression of intrinsic cell-fate determinants. These results identify Ryk as a multi-functional receptor that is able to transduce extrinsic cues into progenitor cells, promote GABAergic neuron formation, and inhibit oligodendrogenesis during ventral embryonic brain development.

**KEY WORDS: Ryk, Oligodendrocyte, GABAergic neuron, Mouse**

## INTRODUCTION

Multipotent neural progenitor cells (NPCs) located in the ventral ventricular zone (VZ) of the telencephalon follow complex differentiation paths. Two distinct types of neural cells, the inhibitory GABAergic neurons and the oligodendrocytes, that eventually help populate the dorsal telencephalon, arise from this spatially, temporally and molecularly delimited 'common' pool of NPCs (Kessaris et al., 2006; Le Bras et al., 2005; Miller, 2002; Wonders and Anderson, 2006; Yue et al., 2006). Generation of these different cell types in proper ratios and at correct times is important for normal development of the telencephalon. Both extrinsic and intrinsic molecular regulators combine to assign uncommitted progenitor cells to specific neural lineages and facilitate their differentiation. Major progress has been made in understanding the intrinsic transcriptional regulators of cell-fate determination. However, how these intrinsic factors are integrated with extrinsic signals at the progenitor cell level to regulate the spatiotemporal pattern of neuro/gliogenesis is less well understood.

Mouse embryonic neurogenesis begins around E8.5 and peaks around E14.5 (Bayer and Altman, 1991). GABAergic neurons originate from the subpallium, including the medial ganglionic eminence (MGE), the lateral ganglionic eminence (LGE) and the anterior entopeduncular area (AEP) (Wonders and Anderson, 2006). Postmitotic GABAergic neurons migrate tangentially to the cortex and the basal ganglia, and then integrate into the cortical projection neuron network, eventually maturing postnatally (Wonders and Anderson, 2006). Oligodendrocyte development in the mouse telencephalon occurs in three distinct waves. Oligodendrocyte precursor cells (OPCs) of the first wave appear in the MGE and AEP at around E12.5 (Kessaris et al., 2006; Olivier et al., 2001; Pringle and Richardson, 1993; Spassky et al., 2001; Tekki-Kessaris et al., 2001), arrive in the cortex at around E16, and mature into myelinating oligodendrocytes postnatally (Richardson et al., 2006). A second wave of OPCs originates from the LGE and/or caudal ganglionic eminences at around E14.5 (Kessaris et al., 2006). Third, OPCs have also been observed to emerge in the dorsal telencephalon during postnatal stages (Gorski et al., 2002; Kessaris et al., 2006; Yue et al., 2006). Compared with dorsal telencephalic NPCs, which have been intensely studied, little is known about the regulation of NPC differentiation in the ventral telencephalon and the neuron-versus-glia fate decisions made in the ventral forebrain.

β-Catenin-mediated canonical Wnt signaling has traditionally been considered to be crucial for the development of the dorsal telencephalon, including dorsal NPC proliferation, patterning and neuronal differentiation (Galceran et al., 2000; Grove et al., 1998; Hirabayashi et al., 2004; Israsena et al., 2004; Lee et al., 2000). Recent discoveries show that β-catenin-dependent Wnt signaling is activated in the ventral telencephalon (Gulacsi and Anderson, 2008; Shimizu et al., 2005; Ye et al., 2009) and is associated with ventral tissue growth (Gulacsi and Anderson, 2008). Moreover, β-catenin-dependent Wnt signaling has recently been shown to play a crucial inhibitory role in specification and maturation of oligodendrocytes in the telencephalon (Fancy et al., 2009; Ye et al., 2009). Thus, a more complex role for Wnt signaling in growth and differentiation of ventral NPCs is beginning to be appreciated. However, questions that include whether Wnt signaling is involved

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in ventral neuronal fate specification, whether β-catenin-independent Wnt signaling is involved in the development of the ventral telencephalon and whether Wnt signaling acts on different lineages concurrently at the progenitor level remain unanswered.

Discovery of the Wnt receptor Ryk have revealed further complexities to the Wnt signaling network (Inoue et al., 2004; Lu et al., 2004). Ryk has been shown to have various functions in the CNS; for example, Ryk is required for Wnt3a-induced neurite outgrowth of dorsal root ganglion neurons (Lu et al., 2004), for Wnt5a-induced axon outgrowth (Li et al., 2009), for repulsion of cortical axon guidance by Wnt5a (Keeble et al., 2006), for Wnt3-mediated repulsive axon guidance (Schmitt et al., 2006) and for Wnt3-induced neuronal differentiation of dorsal telencephalic NPCs (Lyu et al., 2008).

Immunohistochemical analysis showed that Ryk is expressed in Nestin-positive progenitors, in neurons and in oligodendrocyte progenitors in the rodent central nervous system (Kamitori et al., 2002; Lyu et al., 2008). These data strongly indicate that Wnt signaling acts through Ryk receptors expressed on NPCs to regulate NPC differentiation; however, it is not known whether regulating neuronal differentiation is the only function of Ryk-mediated Wnt signaling in the developing brain.

Here, we report that during development of the ventral telencephalon, Ryk receptor regulates cell-fate choice between the oligodendrocytic and GABAergic neuronal differentiation pathways in ventral progenitor cells. Ryk does this through a cell-autonomous mechanism by regulating the expression of the key cell-fate determinants Dlx2 and Olig2. We further demonstrate that Ryk receptor is responsible for Wnt3a-induced promotion of GABAergic neuronal differentiation and inhibition of oligodendrocyte differentiation. Ryk intracellular domain (ICD) is required and sufficient to suppress oligodendrocyte differentiation and induce GABAergic neuronal differentiation, suggesting that nuclear signaling of Ryk-ICD governs the neuronal versus oligodendroglial differentiation of NPCs.

## MATERIALS AND METHODS

### Immunohistochemistry and immunocytochemistry

For immunohistochemistry, embryonic brains were dissected and fixed in 4% parafomaldehyde (PFA) at 4°C and cryoprotected in 30% sucrose. Samples were embedded and frozen in Tissue Tek OCT compound, and sections were then cut at 12 μm. For immunocytochemistry, cell cultures were fixed with 4% PFA in PBS for 15 minutes and immunostained after permeabilization in 0.2% Triton X-100. Immunostaining for membrane proteins was performed without prior permeabilization.

The following antibodies were used: anti-β-galactosidase (1:1000, Abcam); anti-nestin (1:400, BD); anti-tubulin III (1:1000, Sigma-Aldrich); anti-glial fibrillary acidic protein (GFAP) (1:1000, Sigma-Aldrich); anti-Olig2 (1:400, Chemicon); anti-NG2 (1:400, Millipore); anti-PDGFRα receptor (1:300, Santa Cruz); anti-O4 (1:200, Chemicon); anti-galactosidase-C (GAL-C) (1:400, Sigma-Aldrich); anti-myelin basic protein (MBP) (1:400, Abcam); anti-Nkx2.1 (1:300, Millipore); anti-Islet1 (1:100, Hybridoma Bank); anti-Shh (1:1000, Hybridoma Bank); anti-Dlx2 (1:1000); anti-Lhx6 (1:1000, a kind gift from Dr Vassilis Pachinis, NIMR, London, UK); anti-GABA (1:3000, Sigma); anti-Gad67 (1:1000, Millipore); anti-calbindin (1:1000, Swant); Gsh2 antibody (1:2000, a kind gift from K. Campbell, University of Cincinnati, OH, USA). For BrdU labeling, cells were treated with 2 N HCl for 30 minutes and detected by using mouse anti-BrdU (1:50, Becton Dickinson) or rat anti-BrdU (1:350, Accuratech) antibody. TUNEL assays were performed using the In Situ Cell Death Detection Kit (Roche) following the manufacturer's instructions. Immunolabeled cells were detected by using fluorescently-labeled secondary antibodies (1:300, Jackson ImmunoResearch Laboratories). Viable cells were identified using Hoechst nuclear staining.

### Neural progenitor cell (NPC) culture

NPCs were derived from E14.5 mouse telencephalons. Tissues were dissected and dissociated as described previously (Reynolds and Weiss, 1996; Schmitt et al., 2006). Cells were cultured in serum-free Neurobasal medium (Invitrogen) supplemented with mouse recombinant epidermal growth factor (20 ng/ml, Sigma-Aldrich), basic fibroblast growth factor (20 ng/ml, R&D), B27 (Invitrogen), 1% penicillin-streptomycin (Sigma-Aldrich), 1% L-glutamine and 1% glutamax in T25 culture flasks (Nunc) in a humidified 5% $CO_2$/95% air incubator at 37°C. For differentiation, neurospheres were dissociated into single cells and plated onto poly-L-lysine/laminin-coated chamber slides (Nunc) at ~$10^4$ cells per well in the presence of mitogen. After 1 day, medium was changed to NPC medium without mitogen. After several additional days, cells were analyzed by immunocytochemistry.

### Clonal assay

In the clonal assay, wild-type and knockout NPCs were mechanically dissociated into single cells and grown as clonal colonies in the presence of 0.9% (w/v) methylcellulose matrix under proliferation conditions for 1 week. Individual neurospheres were then picked and transferred to 24-well plates (one neurosphere per well) and cultured for another week. These colonies were collected and plated onto plastic chambers. Subsequently, differentiation was carried out as described above. Six days later, differentiation of neurons and glia of individual clones were examined by immunocytochemistry. More than 100 clones were examined.

### Animals

All mice were maintained and handled according to the guidelines and regulations of the Institutional Animal Care and Use Committee and the National Institutes of Health. Maintenance and genotyping of $Ryk^{+/-}$ and $Ryk^{-/-}$mice were carried out as described previously (Halford et al., 2000). Embryos and pups of both wild-type and knockout mice were collected from timed, mated pregnant females.

### BrdU labeling

For in vivo BrdU labeling, time-mated pregnant females were injected intraperitoneally with 50 μg per g body weight of 5-bromo-2′-deoxyuridine (BrdU) (Sigma). Mice were culled 30 minutes later and embryos were collected, fixed in 4% parafomaldehyde and embedded in OCT compound for sectioning. Sections were stained with anti-BrdU antibody. For in vitro BrdU labeling, dissociated NPCs were plated onto coated slides in the presence of mitogen. Twenty-four hours later, mitogen was removed by changing the medium, and cells were treated with BrdU (10 μM) for 3 hours and cultured for an additional 2 days to allow NPC differentiation.

### Quantitative RT-PCR

Total RNA was isolated using the RNeasy Mini Kit (Qiagen). cDNA synthesis was carried out with 500 ng of total RNA using the SuperScript III qRT-PCR kit (Invitrogen). Real-time quantitative RT-PCR (qRT-PCR) was performed with an ABI PRISM 7900 Sequence Detection System using SYBR Green Master Mix (ABI). Samples were run in triplicate and relative levels of each mRNA were examined by comparing Cycle Threshold (Ct) values for each reaction among samples using the *ACTB* gene as a reference. Relative mRNA expression levels were measured from at least three independent experiments.

### Statistical analysis

Quantification was obtained from more than three individual experimental groups. *P* values were determined by Student's two-tailed *t*-test between groups.

## RESULTS

### Ryk is highly expressed in NPCs in the MGE and LGE of the developing brain

We investigated the expression of Ryk in NPCs in vivo by performing immunohistochemistry on coronal sections of brains from wild-type, heterozygous and homozygous Ryk knockout mice at various embryonic stages. In the Ryk knockout allele, part of the

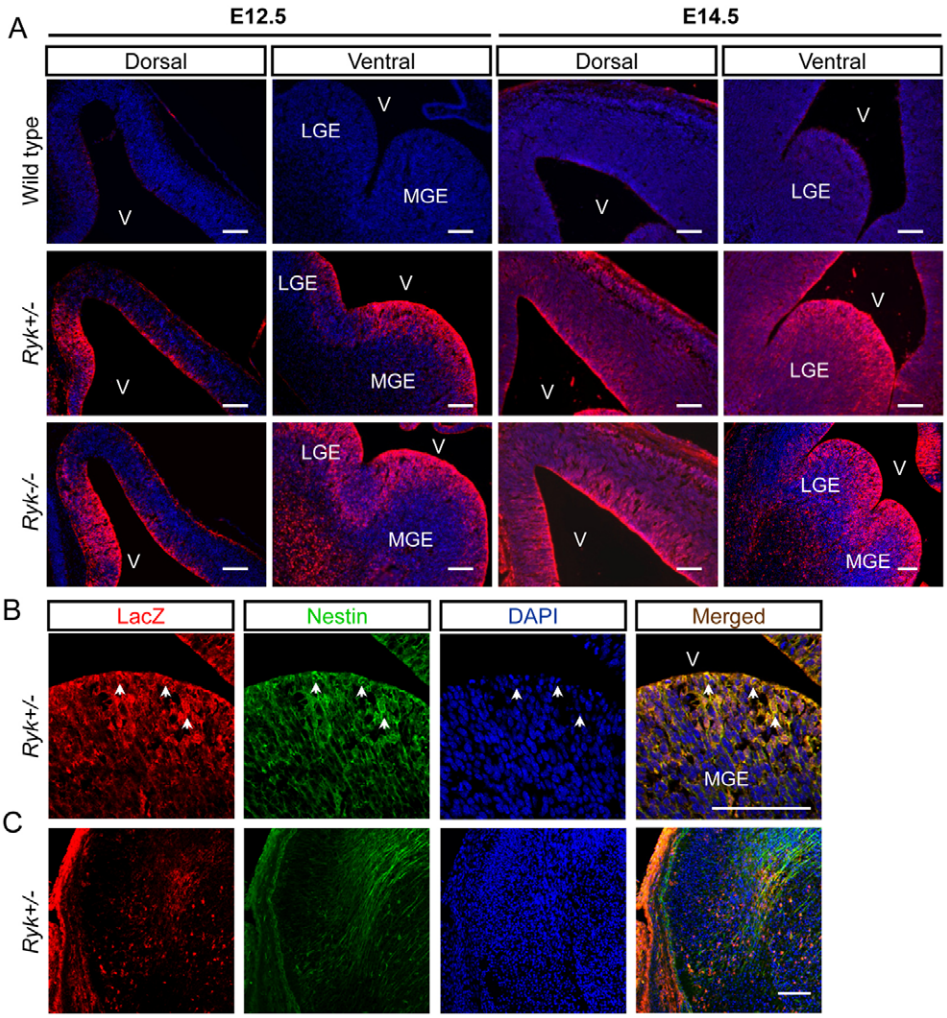



**Fig. 1. Ryk expression in embryonic mouse brain.** (**A**) β-Galactosidase (*lacZ*) staining of coronal sections of E12.5 and E14.5 wild-type, heterozygous and knockout mouse brains. Nuclei are stained with DAPI. V, ventricular zone; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence. (**B**) Co-immunostaining of *lacZ* and Nestin plus DAPI staining in E14.5 heterozygous brain in the ventricular zone of the LGE region indicates Ryk and Nestin co-expression (arrowheads). (**C**) Co-immunostaining of *lacZ* and Nestin plus DAPI staining of E14.5 heterozygous brains in the mantle zone of LGE indicates that Ryk is expressed by Nestin-negative differentiated cells. Scale bars: 100 μm.

Ryk gene from exon 2 to exon 6 was replaced by an expression cassette *lacZ* gene; thereby, the endogenous Ryk promoter drives expression of *lacZ*, allowing identification of Ryk-expressing cells (Halford et al., 2000). *lacZ* staining and, by inference, Ryk expression in the mouse telencephalon is detectable at E10.5 (data not shown), peaks at E12.5 and is sustained until E14.5 (Fig. 1A); it then decreases at around E16.5 and is barely detectable by E18.5 (see Fig. S1 in the supplementary material). Staining at E12.5 and E14.5 indicates that Ryk is highly expressed in cells in the VZ, in both the dorsal and ventral regions (Fig. 1A). Particularly high expression was detected in the MGE and LGE from E12.5 to E14.5 (Fig. 1A). Ryk was also expressed in the cortical plate, which consists mainly of newly differentiating neurons at E14.5 (Fig. 1A), indicating that Ryk is expressed in both proliferating mitotic and postmitotic cells at this stage. *lacZ* expression in the VZ of the MGE and LGE showed colocalization with Nestin (Fig. 1B), a marker of NPCs, suggesting that Ryk is expressed by the primitive ventral progenitor pool. Nestin expression was equivalent between heterozygous and homozygous knockout mice at E14.5 (data not shown). *lacZ* is also expressed in Nestin-negative cells located in the subventricular zone (SVZ) and mantle zone (Fig. 1C), suggesting that Ryk is expressed in a differentiated cell population derived from ventral VZ progenitor cells. The finding of Ryk expression in ventral NPCs raised the possibility that Ryk participates in the development of ventrally derived cell lineages, including oligodendrocytes and GABAergic neurons.

## Ryk knockout mice exhibit increased oligodendrogenesis

By observing the patterns of Ryk expression, we proceeded to test whether Ryk mutants have any defects in oligodendrogenesis. Using immunohistochemistry analysis of OPC markers, we found that Ryk knockout mice exhibited increased numbers of OPCs and pro-oligodendrocytes in the forebrain throughout embryonic development. Staining for PDGFRα, one of the earliest OPC markers, in E12.5 brain sections revealed significantly more PDGFRα+ cells in the mantle zone of the MGE of knockout mice than of wild-type mice (Fig. 2A,B). Analysis of Olig2 staining showed that Olig2 expression was enhanced in the MGE of the mutant (Fig. 2A). At E18.5, 2.5-fold more PDGFRα+ cells were seen in both the cortex and striatum of knockout mice compared with wild-type controls (Fig. 2C,D). At E18.5, staining of NG2, another OPC marker, showed a twofold increase in the number of NG2+ cells in the cortex of knockout mice compared with the control (Fig. 2C,D). Immunostaining of oligodendrocyte markers in P0 mouse brains showed that the numbers of cells expressing NG2, O4 and GalC (pro-oligodendrocyte markers) were all significantly increased in the cortex and striatum of knockout mice compared with wild type (Fig. 2E,F). Besides OPC markers, we also examined expression of the mature oligodendrocyte marker myelin basic protein (MBP), which begins to be expressed in myelinating oligodendrocytes 1 week after the P0 stage. No MBP staining was detected in either wild-type or knockout P0 mice (data not shown).

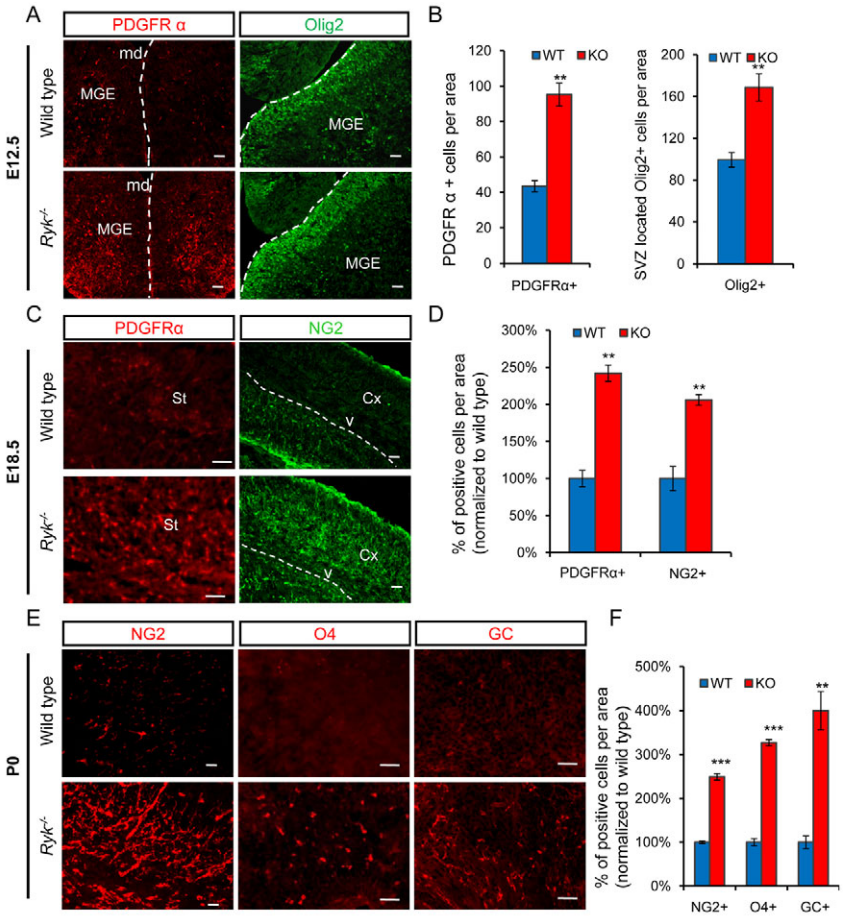



**Fig. 2. Ryk knockout mice exhibit increased oligodendrogenesis during brain development.** (**A**) PDGFRα and Olig2 immunostaining of the MGE region of E12.5 wild-type and Ryk knockout brains. Broken line indicates the VZ boundary. (**B**) Quantification of PDGFRα+ and SVZ-located Olig2+ cells per area. (**C**) PDGFRα immunostaining of striatal sections and NG2 immunostaining of cortical sections of E18.5 brains. v, ventricular zone; St, striatum; Cx, cortex. (**D**) Quantification of PDGFRα+ and NG2+ cells per area. (**E**) NG2, O4 and GC expression in P0 brains. (**F**) Quantification of NG2+, O4+ and GC+ cells per area in P0 brains. Scale bars: 50 μm. $^{**}P<0.01$, $^{***}P<0.001$. (B,D,F) Results are mean ± s.e.m.

We next investigated whether the increase in production of the first wave of OPCs observed in the MGE region of E12.5 Ryk knockout mice is caused by altered timing of differentiation. We examined the timing of the first wave oligodendrogenesis by performing PDGFRα staining on sections of E9.5, E10.5 and E11.5 wild-type and knockout samples. No PDGFRα+ cells were detected in wild-type or knockout E9.5 and E10.5 brains (see Fig. S2A,B in the supplementary material). The first detectable PDGFRα+ cells in both wild-type and knockout brains were found in E11.5 samples. Interestingly, the numbers of PDGFRα+ cells per section in knockout mice were double those in wild type (see Fig. S2C,D in the supplementary material). Therefore, our data suggest that the timing of MGE-derived oligodendrogenesis is not significantly changed, but production of first-wave OPCs is increased in the mutant.

Based on the fact that oligodendrogenesis occurs as three distinct waves, we examined whether the second wave of oligodendrogenesis originating in the LGE is affected in Ryk knockout mice. Immunostaining of Olig2 at E14.5 in the LGE region showed significantly more Olig2+ cells migrate out of the LGE VZ into the SVZ and mantle zone in the knockout mice compared with controls (see Fig. S3A in the supplementary material). We then proceeded to perform BrdU injection of 15.5-day pregnant mother mice and analyzed the OPCs differentiated from these labeled cells 2 days later. NG2 and BrdU double staining was carried out to identify OPCs that were labeled by BrdU at E15.5. Most NG2+/BrdU+ OPCs were detected in the septum area (data not shown). Interestingly, quantification of NG2+/BrdU+ cells showed a twofold increase in the knockout mice compared with wild-type controls, suggesting that more OPCs are being produced at E15.5 in the mutant (see Fig. S3B in the supplementary material). These results demonstrate that deletion of the Ryk gene causes increased numbers of OPCs to be generated in both the first and second waves of OPC production in the ventral telencephalon.

## Formation of GABAergic neurons is reduced in Ryk knockout mice

It has been shown using clonal analysis that OPCs and GABAergic neurons probably derive from a common ventral telencephalic progenitor (He et al., 2001; Yung et al., 2002). Given the substantial increase in oligodendrocyte numbers in Ryk knockout mice, we next examined whether production of GABAergic neurons is also affected in the mutant.

We analyzed neuron production in the ventral forebrain through immunostaining of the GABAergic neuronal markers GAD67 and GABA, and found a significant reduction in expression of GAD67 and GABA in the striatum and septum area of Ryk knockout mice at E14.5 compared with controls (Fig. 3A). Reduced numbers of migrating GABA+ cells were found in the piriform cortex of Ryk knockout mice (Fig. 3A). We also observed reduced expression of Dlx2 in the ventral MGE and LGE region of E14.5 Ryk knockout brains relative to controls (Fig. 3B). Dlx1 and Dlx2 (Dlx1/2) have been shown to play a crucial role in controlling neuro/oligodendrogenesis in the developing telencephalon (Parras et al., 2007; Petryniak et al., 2007): Dlx1/2 mutant mice showed a dramatic increase in OPC formation at the expense of GABAergic neuron differentiation. In Ryk knockout mice, expression of the

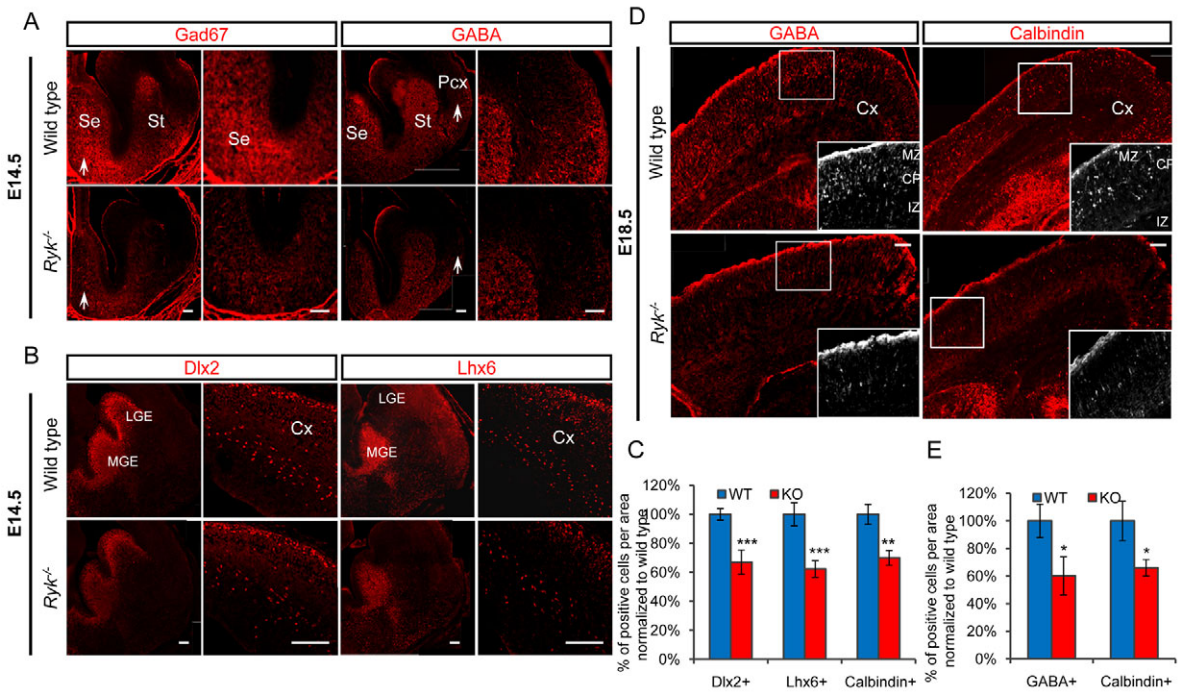



**Fig. 3. Impaired GABAergic neuron development in Ryk knockout mice.** (**A**) Immunostaining of GABAergic neuronal markers GAD67 and GABA in the ventral telencephalon of E14.5 wild-type and Ryk knockout mice. Areas indicated by arrowheads are enlarged on the right. Se, septum; St, striatum; Pcx, piriform cortex. (**B**) Immunostaining of Dlx2 and Lhx6 in the ventral telencephalon (left panels) and cortex (right panels) of E14.5 wild-type and Ryk knockout mice. (**C**) Histogram depicts the percentage of Dlx2+, Lhx6+ and calbindin+ cells in the knockout E14.5 cortex relative to wild-type cortex. (**D**) Immunostaining of GABA and calbindin in the cortex of E18.5 wild-type and Ryk knockout mice; white boxes indicate the insets. Cx, cortex; CP, cortical plate; MZ, marginal zone; IZ, intermediate zone. (**E**) Histogram depicts the percentage of GABA+ and calbindin+ cells in the knockout E18.5 cortex relative to wild-type cortex. Scale bars: 100 μm. *$P$<0.05, **$P$<0.01, ***$P$<0.001. (C,E) Results are mean ± s.e.m.

GABAergic neuronal marker Lhx6 was also found to be reduced (Fig. 3B). Moreover, the numbers of migrating Dlx2+, Lhx6+, calbindin+ (GABAergic neurononal marker) cells in the piriform cortex were also significantly reduced in Ryk knockout mice (Fig. 3B,C). As a consequence of impaired neuron formation at this early stage, labeling for GABA and calbindin revealed a significant reduction in the density of cortical GABAergic neurons in the cortex of Ryk knockout mice at E18.5 (Fig. 3D,E). GABAergic neurons were also found to be generated from the olfactory bulb (OB) and CGE region. We observed a moderate reduction in GABA+ cells located in the knockout OBs (see Fig. S4 in the supplementary material) but no significant change in the CGE region (data not shown). Thus, increased OPC formation occurs concomitantly with reduced GABAergic neuron formation in the ventral telencephalon of Ryk knockout mice. Our data suggest that Ryk promotes GABAergic neuron generation while repressing oligodendrogenesis by regulating Dlx2 expression.

## Dorsal-ventral patterning is preserved in Ryk knockout mice

The significant impact of Ryk gene deletion on generation of oligodendrocytes and GABAergic neurons raises the possibility that the dorsoventral (DV) patterning of progenitor domains is altered in Ryk mutant embryos. We first examined the expression of Shh, which is responsible for induction and specification of ventral tissue (Ericson et al., 1995). In Ryk knockout mice, no noticeable change was observed in either the level or spatial distribution pattern of Shh expression at E12.5 (see Fig. S5A in the supplementary material). Furthermore, we found that Pax6 and Gsh2 (Campbell, 2003) at E12.5 and E14.5 showed normal expression patterns in the dorsal VZ and ventral VZ in Ryk knockout mice (see Fig. S5B,C in the supplementary material), suggesting a normal position boundary between dorsal and ventral progenitors. The MGE marker Nkx2.1 and the LGE marker Islet1 (Campbell, 2003) both appeared to be expressed normally in Ryk knockout embryos at E12.5 and E14.5 compared with controls (see Fig. S5B,C in the supplementary material). Taken together, these results suggest that the increased production of OPCs and decreased neuron formation in Ryk mutant mice is not due to altered DV patterning of the telencephalon.

## Cell proliferation and cell survival of Ryk knockout mice are not affected

We next examined whether progenitor proliferation or cell survival is affected in Ryk mutant embryos. We first detected S-phase cells by administration of BrdU to 12.5-day pregnant mother mice 30 minutes before sacrifice. As expected, BrdU-labeled S-phase cells in the forebrain of E12.5 embryos were located basally in the VZ, whereas robust staining of Ki67, a marker of proliferating cells, was detected in the apical layer of the VZ (Fig. 4A). The number of BrdU-labeled S-phase cells and Ki67+ proliferating cells in the MGE was not visibly altered by the absence of Ryk (Fig. 4A,B).

To further examine proliferation versus differentiation, we conducted immunohistochemical staining of BrdU and PDGFRα. Most PDGFRα+ cells were BrdU-negative in both wild-type and knockout brains (Fig. 4C). Whereas the numbers of BrdU-labeled

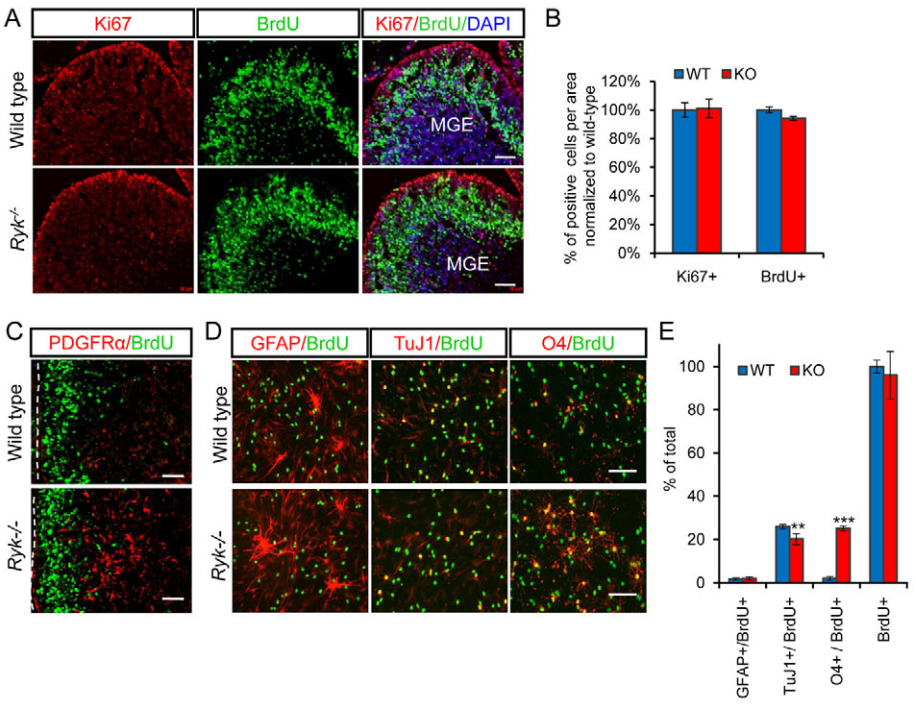


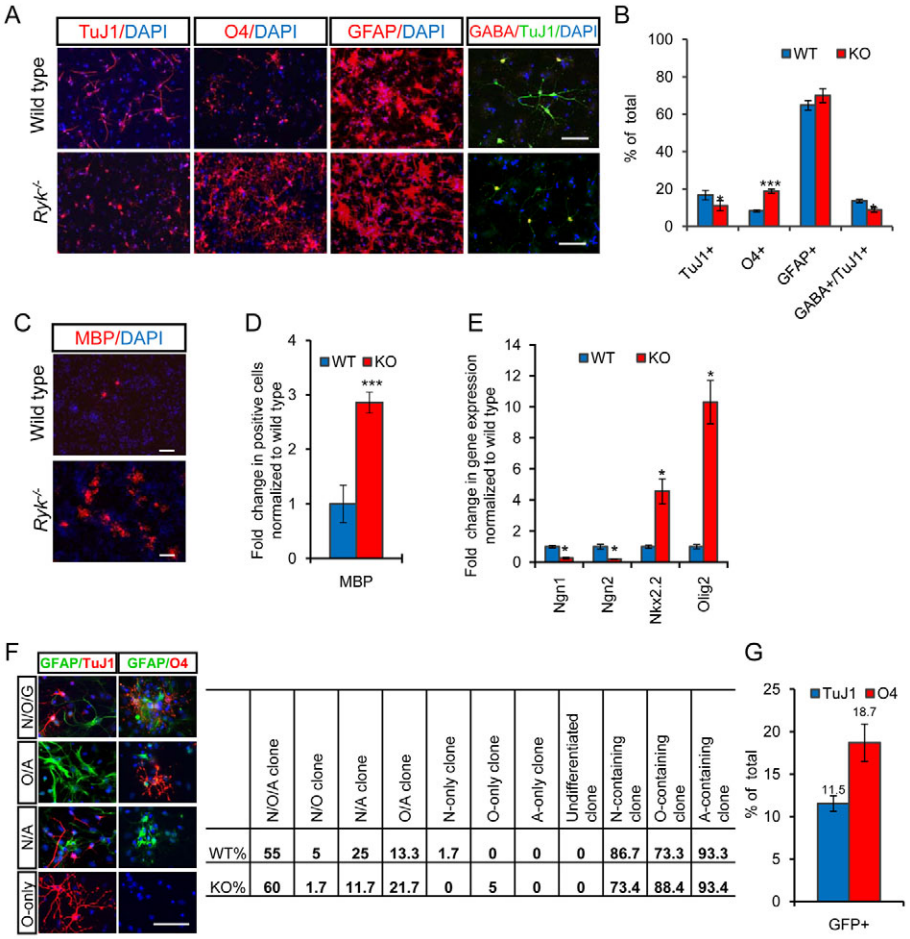

**Fig. 4. Enhanced oligodendrocyte differentiation without impaired proliferation in Ryk knockout mice.** (**A**) Co-immunostaining of Ki67 and BrdU plus DAPI staining in the MGE region of E12.5 brains. (**B**) Quantification of percentage of Ki67+ and BrdU+ cells in Ryk knockout mice relative to wild type. (**C**) Co-immunostaining of PDGFRα and BrdU in the MGE of E12.5 brains. Broken line is the VZ boundary. (**D**) Co-immunostaining of GFAP, TuJ1, O4 and BrdU in dissected telencephalic cells treated with BrdU for 3 hours and cultured under differentiation conditions for 2 days. (**E**) Quantification of the percentage of GFAP+/BrdU+, TuJ1+/BrdU+, O4+/BrdU+ cells and total BrdU+ cells ($n$=3). Scale bars: 50 μm. (B,E) Results are mean ± s.e.m. $^{**}P<0.01$, $^{***}P<0.001$.

proliferating cells were similar between wild-type and knockout mice, the number of PDGFRα+ oligodendrocyte precursors in the MGE was significantly increased in E12.5 Ryk knockout mouse embryos (Fig. 4C). As OPCs are mitotic cells, increased generation of OPCs in the MGE region can be due to enhanced proliferation of OPCs, we have performed double immunostaining of PH3 (phosphorylated histone 3, an M phase marker) and Olig2 at E12.5; however, the percentage of PH3+/Olig2+ cells in mantle located Olig2+ cells is not changed (data not shown), which suggested that OPC proliferation is probably not the cause of the increased OPCs induction. To further examine the role of Ryk role in regulating NPC differentiation rather than proliferation, we performed in vitro culture of primary NPCs. We isolated NPCs from both wild-type and mutant E14.5 telencephalon, plated them onto coated slides, treated the cells with BrdU for 3 hours after removing the mitogen, and cultured the cells under differentiation conditions for an additional 2 days. During the 3-hour BrdU treatment, newborn cells were labeled. The cell fates of these BrdU-labeled cells during differentiation were evaluated by staining for the lineage markers GFAP, TuJ1 and O4 (Fig. 4D). Whereas the percentage of GFAP+/BrdU+ cells among total BrdU+ cells was not changed in knockout cultures (Fig. 4E), the percentage of TuJ1+/BrdU+ cells was decreased and the proportion of O4+/BrdU+ cells was significantly increased (Fig. 4E), suggesting that, in the absence of Ryk, greater numbers of NPCs were fated to become oligodendrocytes rather than neuronal cells. Overall, our in vivo and in vitro results suggest that Ryk gene deletion affects differentiation instead of proliferation of NPCs in the ventral telencephalon.

We next employed TUNEL staining to assess apoptosis in the MGE at E12.5 in vivo. Few apoptotic cells were detected in both wild-type and knockout brains at this stage, and there was no significant difference between the two groups (see Fig. S6A in the supplementary material). Similar results were also found in the LGE region at E14.5 (data not shown). To rule out the possibility of different rates of apoptosis in neuronal versus oligodendroglial lineages, we performed TUNEL analysis in combination with staining of the lineage markers Nestin, GFAP, TuJ1 and O4 on differentiated cell cultures. The percentages of apoptotic cells showed no significant difference between the neural lineages (see Fig. S6B in the supplementary material).

## Ryk regulates NPC differentiation in a cell-autonomous fashion

We further examined the role of Ryk in cell-fate specification by using neurosphere culture. NPCs were isolated from the telencephalon of E14.5 wild-type and Ryk knockout mice, and maintained in proliferation conditions in the presence of epidermal growth factor (EGF) and basic fibroblast growth factor (FGF). Neurospheres from floating culture were then plated on slides and cultured under differentiation conditions. On differentiation day 2, most cells were Nestin+ in both wild-type and Ryk-knockout cell cultures, with no noticeable morphological differences between the two cultures (data not shown). However, by differentiation day 3, the numbers of NG2+ and Olig2+ cells were increased in knockout cell culture compared with wild-type controls by 40% (see Fig. S7A in the supplementary material). On differentiation day 6, the number of TuJ1+ cells was decreased in the Ryk knockout cell culture. Tuj1 is an early neuron marker. In addition, TuJ1+ cells exhibited an immature shape with shortened dendrites in the Ryk-knockout cell culture (Fig. 5A). Consistent with increases of NG2+ and Olig2+ cells, the number of O4+ cells was also significantly increased in Ryk-knockout cultures (Fig. 5A). However, the number and morphology of GFAP+ astrocytes were similar in Ryk-knockout and wild-type cultures (Fig. 5A). Quantitative analysis of cells of all three lineages (i.e. neurons, oligodendrocytes and astrocytes) on differentiation day 6 confirmed the visual observations (Fig. 5B). We also examined the expression of the mature-oligodendrocyte marker MBP in vitro: immunostaining of NPC cultures on differentiation day 9 showed that the number of MBP+ cells was increased by 2.5-fold in knockout cells compared with wild-type controls (Fig. 5C,D). These results demonstrate that




| | N/O/A clone | N/O clone | N/A clone | O/A clone | N-only clone | O-only clone | A-only clone | Undifferentiated clone | N-containing clone | O-containing clone | A-containing clone |
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| WT% | 55 | 5 | 25 | 13.3 | 1.7 | 0 | 0 | 0 | 86.7 | 73.3 | 93.3 |
| KO% | 60 | 1.7 | 11.7 | 21.7 | 0 | 5 | 0 | 0 | 73.4 | 88.4 | 93.4 |

**Fig. 5. Ryk knockout NPC cultures give rise to reduced numbers of neurons and increased numbers of oligodendrocytes though a cell-autonomous mechanism.** (**A**) Expression of TuJ1, O4, GFAP and GABA in wild-type and Ryk knockout cell cultures on differentiation day 6 (D6). (**B**) Quantification of TuJ1+, O4+, GFAP+ and TuJ1+/GABA+ cells. (**C**) MBP expression in wild-type and knockout cell cultures at D9. (**D**) Histograms show normalized fold changes in MBP+ cells. Results are from three separate pairs of wild-type and knockout mice. (**E**) Expression of Ngn1, Ngn2, Nkx2.2 and Olig2 at D6 in wild-type and knockout cell cultures, as determined by quantitative RT-PCR and normalized. (**F**) Neurospheres subjected to the clonal assay: different kinds of clones were identified by immunostaining for GFAP/TuJ1 and GFAP/O4. Images on the left show four different types of clones: tri-potent clones (N/O/A), bi-potent clones (O/A, N/A) and uni-potent clones (O-alone). The table on the right compares wild-type and Ryk knockout NPCs in the clonal assay. The parameters used to evaluate the differentiation properties of each clone are shown on the top row. The percentage of clones for each parameter is listed below for both wild-type and Ryk knockout cells. More than 100 clones were tested. (**G**) Mixtures of knockout NPCs transduced with a GFP-expressing construct and an excess of wild-type NPCs were cultured under differentiation conditions at D6 and then subjected to immunostaining for TuJ1 and O4. Percentages of GFP+/ TuJ1+ and GFP+/O4+ cells were plotted (±s.e.m.). Scale bars: 100 μm. **P*<0.05, ****P*<0.001. (B,D,E) Results are mean ± s.e.m.

Ryk signaling inhibits oligodendrocyte lineage differentiation during NPC differentiation in vitro. This observation is further supported by expression profiling of neurogenesis and oligodendrogenesis genes. Quantitative PCR of NPC cultures on differentiation day 6 showed a significant increase in expression of Nkx2. 2 and Olig2, both of which control oligodendrocyte differentiation, and a decrease in expression of Ngn1 and Ngn2, which regulate neuronal differentiation (Fig. 5E). Together, results from these in vitro experiments demonstrate that Ryk inhibits differentiation of NPCs into oligodendrocytes.

The fact that the Ryk knockout mice exhibited opposite phenotypes with respect to neuronal differentiation and oligodendroglial differentiation raises the possibility that the altered oligodendrocyte differentiation pattern is due to a cell-non-autonomous mechanism that is secondary either to defects of tissues other than NPCs or to the defect in neuronal differentiation. To address the first aspect of this issue, we carried out clonal assays to examine whether the altered differentiation pattern occurs at the progenitor level (Gritti et al., 2008; Reynolds and Weiss, 1996; Sugimori et al., 2007). We let both wild-type and knockout single neural progenitor cells grow as clonal colonies in methylcellulose matrix, and subsequently, individual colonies were isolated and subjected to differentiation assay. Differentiated cells were detected and analyzed by immunostaining with lineage markers TuJ1, O4 and GFAP (Fig. 5F). Under our conditions, three types of clones can be found: tri-potent clones (N/O/A: N, neuron; O, oligodendrocyte; A, astrocyte); bi-potent clones (N/O, N/A and O/A clones); and very few uni-potent clones (N-alone, O-alone and A-alone clones) (Fig. 5F). Because each clone or each clonal neurosphere is formed without any contact with other cells, the differentiation properties reflect the intrinsic characteristics of the initial progenitor. The percentage of tri-potent clones in total was

similar between wild-type and knockout cultures; however, N/A bi-potent clones decreased 53% whereas O/A bi-potent clones increased 63% in the knockout cultures (Fig. 5F). Moreover, uni-potent O-alone clones, which can differentiate only into oligodendrocytes, were significantly increased, to 5%, in the knockout culture compared with the wild-type culture, which contained 0% (Fig. 5F). In general, the number of N-containing clones was reduced in the knockout culture by 15% and the number of O-containing clones was increased by 21% (Fig. 5F). The number of A-containing clones was not changed (Fig. 5F). Therefore, the results demonstrate that the observed opposite changes in the generation of neurons and oligodendrocytes in the Ryk knockout NPC populational assay are indeed attributable to abnormal cell-fate commitment of NPCs at the progenitor level. These data also indicate that defects in Ryk mutants occur in NPCs, not secondary to abnormality of other cell types or tissues.

The second aspect of the cell-non-autonomous possibility is the potential existence of a feedback signal released from differentiated neurons that can inhibit oligodendrocyte formation and lead to the opposite phenotypes of neuron and oligodendrocyte differentiation in knockout mice. To address this, we labeled knockout NPCs with a GFP-expression construct using lentivirus and mixed them with an excess amount of unlabeled wild-type NPCs, then cultured them under differentiation conditions for 6 days. Lentiviral infection efficiency for knockout NPCs reached 100% in this case (see Fig. S7B in the supplementary material). Immunostaining of the lineage markers TuJ1 and O4 in the culture showed that GFP-labeled knockout NPCs differentiated into 11.5% (±0.9%) TuJ1+ neurons and 18.7% (±2.2%) O4+ oligodendrocytes (Fig. 5G; compare with 5B), suggesting these cells exhibited the same differentiation pattern as the pure knockout cell culture (TuJ1+11.1%, O4+18.8%). Thus, wild-type cells cultured in the same dish did not rescue the abnormal cell-fate commitment of knockout cells to any extent. Therefore, it is unlikely that differentiated neuronal cells release any type of inhibitory signal to suppress oligodendrocyte formation and change the differentiation pattern in the knockout NPCs. In summary, our data demonstrate that the altered cell-fate commitment in Ryk knockout mice occurs in progenitors, and is not secondary to abnormality of other cell types or to defects in any cell-cell interactions, rather it is a result of a change of cell-fate decision.

## The Ryk receptor is required for Wnt3a-induced GABAergic neuronal differentiation and inhibition of oligodendrocyte differentiation

To test the hypothesis that Ryk mediates Wnt signaling in modulating neuronal versus glial cell-fate specification, we treated wild-type and knockout NPC cultures with Wnt3a recombinant protein during the differentiation assay. Wnt3a has been shown to bind to Ryk receptor to promote neurite outgrowth (Lu et al., 2004), and Wnt3a can induce neuronal differentiation of NPCs in both the forebrain and spinal cord (Kalani et al., 2008; Lie et al., 2005; Muroyama et al., 2002; Yu et al., 2006). Wnt3a has recently been shown to upregulate gene expression of the oligodendrocyte inhibitors Id2 and Id4, and to downregulate expression of myelin genes such as MBP in hippocampus-derived adult neural progenitor cells and oligodendroglial cell cultures (Ye et al., 2009). We found that exogenous Wnt3a treatment increased the number of TuJ1+ neurons significantly in the wild-type culture but had a much smaller effect in the knockout culture (see Fig. S8 in the supplementary material). Blocking the interaction between Ryk receptor and endogenous Wnts in the medium by using Ryk antibody led to a reduction of TuJ1+ cells to the same level observed in the knockout cell culture (see Fig. S8 in the supplementary material). Interestingly, Wnt3a treatment increased the percentage of GABAergic neurons which were GABA+/TuJ1+ by 50% in the wild-type cell culture, but had no such stimulatory effect on knockout cell cultures (Fig. 6A), suggesting that Ryk receptor is required for GABAergic neuron differentiation induced by Wnt3a. We then asked whether the interaction between Wnt3a and Ryk receptor is crucial for this phenomenon. We found that applying Ryk antibody not only brought down the percentage of GABA+/TuJ1+ neurons in the wild-type cell culture to the level observed in the knockout culture, but also almost completely eliminated Wnt3a-induced generation of GABA+/TuJ1+ neurons in the wild-type culture (Fig. 6A). Therefore, we conclude that Ryk receptor plays a crucial role in Wnt3a-induced differentiation of GABAergic neurons in vitro.

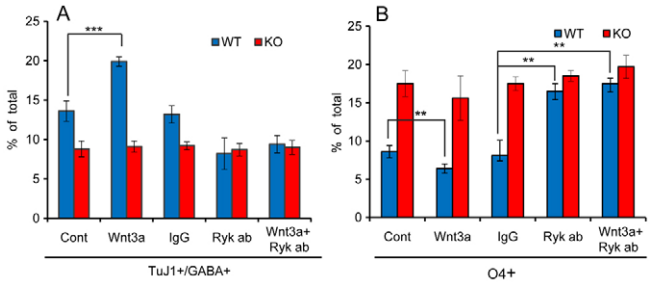


**Fig. 6. Ryk receptor is required for Wnt3a-induced GABAergic neuron differentiation and inhibition of oligodendrocyte differentiation.** NPC cultures were treated with Wnt3a recombinant proteins at a concentration of 100 ng/ml under differentiation conditions; on differentiation day 6, differentiated cell cultures were analyzed by immunostaining with markers. To block Ryk receptor and Wnt protein interaction, 200 ng/ml Ryk antibody (ab) was applied. As a control, 200 ng/ml IgG was applied to the NPC culture. (**A**) Ryk receptor is required for GABAergic neuron differentiation stimulated by Wnt3a. GABAergic neurons were identified by co-immunostaining of TuJ1 and GABA. (**B**) Ryk receptor is crucial for Wnt3a-induced suppression of oligodendrocyte differentiation demonstrated by O4 immunostaining. $^{**}P<0.01$, $^{***}P<0.001$. Results are mean ± s.e.m.

Wnt3a treatment induced the opposite effect on O4+ oligodendrocyte differentiation. A 25% decrease in O4+ cells was found following Wnt3a treatment of the wild-type culture. Such a suppressing effect of Wnt3a, however, was not significant in the knockout cell culture (Fig. 6B). As expected, applying Ryk antibody doubled the production of O4+ cells, yielding numbers very close to the level observed in the knockout cell culture (Fig. 6B). Application of Ryk antibody also eliminated the inhibitory effect of Wnt3a. Taken together, our data indicate that Ryk receptor plays a crucial role in Wnt3a-induced inhibition of oligodendrocyte differentiation.

## Ryk ICD is required and sufficient for promoting GABAergic neuron differentiation and suppressing oligodendrocyte differentiation

Previously, we have discovered that cleavage of Ryk and subsequent nuclear localization of the cleavage product, Ryk ICD, constitutes a novel mechanism of Wnt signaling (Lyu et al., 2008). To investigate the role of Ryk nuclear signaling in regulating GABAergic versus oligodendrocyte differentiation, constructs expressing (1) wild-type Ryk, (2) Ryk NLS-ICD (in which ICD is

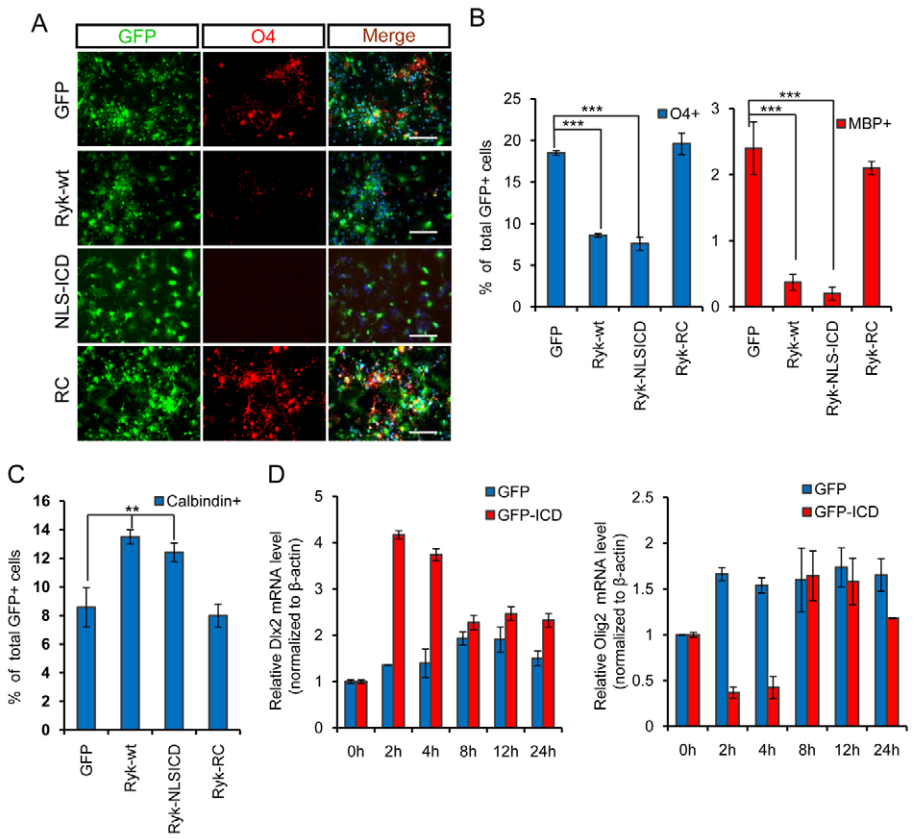


**Fig. 7. Ryk ICD is sufficient and required to mediate the cell-fate change.** (**A**) Ectopic expression of wild-type Ryk or Ryk NLS-ICD rescues the excess oligodendrocyte formation in the knockout cultures. Knockout NPCs transduced with lentivirus expressing either control GFP, wild-type Ryk, ICD with nuclear localization signal (Ryk NLS-ICD) or uncleavable Ryk mutant (Ryk RC) were cultured under differentiation conditions for 6 days. Differentiated NPC cultures were then analyzed by immunostaining with GFP O4. (**B**) Percentages of O4+/GFP+ and MBP+/GFP+ cells in total GFP+ cells are shown (±s.e.m.) in the histograms. (**C**) Differentiated NPC cultures from A were analyzed by immunostaining with calbindin. Percentages of calbindin+/GFP+ cells in total GFP+ cells are shown (±s.e.m.) in the histograms. (**D**) Quantitative PCR showed that inducible ectopic expression of Ryk ICD regulates the expression of Dlx2 and Olig2 in NPC culture under proliferation conditions with the expression of inducible GFP as a control. Cells were collected after doxycycline (Dox) treatment at 0 hours, 2 hours, 4 hours, 8 hours, 12 hours and 24 hours. Scale bars: 100 μm. $^{**}P<0.01$, $^{***}P<0.001$. (D) Results are mean ± s.e.m.

fused with a nuclear localization signal peptide), (3) Ryk RC, which is a chimeric construct that cannot be cleaved, or (4) control GFP alone, were transduced into Ryk knockout NPCs by lentivirus. Transduced NPCs were cultured under differentiation conditions for 6 days (Fig. 7A). As expected, overexpression of wild-type Ryk was sufficient to rescue the excess oligodendrocyte differentiation from knockout cultures, as shown by O4 staining compared with the control (Fig. 7A). Interestingly, transduction of Ryk NLS-ICD, like wild-type Ryk, led to more than an 80% reduction in O4+ oligodendrocyte differentiation compared with the control (Fig. 7A). However, Ryk RC-transduced cells gave rise to similar numbers of O4+ cells as the control (Fig. 7A). Quantification of O4 and GFP double positives cells further supports that cleavage of Ryk is important for the role of Ryk in oligodendrogenesis, and nuclear localization of Ryk ICD is sufficient to inhibit oligodendrogenesis (Fig. 7B, left panel). A similar result was obtained from MBP immunostaining (Fig. 7B, right panel). Moreover, while repressing differentiation of O4+ cells, overexpression of wild-type Ryk and Ryk NLS-ICD can rescue the defect in differentiation of GABAergic neurons detected by calbindin immunostaining, but not Ryk RC (Fig. 7C). These data demonstrate that Ryk ICD-mediated nuclear signaling is required and sufficient for suppression of oligodendrocyte lineage differentiation and promotion of the GABAergic neuron lineage from NPCs.

To investigate whether ICD-mediated Ryk nuclear signaling regulates the expression of key genes involved in cell-fate specification of GABAergic neurons and oligodendrocytes, Ryk knockout NPCs were cultured under proliferation conditions and transduced with a construct expressing doxycycline-inducible Ryk ICD tagged with GFP or a construct expressing doxycycline-inducible GFP only as a control, and time-dependent changes in the mRNA levels of Dlx1 and Olig2 were examined (Fig. 7D). Two hours after application of doxycycline, Dlx2 gene expression was stimulated by fourfold compared with the control; however, Olig2 gene expression was inhibited by twofold in the same time window (Fig. 7D). This suggests that the end effect of Ryk nuclear signaling in the cell-fate determination of NPCs is to regulate expression of key differentiation genes.

## DISCUSSION

Although regulation of fate determination of NPCs has been intensely studied, very little is known about how the production of neuronal versus oligodendroglial cells is balanced in the common progenitors and how cell-fate decisions are made and regulated by extrinsic signaling in the ventral telencephalon. In this paper, we demonstrate that a novel function of Ryk-mediated Wnt signaling regulates the cell-fate decision of ventral NPCs to differentiate as either GABAergic neurons or oligodendrocytes during telencephalon development.

### Evidence that Ryk receptor regulates neuronal versus oligodendroglial cell fate

We previously reported that Ryk plays a crucial role in promoting neuronal differentiation in the dorsal telencephalon during corticogenesis (Lyu et al., 2008). Here, we provide three lines of evidence that Ryk receptor also has a central role in repressing oligodendrocyte differentiation while promoting GABAergic neuronal differentiation in the ventral telencephalon. First, the first wave of MGE-derived OPC production is increased significantly in Ryk knockout cortices from E11.5 to E12.5 without changing the differentiation timing and location. The LGE-derived second wave of oligodendrogenesis is also likely to be enhanced. Second, development of MGE- and LGE-derived GABAergic neurons is

impaired in Ryk knockout mice from E14.5 to E18.5, as evidenced by in vivo analysis of GABAergic neuron markers and expression of genes required for determination of GABAergic neuronal cell fate. Third, excessive production of oligodendrocytes is observed in primary cultures and clonal neurosphere assays of Ryk knockout NPCs. We also found a significant reduction in GABA+/TuJ1+ neurons but a significant increase in O4+ oligodendrocytes in the knockout cell culture. These results have revealed an unexpected function of Ryk receptor in controlling GABAergic neurogenesis and oligodendrogenesis during embryonic brain development. This suggests that normal development of the ventral forebrain requires Ryk receptor to promote neurogenesis while restricting concurrent oligodendrogenesis.

In principle, the aforementioned phenotypes of Ryk mutant NPCs could be a consequence of secondary effects of altered DV patterning, abnormal proliferation or maturation of ventral cells, cell death or non-cell-autonomous effects. Our in vivo and in vitro data do not support any of these possibilities. Rather, our in vivo and in vitro data collectively support a model in which Ryk regulates the fate choice between GABAergic neurons and oligodendrocytes of ventral telencephalic NPCs.

Our data also suggest that Ryk may regulate cell fate by modulating the expression of Dlx2 and Olig2. Members of the Dlx gene family are known to be key factors in GABAergic interneuron specification, migration and differentiation (Cobos et al., 2007; Panganiban and Rubenstein, 2002). Dlx1/2 were first reported as homeobox transcriptional factors involved in coordinating GABAergic neuron versus oligodendrocyte specification in bi-potent progenitor cells (Petryniak et al., 2007). Although whether Ryk receptor can directly regulate expression of Dlx2 is currently unknown, our in vivo and in vitro data suggest that extrinsic signal molecules and receptors may work through transcriptional factors to regulate progenitor cells in order to coordinate differentiation of different lineages. To our knowledge, Ryk is the first receptor shown to coordinate OPC and neuron specification in the mammalian telencephalon.

## Activity of Ryk-mediated Wnt signaling in the ventral telencephalon

Because of the cell-fate-change phenotype that we observed in Ryk knockout mice and NPC cultures, and the fact that Ryk receptor is known to bind multiple Wnt proteins (e.g. Wnt1, Wnt3, Wnt3a, Wnt4, Wnt5a), we sought to identify the Wnt ligand(s) responsible for this effect by employing a series of Wnt treatment experiments. Wnt3a is known to promote neuronal differentiation of NPCs of the forebrain and spinal cord (Kalani et al., 2008; Lie et al., 2005; Muroyama et al., 2002; Yu et al., 2006). Our in vitro study showed that Wnt3a promotes GABAergic neuron differentiation and that Ryk receptor is required for this effect.

Previous studies have shown that Wnt signaling inhibits differentiation and maturation of oligodendrocytes in both the spinal cord and telencephalon, and that the β-catenin-mediated canonical pathway has been implicated in this action (Fancy et al., 2009; Shimizu et al., 2005; Ye et al., 2009). Our study shows that Ryk receptor also plays a crucial role in Wnt3a-induced inhibition of oligodendrocyte differentiation. Therefore, downstream of Wnt, both the β-catenin-mediated canonical and Ryk receptor-mediated atypical Wnt signaling pathways appear to be involved in repressing oligodendroglial cell-fate choice. Our data illustrate that Wnt proteins act through Ryk to promote neurogenesis in both the dorsal and ventral telencephalon and to antagonize simultaneously oligodendroglial fate in the early stages of brain development.

## Ryk-ICD regulates both neuronal and oligodendroglial cell differentiation

How does Wnt3a influence the differentiation of NPCs? Our previous data indicate that cleavage of Ryk and ICD nuclear localization are required for Wnt3-induced neuronal differentiation (Lyu et al., 2008). In the present study, we found that, during Wnt3a-induced GABAergic neurogenesis, both Ryk wild-type and Ryk NLS-ICD, but not the non-cleavable form Ryk RC, can rescue the supernumerary formation of oligodendrocytes in Ryk mutant cell culture. Therefore, we conclude that ICD-dependent Ryk signaling serves as a common mechanism for both promoting neuronal differentiation and inhibiting oligodendrocyte differentiation. However, Ryk signaling does not seem to affect the level of β-catenin or the activity of TCF significantly in NPCs in vitro (Lyu et al., 2008), suggesting that Ryk ICD-mediated β-catenin-independent signaling induces GABAergic neuron generation and represses oligodendrocyte specification.

Our study reveals for the first time that Ryk-mediated atypical Wnt signaling is involved in the neuronal differentiation from the dorsal pallium and also in differentiation of ventral cell types, including GABAergic neurons and oligodendrocytes, during forebrain development. Our results provide strong evidence that Ryk signaling is crucial for controlling the neuron-versus-oligodendrocyte fate decision. This activity in ventral NPCs may promote both dorsal and ventral neurogenesis while antagonizing the induction of other cell types from the ventral NPCs during early brain development.

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### Competing interests statement

The authors declare no competing financial interests.

### Supplementary material

Supplementary material for this article is available at
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