Ror family receptor tyrosine kinases regulate the maintenance of neural progenitor cells in the developing neocortex

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Abstract
The Ror family receptor tyrosine kinases (RTKs), Ror1 and Ror2, have been shown to play crucial roles in developmental morphogenesis by acting as receptors or co-receptors to mediate Wnt5a-induced signaling. Although Ror1, Ror2 and Wnt5a are expressed in the developing brain, little is known about their roles in the neural development. Here we show that Ror1, Ror2 and their ligand Wnt5a are highly expressed in neocortical neural progenitor cells (NPCs). Small interfering RNA (siRNA)-mediated suppression of Ror1, Ror2 or Wnt5a in cultured NPCs isolated from embryonic neocortex results in the reduction of βIII-tubulin-positive neurons that are produced from NPCs possibly through the generation of T-box brain 2 (Tbr2)-positive intermediate progenitors. BrdU-labeling experiments further reveal that the proportion of proliferative and neurogenic NPCs, which are positive for neural progenitor cell marker (Pax6) but negative for glial cell marker (glial fibrillary acidic protein; GFAP), is reduced within a few days in culture following knockdown of these molecules, suggesting that Ror1, Ror2 and Wnt5a regulate neurogenesis through the maintenance of NPCs. Moreover, we show that Dishevelled 2 (Dvl2) is involved in Wnt5a–Ror1 and Wnt5a–Ror2 signaling in NPCs, and that suppressed expression of Dvl2 indeed reduces the proportion of proliferative and neurogenic NPCs. Interestingly, suppressed expression of either Ror1 or Ror2 in NPCs in the developing neocortex results in the precocious differentiation of NPCs into neurons, and their forced expression results in delayed differentiation. Collectively, these results indicate that Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways play roles in maintaining proliferative and neurogenic NPCs during neurogenesis of the developing neocortex.

Key words: Wnt5a, Ror1, Ror2, Signaling, NPCs, Neurogenesis

Introduction
In the developing neocortex, a large number of neurons are generated from neural progenitor cells (NPCs) during a specified period of time (around embryonic day 13 to 18; E13–E18) in the mouse. In this process, NPCs self-renew and simultaneously generate neurons, and a balance between proliferation and differentiation of NPCs is crucial for generating a sufficient number of neurons to properly form a functional neocortex. The cellular proliferation and differentiation are competitively regulated in the respective NPCs, and several transcription factors, including *Hes*-family and *neurogenin* (*Ngn*)-family genes, have been shown to play crucial roles in determining whether NPCs continue to proliferate or exit from the cell cycle (Guillemot, 2007; Miyata et al., 2010; Ross et al., 2003). However, the molecular basis of the regulation of a balance between proliferation and differentiation during the neurogenic period is not well understood.

The Wnt family of secreted glycoproteins have been shown to regulate diverse biological processes, including cell proliferation, differentiation, migration, polarity and axonal outgrowth (Cadigan and Nusse, 1997; Nelson and Nusse, 2004). In mammals, 19 Wnt-family genes have been identified to date, and several Wnt genes are expressed in the developing neocortex (Fougerousse et al., 2000; Keeble et al., 2006; Lee et al., 2000; Lyu et al., 2008). Several lines of evidence demonstrates that Wnt–β-catenin–TCF signaling (i.e. canonical Wnt signaling) plays essential roles in coordinating neocortical neurogenesis by regulating proliferation and differentiation of NPCs through the induction of various transcription factors including *cyclin D1*, *Hes1*, *Ngn1*, *Ngn2* and *N-myc* (Hirabayashi et al., 2004; Kuwahara et al., 2010; Shimizu et al., 2008). In fact, canonical Wnt signaling appears to shift the balance between proliferation and differentiation of NPCs, depending on developmental stages and/or cellular context of NPCs (Hirabayashi et al., 2004; Israsena et al., 2004; Munji et al., 2011; Viti et al., 2003; Woodhead et al., 2006).

Several members of the Wnt-family ligands can activate Wnt signaling independently of β-catenin (i.e. noncanonical Wnt signaling) (Semenov et al., 2007; Veeman et al., 2003), and Wnt5a is a representative of the noncanonical Wnt ligands (Bryja et al., 2007; Gonzalez-Sancho et al., 2004; Schulte et al., 2005). It has been shown that Ror2, a member of the Ror family of receptor tyrosine kinases (RTKs), acts as a receptor or co-receptor for Wnt5a to mediate inhibition of Wnt–β-catenin signaling and activation of Wnt–JNK signaling, leading to polarized cell migration (Mikels and Nusse, 2006; Nishita et al., 2003; Nomachi et al., 2008; Oishi et al., 2003). Dishevelled (Dvl) can mediate both the β-catenin-dependent and β-catenin-independent Wnt signaling pathways. We have recently shown...
that Ror2 mediates Wnt5a-induced phosphorylation and polymerization of Dvl in the β-catenin-independent manner (Nishita et al., 2010). In addition, Ror1, another member of the Ror family of RTKs, has recently been suggested to be an alternative receptor for Wnt5a (Fukuda et al., 2008; Grumolato et al., 2010; Sato et al., 2010). Like Wnt5a, both Ror1 and Ror2 are expressed in the developing nervous system, including the neocortex, in mice (Al-Shawi et al., 2001; Matsuda et al., 2001; Oishi et al., 1999). Although Ror2 as well as Ror1 have been implicated in the regulation of neurite extension and synapse formation of hippocampal neurons (Paganoni et al., 2010; Paganon and Ferreira, 2005), the roles of Ror1, Ror2 and Wnt5a in the neocortex, in particular their roles in neocortical neurogenesis, are still unclear.

Here we show that Ror1, Ror2 and their ligand Wnt5a are expressed in NPCs of the developing neocortex, and that Wnt5a transduces its signal to Dvl2 through Ror1 and Ror2 in NPCs without affecting β-catenin–TCF signaling. We further provide evidence indicating that Wnt5a–Ror1 and Wnt5a–Ror2 signaling can function to regulate neurogenesis through the maintenance of proliferative and neurogenic NPCs.

Results
Ror1, Ror2 and their ligand Wnt5a are expressed in neocortical NPCs
To determine stage-dependent expression of the genes encoding the Ror family RTKs and their ligand Wnt5a in the developing neocortex, we examined expression levels of Ror1, Ror2 and Wnt5a mRNAs in the mouse neocortex of different developmental stages by using reverse transcription PCR (RT-PCR). Ror1 and Ror2 mRNAs were detected abundantly at the earlier stages of the neocortex at which nestin, a specific marker of NPCs, is expressed, and were decreased at the later stages at which βIII-tubulin and glial fibrillary acidic protein (GFAP), specific markers of neurons and astrocytes, respectively, are expressed. Wnt5a mRNA was constantly detected during the development of the neocortex (Fig. 1A). Even at a neonatal stage, Ror1 and Ror2 mRNAs were detected abundantly in the tissue samples from the ventricular zone (VZ), in which NPCs have been shown to be exclusively localized, whereas their expression levels were lower in those from the cortical plate (CP) (Fig. 1B), suggesting that both Ror1 and Ror2 are selectively expressed in NPCs.

Ror1, Ror2 and Wnt5a were all expressed significantly in primary cultured NPCs in the presence of basic fibroblast growth factor (bFGF), which maintains NPCs in an undifferentiated state, whereas reduced expression of Ror1, Ror2 and Wnt5a was observed in the cells cultured in the absence of bFGF, a condition that allows NPCs to differentiate into neurons and glial cells (Fig. 1C,D). We first wanted to examine whether Ror1 and Ror2 are coexpressed in the respective NPCs, but failed to do so because an antibody against Ror1 suitable for immunocytochemical analysis is currently unavailable. Therefore, we examined whether Ror1 and Ror2 are expressed in at least partially purified NPCs. For this purpose, we performed fluorescence-activated cell sorting (FACS) under stringent gating conditions (Fig. 1E, inset) to isolate NPCs with an antibody recognizing the extracellular region of CD133, a cell surface marker of embryonic NPCs (Pfenninger et al., 2007), because cultured NPCs might be heterogeneous populations at their differentiation states even in the presence of bFGF. Nestin and Hex5 genes, tightly associated with NPCs, were highly expressed in cells expressing CD133 at a higher level (CD133 high) compared with cells expressing CD133 at a lower level (CD133 low) (Fig. 1E), indicating that CD133 high cell populations predominantly contain NPCs. As expected, higher expression levels of Ror1 and Ror2 mRNAs were detected in CD133 high, but not CD133 low populations (Fig. 1E). We further sorted cells by using an anti-Ror2 antibody, which recognizes the extracellular portion of Ror2. CD133, Nestin, Hex5 and Ror1 mRNAs were expressed at higher levels in Ror2 high populations than in Ror2 low ones (Fig. 1F). These results indicate that both Ror1 and Ror2 are expressed predominantly in NPCs. Because a sustained amount of Ror1, but not Ror2, was expressed at the later stages of the neocortex, where GFAP-positive astrocytes are robustly generated (Fig. 1A), we examined expression levels of Ror1, Ror2 and Wnt5a mRNAs in cultured astrocytes. Expression of Ror2 was marginally detectable in astrocytes, whereas expression of Ror1 and Wnt5a was detectable at high levels, comparable with those in NPCs (supplementary material Fig. S1A), suggesting a possible role of Wnt5a–Ror1 signaling in astrocytes.

We next examined whether Ror1 and Ror2 expressed in NPCs are required for Wnt5a-induced signaling events. Immunoblotting analysis revealed that the phosphorylation-dependent delayed electrophoretic mobility of Dvl2, a well-known surrogate marker of Wnt5a signaling (Nishita et al., 2010), was significantly inhibited by treatment of NPCs with either Ror1 or Ror2 siRNA (Fig. 1G). Consistent with the previous report (Grumolato et al., 2010), we also observed the delayed electrophoretic mobilities of Ror1 and Ror2 following Wnt5a stimulation of NPCs (Fig. 1G). In astrocytes, in which Ror1 is expressed dominantly, Ror1 siRNA, but not Ror2 siRNA, inhibited phosphorylation of Dvl2 induced by Wnt5a stimulation (supplementary material Fig. S1B), indicating that Ror1 can mediate Wnt5a signaling independently of Ror2. Collectively, these results suggest that both Ror1 and Ror2 in NPCs play important roles in mediating Wnt5a-induced signaling.

Ror1, Ror2 and Wnt5a regulate production of neurons from NPCs
To examine the roles of Ror1 and Ror2 in NPCs, we analyzed the effects of suppressed expression of Ror1 and Ror2 on neurogenesis by using in vitro cultured NPCs. The amounts of βIII-tubulin were decreased in NPCs treated with either Ror1 or Ror2 siRNAs under a differentiating condition (Fig. 2A,B). Immunostaining analysis also revealed that the treatment of NPCs with either Ror1 or Ror2 siRNA decreased the proportion of βIII-tubulin-positive neurons (Fig. 2C,D). Five days after transfer to differentiation medium, βIII-tubulin-positive neurons generated from cells treated with a control siRNA were assembled as clusters, possibly reflecting characteristics of neurons generated continually from NPCs. The number of clustered neurons was significantly decreased when the cells were treated with either Ror1 or Ror2 siRNA (Fig. 2E). Furthermore, similar effects on the generation of neurons were observed when Wnt5a expression in NPCs was suppressed by Wnt5a siRNAs (Fig. 2F–I).

In the neurogenic stage of the neocortex, an NPC can produce a number of neurons through the transient generation of intermediate progenitor cells (IPCs) (Guillemot, 2005). Thus, we next examined the roles of Ror1 and Ror2 in the generation of IPCs. IPCs can be specifically stained with an antibody against T-box brain 2 (Tbr2) (Englund et al., 2005). Three days after
transfer to differentiation medium, Tbr2-positive IPCs were also observed as clusters in cells treated with control siRNA (Fig. 2J) and were localized in close vicinity to the clustered βIII-tubulin-positive neurons (data not shown). The proportion of these cells was remarkably decreased following suppressed expression of Ror1, Ror2 or Wnt5a (Fig. 2J,K). These results indicate that Wnt5a–Ror1 and Wnt5a–Ror2 signaling contribute to the production of neurons from IPCs by continuous proliferation of NPCs (see below). Because cultured NPCs generate neurons first, and astrocytes subsequently (Qian et al., 2000), we also examined the effects of suppressed expression of Ror1 or Ror2 on astrogenesis by immunostaining with an antibody against GFAP. Interestingly, the treatment of NPCs with either Ror1 or Ror2 siRNAs resulted in a marked increase in the proportion of GFAP-positive cells (supplementary material Fig. S2A,B), suggesting that both Ror1 and Ror2 play a crucial role in inhibiting differentiation of NPCs into astrocytes. Therefore, the decreased neurogenesis in cultured NPCs treated with either Ror1 or Ror2 siRNAs might be due to the precocious differentiation of NPCs into astrocytes.

Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways regulate maintenance of the proliferative and neurogenic state of NPCs

It has been well documented that the number of neurons generated from NPCs depends on a balance between proliferation and
differentiation of NPCs (Miyata et al., 2010). Thus, we performed BrdU-labeling experiments to examine whether Wnt5a–Ror1 and Wnt5a–Ror2 signaling can regulate cell proliferation. In these experiments, NPCs treated with the respective siRNAs were cultured for 48 hours in the absence of bFGF, and then further labeled with BrdU for 24 hours. Among cells treated with control siRNAs, BrdU-positive cells exhibited clustered distribution, as seen for βIII-tubulin-positive neurons (Fig. 3A, Fig. 2C,G). By contrast, the scattered distribution and decreased proportion of BrdU-positive cells were detected following suppressed expression of Ror1, Ror2 or Wnt5a (Fig. 3A,B).

The decreased proportion of BrdU-positive cells in these cells was possibly due to a reduction of cell cycle retaining NPCs (i.e. proliferative NPCs) or of the growth rate of NPCs. To examine whether Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways are involved in maintaining proliferative NPCs, we next performed BrdU pulse-chase experiments together with immunostaining with Ki67, a marker for proliferating cells. In this experiment, NPCs treated with the respective siRNAs were labeled with BrdU for 3 hours in the presence of bFGF, transferred to differentiation medium and further cultured for 48 hours. The cell cycle retention index was determined as the percentage of
BrdU-positive (BrdU⁺) cells that still retained expression of Ki67 (100 × Ki67⁺/BrdU⁺/BrdU⁺) 48 hours after BrdU pulse labeling. Higher retention indices reveal that more cells remain as proliferative NPCs. Treatment of cells with siRNAs for Ror1, Ror2 and Wnt5a resulted in significant reduction of the cell cycle retention indices (Fig. 3C,D), indicating that Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways play roles in maintaining the proliferative NPCs.

To confirm the specific effects of these siRNAs on NPCs, BrdU-labeled cells were co-stained with Pax6, a rough marker for NPCs and GFAP. We monitored BrdU- and Pax6-double positive and GFAP-negative cells as proliferating NPCs, because Pax6 is expressed in both NPCs and astrocytes (Sakurai and Osumi, 2008). The proportion of these cells was drastically reduced following treatment with the respective siRNAs (Fig. 4A,B). On the other hand, mature astrocytes, expressing GFAP at high levels, failed to incorporate BrdU within 24 hours under our culture condition, and some immature astrocytes, expressing GFAP at low levels, were labeled with BrdU under the same condition (Fig. 4C). Importantly, there was no apparent difference in the proportion of BrdU- and GFAP-double positive cells following treatment with the respective siRNAs (Fig. 4D). These results indicate that the effects of the respective siRNAs for Ror1, Ror2 and Wnt5a on cell proliferation are due to their direct action on NPCs, but not on astrocytes, and further emphasize that Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways play roles in maintaining the proliferative NPCs. In addition, some BrdU-labeled cells were co-stained with βIII-tubulin (Fig. 4E), and these BrdU- and βIII-tubulin-double positive cells represent newly generated neurons within

Fig. 3. Ror1, Ror2 and Wnt5a are required for maintenance of the proliferative state of NPCs. (A) NPCs were transfected with the indicated siRNAs and cultured in differentiation medium. After 2 days in culture, cells were treated with BrdU (10 μM) for 24 hours and then immunostained with the antibody against BrdU (red) and counterstained with DAPI (blue). Scale bar: 100 μm. (B) Quantification of the percentage of BrdU-positive cells. (C) NPCs were transfected with the indicated siRNAs, treated with BrdU for 3 hours and then transferred to differentiation medium. After 2 days in culture, cells were immunostained with antibodies against BrdU (green) and Ki67 (red). Scale bar: 20 μm. (D) Quantification of the percentage of Ki67-positive cells among the BrdU-positive cells.
24 hours after an addition of BrdU. The proportion of these double-positive cells was significantly decreased following treatment with siRNAs for Ror1, Ror2 or Wnt5a (Fig. 4F), suggesting that Wnt5a–Ror1 and Wnt5a–Ror2 signaling can enhance neuronal production through the maintenance of neurogenic NPCs.

We next examined whether Ror1 and Ror2 function redundantly in NPCs. To this end, we compared the effects of downregulation of Ror1, Ror2 or both in NPCs on the cellular responses of NPCs. As shown in supplementary material Fig. S3A–E, slightly enhanced effects on neurogenesis, astrogenesis and the maintenance of proliferative NPCs were observed reproducibly in the double-knockdown cells compared with single-knockdown cells. However, the differences were not statistically significant. This might be due to the limited capacity of late-stage NPCs (isolated from E14.5 neocortex) to generate neurons. Because early-stage NPCs are maintained in a more undifferentiated state and generate more neurons rather than astrocytes, we also examined the effects of the double knockdown by using NPCs isolated from E11.5 neocortex. Interestingly, the simultaneous suppression of both Ror1 and Ror2 in these NPCs showed significant additive effects on neurogenesis, astrogenesis and the maintenance of proliferative NPCs (supplementary material Fig. S3F–J). Taken together, these results indicate that Ror1 and Ror2 in NPCs function redundantly.

The self-renewal ability of NPCs has been thought to play an essential role in maintaining the proliferative and neurogenic state of NPCs. Therefore, we assessed the self-renewal ability of NPCs using neurosphere formation analysis. Cells treated with the respective siRNAs were grown as neurospheres for 1 week. The numbers and sizes of neurospheres were significantly reduced following treatment with siRNAs for Ror1, Ror2 or Wnt5a compared with treatment with control siRNA (Fig. 5A–C). It has been reported that Notch signaling plays an essential role in maintaining an undifferentiated state of NPCs (Imayoshi et al., 2010). Interestingly, the expression of Hes5, a downstream target gene of Notch signaling, was also suppressed following treatment with the respective siRNAs (Fig. 5D). These findings support our notion that Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways play important roles in maintaining the proliferative and neurogenic NPCs.

Dvl2 mediates Wnt5a–Ror1 and Wnt5a–Ror2 signaling in NPCs in a β-catenin-independent manner

It has been shown that Dvl is phosphorylated following stimulation of several cultured cells with either Wnt3a or Wnt5a (Bryja et al., 2007; Gonzalez-Sancho et al., 2004; Schulte et al., 2005). We have also recently reported that Ror2 mediates Dvl2 phosphorylation induced by Wnt5a, but not Wnt3a (Nishita et al., 2010). In our present study we also found that both Ror1 and Ror2 can mediate Wnt5a-induced Dvl2
phosphorylation in NPCs (Fig. 1E). Intriguingly, phosphorylation of Dvl2 detected in cultured NPCs can be inhibited by treatment with siRNAs for Ror1, Ror2 or Wnt5a (Fig. 6A), indicating that endogenously expressed Wnt5a as well as Ror1 and/or Ror2 can transduce their signals to Dvl2 in NPCs. To investigate the role of Dvl2 in the regulation of proliferation and differentiation of NPCs, we treated NPCs with Dvl2 siRNA (Fig. 6B). The proportion of generated neurons (Fig. 6C–E) and of BrdU-positive proliferating cells (Fig. 6F,G) under a differentiating condition was decreased following suppressed expression of Dvl2 in NPCs. In addition, expression levels of Hes5 transcripts were inhibited at least partly by suppressed expression of Dvl2 (Fig. 6H). These results suggest that Dvl2 plays a role in maintaining the proliferative and neurogenic NPCs by acting as one of downstream effectors in Wnt5a–Ror1 and Wnt5a–Ror2 signaling.

Wnt–β-catenin signaling has also been implicated in the regulation of proliferation and differentiation of NPCs (Viti et al., 2003; Hirabayashi et al., 2004; Israsena et al., 2004; Woodhead et al., 2006; Munji et al., 2011). Considering the findings that Dvl2 can mediate Wnt–β-catenin signaling as well as Wnt5a-induced signaling, and that Wnt5a–Ror2 signaling mediates inhibition of β-catenin–TCF signaling (Mikels and Nusse, 2006), it is of importance to clarify whether Wnt5a–Ror1 and/or Wnt5a–Ror2 signaling can affect β-catenin–TCF signaling in NPCs by using the TopFlash reporter (TCF–LEF-driven luciferase reporter) assay. Treatment of NPCs with Wnt3a, but not Wnt5a, resulted in a robust increase in the TopFlash reporter activity (supplementary material Fig. S4A). Furthermore, this Wnt3a-induced activation was unaffected by suppressed expression of either Ror1 or Ror2 (supplementary material Fig. S4B). In cultured NPCs, we could detect very low levels of basal TopFlash reporter activity, and this basal activity was unaffected by suppressed expression of either Ror1 or Ror2 (supplementary material Fig. S4B). These findings indicate that Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways act in a β-catenin-independent manner in NPCs.

**Ror1 and Ror2 play roles in maintaining NPCs within the neocortical VZ**

We next examined whether Ror1 and/or Ror2 play important roles in maintaining NPCs in the developing neocortex in vivo. To this end, we employed an in utero electroporation method to selectively introduce the respective plasmid vectors into NPCs in the VZ of the neocortex. We used a vector-based artificial microRNA (miRNA) expression system for the knockdown analysis. The efficiency of knockdown was first confirmed by transfection of the respective miRNA vectors (miR-Ror1 and miR-Ror2) into mouse NIH3T3 cells that express both Ror1 and Ror2 endogenously. Because miR-Ror1 #1, miR-Ror2 #1 and miR-Ror1 #1+Ror2 #1 efficiently reduced the expression of Ror1, Ror2 and both, respectively (Fig. 7A), these three vectors were used for the following knockdown analyses in vivo. Because these miRNA vectors co-cistronically express the respective miRNAs along with Emerald Green Fluorescent Protein (EmGFP), the EmGFP-expressing cells were considered as miRNA-expressing cells. We introduced the respective miRNA vectors into the brain of E14.5 mouse embryos and analyzed the distribution of miRNA-expressing cells in the neocortex at E17.5. Knockdown of Ror1, Ror2 or both resulted in decreases in cell numbers of EmGFP-expressing cells localized in
the VZ as compared with controls (treated with miR-LacZ) (Fig. 7B; and data not shown). The proportion of Pax6-positive NPCs among EmGFP-expressing cells was also significantly reduced following knockdown of Ror1, Ror2 or both (Fig. 7B,C). We also stained the neocortices with an antibody against Sox2 as an alternative marker of NPCs and observed a reduction in the proportion of Sox2-positive NPCs among EmGFP-expressing cells in the neocortices following knockdown of Ror1, Ror2 or both (supplementary material Fig. S5A,B). Conversely, the proportion of EmGFP-expressing cells localized in the intermediate zone (IZ) and CP was slightly increased following knockdown of Ror2 or of both Ror1 and Ror2 (Fig. 7D). These cells within the IZ and CP were also stained with antibody against βIII tubulin (data not shown), indicating that NPCs failed to maintain their undifferentiated states and, as a result, differentiated into neurons following suppressed expression of Ror1, Ror2, or both. On the other hand, we examined the effects of forced expression of Ror1 or Ror2 on the cell fate specification of NPCs in vivo. It was found that forced expression of either Ror1 or Ror2 restricted cellular distribution within the VZ and increased the proportion of Pax6-positive NPCs (Fig. 8A,B) and of Sox2-positive NPCs (supplementary material Fig. S5C,D). The proportion of cells localized in the CP was markedly reduced following forced expression of either Ror1 or Ror2 (Fig. 8C). Collectively, these results suggest that both Ror1 and Ror2 expressed in NPCs are required for the maintenance of their undifferentiated states within the VZ. Furthermore, miRNA-mediated suppressed expression of Dvl2 (supplementary material Fig. S6A) also results in decreased proportion of Pax6-positive NPCs (supplementary material Fig. S6B,C) of Sox2-positive NPCs (supplementary material Fig. S6D) and of cells localized in the VZ (supplementary material Fig. S6E), indicating that Dvl2 mediates the functions of Ror1 and Ror2 in the maintenance of NPCs in the developing neocortex.

**Discussion**

The Ror family RTKs possess an evolutionally conserved structure among *Caenorhabditis elegans*, *Aplysia*, *Drosophila melanogaster*, *Xenopus laevis*, mice and humans. In *Aplysia* and *D. melanogaster*, *Ror* genes are expressed exclusively in the developing nervous system (McKay et al., 2001; Oishi et al., 1997; Wilson et al., 1993), although their functions are still largely unknown. The *C. elegans* Ror homolog, *Cam-1*, has been implicated in neuronal development, including neuronal migration, axon guidance, and asymmetric division (Forrester et al., 1999; Forrester et al., 2004; Kennerdell et al., 2009; Koga et al., 1999; Song et al., 2010). In mammals, the Ror family RTKs consist of two structurally related proteins, Ror1 and Ror2, and both mouse Ror1 and Ror2 are also expressed in the developing brain (Al-Shawi et al., 2001; Matsuda et al., 2001; Oishi et al., 1999). These findings emphasize possible roles of Ror family RTKs in regulating the formation and function of the mammalian central nervous system. Indeed, it has been shown that both Ror1...
and Ror2 play roles in regulating neurite extension and synapse formation in hippocampal neurons (Paganoni et al., 2010; Paganoni and Ferreira, 2005). However, we could not clearly detect the expression of Ror1 and Ror2 in cultured neurons isolated from neonatal neocortex (data not shown). On the other hand, their expression was readily detectable when cells were isolated from the embryonic neocortex at the earlier stages by the same procedure. These findings indicate that Ror1 and Ror2 are expressed in undifferentiated cells, at least in the neocortex. In this study, we provide evidence indicating that Ror1 and Ror2 are expressed in neocortical NPCs (Fig. 1). Although we failed to show that Ror1 and Ror2 are coexpressed in the respective NPCs, it was found that both Ror1 and Ror2 are highly expressed in purified NPCs (Fig. 1E,F). Furthermore, suppressed expression of Ror1, Ror2 and Wnt5a in NPCs decreased production of neurons during long-term culture (Fig. 2). Our findings indicate that the reduction of neuronal production from NPCs is attributable to the reduction of proliferative and neurogenic NPCs within a few days in culture (Figs 3 and 4). Taken together, the results indicate that Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways play roles in regulating maintenance of the proliferative and neurogenic state of NPCs. This is the first time that the crucial roles of Wnt5a–Ror1 and Wnt5a–Ror2 signaling in neocortical neurogenesis has been demonstrated.

Wnt signaling comprises β-catenin-dependent signaling (canonical Wnt–β-catenin signaling) and β-catenin-independent signaling (noncanonical Wnt signaling). These different signaling pathways are mediated in part through different Wnt receptors or receptor complexes. Whereas Wnt–β-catenin signaling is mediated by LRP6–Frizzled receptor complexes, Wnt5a-induced noncanonical Wnt signaling is mediated by Ror2 or Ror2–Frizzled receptor complex (Green et al., 2008; Minami et al., 2010). Dvl is an essential mediator in both canonical and noncanonical Wnt signaling (Bryja et al., 2007; Gonzalez-Sancho et al., 2004; Schulte et al., 2005). The functions of Dvl might be altered, depending on the context of Wnt receptors or receptor complexes. In fact, we have recently shown that Ror2–Frizzled receptor complex mediates Wnt5a-induced AP-1 activation by regulating Dvl polymerization (Nishita et al., 2010). Ror1 has also been shown to mediate Wnt5a-induced signaling in...
lymphocytic leukemia cells (Fukuda et al., 2008). However, it was uncertain whether Ror1 regulates Dvl following Wnt5a stimulation. In addition, it has not been clarified whether Ror1 expression alone is sufficient to function as a Wnt5a receptor in the absence of Ror2, although it has been reported that Ror1 and Ror2 form a complex and function as a heterodimer in hippocampal neurons (Paganoni et al., 2010). Here, we show clearly that Ror1 as well as Ror2 mediate Wnt5a-induced Dvl phosphorylation in NPCs (Fig. 1), and that Ror1 can mediate Wnt5a signaling independently of Ror2 in astrocytes (supplementary material Fig. S1).

Notch signaling and proneural genes play crucial roles in regulating whether NPCs continue to proliferate or differentiate (Kageyama et al., 2005; Ross et al., 2003). Proneural genes such as Ngn1 and Ngn2, which encode transcription factors of the basic helix–loop–helix (bHLH) class, promote the differentiation of NPCs into more committed cells such as IPCs (Bertrand et al., 2002). Conversely, Notch signaling induces expression of Hes1 and Hes5, which repress expression of proneural genes, and thereby inhibits neural differentiation. We found that Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways are required for the maintenance of an undifferentiated state of NPCs, and that suppressed expression of Ror1, Ror2 and Wnt5a reduces the levels of Hes5 transcripts in NPCs (Fig. 5). Although it can be assumed that the reduction of Hes5 activity leads to enhanced differentiation of NPCs into neurons, our findings indicate that suppression of Ror1 or Ror2 in cultured NPCs decreases the percentage of generated neurons. Therefore, the reduction of Hes5 levels in Ror1- or Ror2-knockdown cells might be due to the decreased proportion of Hes5-expressing NPCs rather than to the decreased expression of Hes5 in NPCs. It should be noted that β-catenin–TCF signaling is capable of inhibiting or promoting the differentiation of NPCs by potentiating transcriptional induction of Hes1 and Hes5 or of Ngn1 and Ngn2, respectively (Hirabayashi et al., 2004; Shimizu et al., 2008). Because Wnt5a–Ror2 signaling can mediate inhibition of β-catenin–TCF signaling (Mikels and Nusse, 2006), it can be envisaged that Wnt5a–Ror1 and/or Wnt5a–Ror2 signaling maintains NPCs through the regulation of β-catenin–TCF signaling. However, Wnt5a–Ror1 and/or Wnt5a–Ror2 signaling does not appear to affect Wnt–β-catenin signaling in NPCs because any apparent alterations were not detected in the amounts of intracellular β-catenin (data not shown) or in TCF/LEF reporter activities following suppressed expression of either Ror1 or Ror2 in NPCs (supplementary material Fig. S4). It can be assumed that Wnt5a–Ror1 and Wnt5a–Ror2 signaling might activate β-catenin-independent signaling pathways such as Wnt–JNK signaling in NPCs without affecting Wnt–β-catenin signaling.

It is important to confirm that Wnt5a–Ror1 and Wnt5a–Ror2 signaling indeed function in the developing neocortex. Our findings using the in utero electroporation method in the developing mouse neocortex reveal that both Ror1 and Ror2 play roles in maintaining NPCs within the VZ (Figs 7 and 8). These functions of Ror1 and Ror2 in the maintenance of NPCs in the neocortex seem to be consistent with the findings obtained from cultured NPCs, although neuronal differentiation of in vivo NPCs appears to be enhanced following suppressed expression of Ror1 and/or Ror2, inconsistent with their effects on the cultured NPCs. The discrepancy found in the effects of the knockdown on neurogenesis in vitro and in vivo might be attributable to the difference in the context (or characteristics) of NPCs (e.g. astrogenic capacity) between them. Indeed, cultured NPCs can generate astrocytes, but NPCs in the neocortex during the embryonic stages cannot generate astrocytes. Expression of Ror2 is probably more prominent than that of Ror1 in the VZ (Al-Shawi et al., 2001). Consistent with the finding, we found that Ror2 knockdown in vivo exhibits a more severe effect on the maintenance of NPCs than Ror1 knockdown in vivo (Fig. 7C). Furthermore, compared with knockdown of Ror1 or Ror2 singly, double-knockdown of Ror1 and Ror2 in vivo showed more
severely affected distribution of cells within the neocortex (Fig. 7D), suggesting that Ror1 and Ror2 might function redundantly to regulate the maintenance of NPCs. The redundant function of Ror1 and Ror2 in NPCs was supported by the results of the double-knockdown experiments using cultured NPCs (supplementary material Fig. S3). However, it remains to be determined whether Ror1 and Ror2 regulate NPCs through the same molecular mechanism.

In conclusion, our present study reveals novel roles of Wnt5a–Ror1 and/or Wnt5a–Ror2 signaling in the maintenance of the proliferative and neurogenic state of NPCs. This regulation might be important for NPC generation of a large number of neurons in the developing neocortex. Furthermore, our results indicate that Wnt5a–Ror1 and Wnt5a–Ror2 signaling pathways act in a Dvl2-dependent and β-catenin-independent manner in NPCs. These observations suggest that, in addition to canonical Wnt–β-catenin signaling, noncanonical Wnt signaling plays a crucial role in the regulation of NPCs. Further study will be required to clarify whether any crosstalk exists between these two signaling pathways.

Materials and Methods

Animals

All the experiments using animals in this study were approved by the Institutional Animal Care and Use Committee (permission numbers P080212 and P100413) and carried out according to the Kobe University Animal Experimentation Regulations.

Antibodies and reagents

Antibody against Ror2 was prepared as described (Kani et al., 2004). The following antibodies were purchased commercially: mouse monoclonal antibodies against βIII-tubulin (TuJ1; Covance, Emeryville, CA), GFAP (GA-5; Sigma-Aldrich, St Louis, MO), β-actin (AC-5; Sigma-Aldrich) and bromodeoxyuridine (BrdU) (BD Pharmingen, San Diego, CA); a rat monoclonal antibody against BrdU (BU1/75; ICR); Abcam, Cambridge, MA); rabbit polyclonal antibodies against Ror1 (4102; Cell Signaling Technology, Danvers, MA), Nestin (Nestin 20, Covance), Dvl2 (3216; Cell Signaling Technology), Thrb (ab23345; Abcam), Pax6 (PRB-278P; Covance), Kid7 (ab15580; Abcam), GFP (ab290; Abcam) and RFP (FM005; MBL, Nagoya, Japan); goat polyclonal antibodies against Wnt5a (AF645; R&D Systems, Minneapolis, MN) and Sox2 (sc-17320; Santa Cruz Biotechnology, Santa Cruz, CA). Mouse purified Wnt5a was purchased from R&D Systems.

Plasmid constructs and siRNAs

Plasmids encoding mouse Ror1 and mouse Ror2 tagged with CFP or GFP at their C termini were constructed by subcloning the Ror1 and Ror2 cDNAs into pECFP and pEGFP plasmid vectors as described previously (Matsuda et al., 2003; Nishita et al., 2006; Oishi et al., 2003). The cDNAs encoding GFP, DsRed, Ror1-CFP and Ror2-GFP were subcloned into pcAGGS plasmid vector to generate pEGF-CFP, pCAG-DsRed, pCAG-Ror1-CFP and pCAG-Ror2-GFP, respectively. The control siRNA #1 (MISSION siRNA Universal Negative Control; Sigma-Aldrich) was used as a negative control for all 21-base pair siRNAs that were purchased from Sigma-Aldrich. The control siRNA #2 (Stealth RNAi Negative Control Medium GC Duplex #2; Invitrogen, Carlsbad, CA) was used as a negative control for Ror2 siRNA #1 (25-bp Stealth siRNA; Invitrogen). For the silencing of Ror1, Ror2 and Dvl2 in the embryonic neocortex, the BLOCK-iT Pol II miR RNA interference technology (Invitrogen) was used. Oligonucleotides were cloned into the pcDNA6.2-GW/EmGF-miR expression vector, which co-cistronically expresses the miRNA and EmGF. Chained sequences of the pre-miRNA insert were constructed according to the manufacturer’s protocol. The cDNAs encoding EmGF and the respective pre-miRNA inserts were subcloned into pCAGGS plasmid vector to generate pCAG-EmGF-miR vectors. All siRNA and miRNA target sequences are listed in supplementary material Table S1.

Cell culture and transfection

NPCs were isolated as follows: cerebral cortices were dissected from ICR mouse embryos at E14.5 and dissociated by incubation with 0.25% trypsin and 0.15 mg/ml DNase I (Sigma-Aldrich) for 10 minutes at 37°C, followed by washing and trituration in DMEM containing 10% (v/v) fetal bovine serum (FBS). The dissociated cells were cultured in DMEM/F12 supplemented with B27 (Invitrogen), 20 ng/ml BFGF (Peprotech, Rocky Hill, NJ) and 20 ng/ml EGF (Peprotech) in uncoated Petri dishes as neurospheres to expand NPCs. The neurospheres were passaged every 3 days. The dissociated cells from neurospheres in the second passage were replated at a density of 5 × 10^5 cells/cm^2 onto poly-L-lysine-coated dishes in DMEM/F12 supplemented with B27 and 10 ng/ml BFGF, and cultured for 24 hours before siRNA transfection. To induce differentiation, the medium was replaced with DMEM/F12 supplemented with B27 and 2% FBS and the cells were further cultured for 3–5 days. In some experiments, dividing cells were labeled with 10 μM BrdU (Sigma-Aldrich) for 3 hours or 24 hours. Primary astrocyte cultures were prepared from cerebral cortices dissected from neonatal mouse embryos. The tissues were dissociated as described above and plated in a flask. One week after seeding in DMEM containing 10% FBS, the flask was shaken at 200 r.p.m. for 20 hours at 37°C to remove mononucleocytede cells. The remaining cells were then seeded at a density of 5 × 10^5 cells/cm^2 and cultured for 48 hours before siRNA transfection. Transfection of siRNAs (each at 20 nM) was performed using Lipofectamine RNAiMax (Invitrogen) according to the manufacturer’s instructions. NIH3T3 cells were cultured in DMEM containing 10% FBS and transfected with expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

Immunoblotting and immunofluorescence microscopy

Cells were lysed in a buffer containing 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 5% (v/v) glycerol, 10 mM NaF, 5 mM EDTA, 1 mM β-aminophenyl methanesulfonyl fluoride (β-APMSF), 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Whole-cell lysates were subjected to analyses by immunoblotting as previously described (Nishita et al., 2006). For immunofluorescence analysis, cells were fixed and stained with the respective antibodies as previously described (Nishita et al., 2006). Fluorescent images were obtained using a laser scanning confocal imaging system (LSM710; Carl Zeiss Microimaging, Thornwood, NY) and processed using ImageJ (National Institutes of Health, Bethesda, MD) and Photoshop CS3 (Adobe, San Jose, CA).

Fluorescence-activated cell sorting

Cells were incubated with rat anti-CD133 (1:50; clone 13A4; Millipore, Billerica, MA) or goat anti-Ror2 (1:50, AF2064; R&D Systems) antibodies in FACS buffer (PBS containing 2 mM EDTA and 0.5% BSA) for 30 minutes on ice, and then washed twice with ice-cold FACS buffer. Subsequently, cells were incubated with secondary antibodies, Alexa Fluor 488 (for anti-rat IgG antibody; Invitrogen) or Alexa Fluor 488 (for anti-goat IgG antibody; Invitrogen) in FACS buffer for 30 minutes on ice, and then washed twice with ice-cold FACS buffer. Cells stained with secondary antibodies alone were used as a negative control. For the determination of each positive region, flow cytometric analysis was performed using Summit version 5.3 (Beckman Coulter, Miami, FL). FACS was performed on MoFlo XDP (Beckman Coulter).

RT-PCR and quantitative RT-PCR

Total RNA was extracted using IsoGen (Nippon Gene, Toyama, Japan), and 0.5 μg of total RNA was subjected to reverse transcription (RT)-PCR or quantitative RT-PCR (qRT-PCR) as previously described (Ren et al., 2011). Relative mRNA levels were determined by qRT-PCR after normalization by 18S ribosomal RNA. Primers used are listed in supplementary material Table S2.

Neurosphere formation analysis

NPCs were transfected with the various siRNAs and cultured for 24 hours. The cells were then detached with 0.05% trypsin and plated in a 24-well uncoated plates at a density of 2 × 10^4 cells per well in DMEM/F12 medium containing 1% (w/v) methylcellulose, B27, 20 ng/ml BFGF and 20 ng/ml EGF. The number of neurospheres with a diameter of over 30 μm was counted at 1 week after plating.

Reporter assay

NPCs were transfected with the different siRNAs. On the next day, cells were transfected with Topflash reporter construct containing TCF-responsive elements along with a renilla luciferase construct, and were further cultured for 48 hours under differentiating conditions. The cells were then treated with Wnt5a (100 ng/ml), Wnt3a (100 ng/ml) or vehicle alone for 4 hours. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s instructions.

In utero electroporation

In utero electroporation was performed as described previously (Saito and Nakatsuji, 2001). Plasmid DNAs were microinjected into the lateral ventricle of E14.5 mouse embryos. The electroporation was performed by using a square wave electroporator (CUY21Edit, Nepagene, Chiba, Japan). Five electrical pulses (40 V, 50-milliseconds duration at 950-millisecond intervals) were delivered. Three days after the electroporation, the brains from embryos were obtained and fixed with 4% paraformaldehyde in PBS at 4°C overnight. After equilibration with 30% (w/v) sucrose in PBS, the fixed tissue was embedded in OCT compound (Sakura Finetek, Tokyo, Japan) and frozen. Coronal sections were prepared by cutting the frozen brain with a cryostat at a thickness of 12 μm. After treatment with blocking buffer...
Data presentation and statistics
For quantification of the percentage of marker-positive cells in vitro, at least 800 cells in 8–12 randomly chosen microscopic fields in each single-staining experiment were counted and at least 200 cells in 10–12 randomly chosen microscopic fields in each double- or triple-staining experiment along with the respective antibodies staining. The values were obtained from at least three independent experiments. For quantification analysis in vivo, 100–200 EmGFP- or DsRed-antibody staining. The values were obtained from at least three independent groups were compared, or using one-way ANOVA followed by Bonferroni’s post-hoc test when more than three groups were analysed.

Acknowledgements
We are grateful to T. Tanoue for instruction on the in utero electroporation method. We also thank K. Yamagata for a critical reading of this manuscript.

Funding
This work was supported by a Grant-in-Aid for Scientific Research (B), a Grant-in-Aid for Young Scientists (B), a Grant-in-Aid for Scientific Research: Research on Innovative Areas, and by the Global Centers of Excellence Program from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and by grants from the Hyogo Science and Technology Association, the Uchira Memorial Foundation and the Astellas Foundation for Research on Metabolic Disorders.

Supplementary material available online at http://jcs.biologists.orglookup/suppl/doi:10.1242/jcs.097782/-/DC1

References


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### Table S1. Target sequences of siRNAs and miRNAs

<table>
<thead>
<tr>
<th>siRNA</th>
<th>Target sequences</th>
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<tr>
<td>Ror1</td>
<td>#1 GGCAAATTCTCCTCCGATT</td>
</tr>
<tr>
<td>Ror1</td>
<td>#2 CCCAATACCTCCTGGCTAT</td>
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<td>Ror2</td>
<td>#1 TCCCATCTTCTGCCACTTCTCTT</td>
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<td>Ror2</td>
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<td>Wnt5a</td>
<td>#1 GCAGATGTAGCCTGTAAGT</td>
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<tr>
<td>Wnt5a</td>
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<td>Dvl2</td>
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<tr>
<td>Dvl2</td>
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<table>
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<td>LacZ</td>
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<tr>
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<td>Ror2</td>
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<tr>
<td>Ror2</td>
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<td>Dvl2</td>
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Table S2. Primer sequences for RT-PCR

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<td>5′-TACGGCTGACAGAATCCATC-3′</td>
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<tr>
<td>Ror2</td>
<td>5′-TGAACACTTGGGAGTGCT-3′</td>
<td>5′-CTGCTTTTCTGTACAACGTGTG-3′</td>
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<tr>
<td>Wnt5a</td>
<td>5′-CAAATAGGCAGCCGAGAGAC-3′</td>
<td>5′-CTCTAGCGTCCACGAACTCC-3′</td>
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<tr>
<td>Nestin (qRT-PCR)</td>
<td>5′-AGGAACCATAAGACACAGGTG-3′</td>
<td>5′-TTCTCAGATGAGAGGTCAGA-3′</td>
</tr>
<tr>
<td>Nestin (qRT-PCR)</td>
<td>5′-TACAGGACTCTGCTGGAGGCTGAGA-3′</td>
<td>5′-CTGGTATCCCAGGAAATGGCAGCTT-3′</td>
</tr>
<tr>
<td>βIII-tubulin</td>
<td>5′-CAGGGCCAAGACAAAGCAG-3′</td>
<td>5′-GAGGACAGAGCCAGTGACG-3′</td>
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<td>GFAP</td>
<td>5′-TCCTTTTGCTGATCAGACTCC-3′</td>
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<td>GAPDH</td>
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