

The Expression Pattern of Nuclear Receptors During Cerebellar Development

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The cerebellum is essential for fine control of movement and posture, and it has been a useful model for studying many aspects of neural development because of its relatively simple anatomy and developmental program. However, the roles of nuclear receptors (NRs) underlying formation of the cerebellum and maintenance of cerebellar functions are still poorly characterized. As a contribution to the Nuclear Receptor Signaling Atlas (NURSA), we employed immunohistochemistry to investigate the expression pattern of 18 NRs in the cerebellum. Ten receptors were demonstrated to be expressed in the postnatal day 21 (P21) cerebellum. Among them, five receptors (COUP-TFI, COUP-TFII, ROR α , ER β , and ERR γ) were expressed at all stages (embryonic stage, P0, P7, and P21) examined. Interestingly, COUP-TFI and COUP-TFII show differential anterior-posterior expression patterns during cerebellar development. Taken together, our results suggest that members of the nuclear receptor superfamily might play importantly physiological roles in the cerebellum. *Developmental Dynamics* 236:810–820, 2007. © 2007 Wiley-Liss, Inc.

Key words: nuclear receptors; cerebellum; expression pattern; NURSA; COUP-TFI; COUP-TFII; ROR α ; ER β ; ERR γ ; anterior-posterior expression patterns

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INTRODUCTION

The cerebellum, and especially the cerebellar cortex, is a highly conserved and well-organized structure in the vertebrate central nervous system (CNS) (Goldowitz and Hamre, 1998). In addition to its well-known role in motor coordination, it also has been implicated in a variety of cognitive and affective functions (Leiner et al., 1993). These functions depend on precise interactions among at least five types of neurons named Purkinje cells, granule cells, Bergmann glia

cells, Golgi cells, and Basket/Stellate cells (Wang and Zoghbi, 2001). Purkinje cells arise from precursors in the ventricular neuroepithelium and migrate outward post-mitotically to reach their final location (Wang and Zoghbi, 2001). Unlike the other cerebellar cells, granule cells, which are the most abundant neurons in the cerebellum, are derived from a separate germinal epithelium known as the rhombic lip. They migrate onto the surface of the cerebellar anlage, where they form the external granule cell

layer (EGL), which is a secondary proliferative zone that continues to divide until about postnatal day 14 (P14) (Voogd and Glickstein, 1998; Wang and Zoghbi, 2001; Wingate, 2001). The post-mitotic descendants migrate inwardly along the Bergmann glia fibers to form the internal granule layer (IGL). The mature granule cells establish extensive synaptic contacts with mossy fibers, Golgi neurons, and Purkinje cells (Komuro and Yacubova, 2003). By the end of the third postnatal week (P21) in the mouse, the EGL

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TABLE 1. Methods to Determine NR Antibody Specificity^a

COUP-TFI	KO mice	VDR	Exogenous overexpression
COUP-TFII	KO mice	MR	Exogenous overexpression
PR	KO mice	SF-1	Exogenous overexpression
AR	KO mice	RAR α	Exogenous overexpression
FXR	KO mice	LXR α	Exogenous overexpression
ERR γ	RNAi knockdown	ER α	Demonstrated by Saji et al. (2000)
RXR α	RNAi knockdown	ER β	Demonstrated by Omoto et al. (2005)
HNF4 α	RNAi knockdown	LRH-1	Demonstrated by Gu et al. (2005)
ROR α	RNAi knockdown	GCNF	Demonstrated by Lan et al. (2003)

^aROR α , RAR-related orphan receptor α ; COUP-TFI, chicken ovalbumin upstream promoter transcription factor I; COUP-TFII, chicken ovalbumin upstream promoter transcription factor II; ER α , estrogen receptor α ; ER β , estrogen receptor β ; ERR γ , estrogen-related receptor γ ; VDR, vitamin D receptor; PR, progesterone receptor; AR, androgen receptor; MR, mineralocorticoid receptor; LRH-1, liver receptor homolog-1; GCNF, germ cell nuclear factor 1; LXR α , oxysterol receptor α ; SF-1, steroidogenic factor-1; FXR, farnesoid X-activated receptor; RAR α , retinoic acid receptor α ; RXR α , Retinoid X receptor α ; HNF4 α , hepatic nuclear factor 4 α .

ceases to exist, and the cerebellum achieves its mature laminated structure consisting of three layers: the molecular layer (ML), the Purkinje cell layer (PCL), and the granular layer (GL) (Voogd and Glickstein, 1998). Furthermore, along the anterior-posterior axis including the ten lobules, the vermis is also divided into the anterior (lobule I to V/VI) and posterior compartment (lobule V/VI to X) (Kuemerle et al., 1997). The existence of this morphological boundary for the anterior and posterior cerebellum is supported by observations of structural abnormalities that are restricted primarily to the anterior or posterior cerebellum lobules in loss of function assays, such as that observed for the *Engrailed-2* and *BETA2/NeuroD1* genes (Ackerman et al., 1997; Kuemerle et al., 1997; Cho and Tsai, 2006). In conclusion, the profound morphological transformations that occur during development make the cerebellum an extremely useful organ for studying neurogenesis.

Nuclear receptors (NRs) comprise a superfamily of conserved transcription factors that play essential roles in diverse biological processes such as embryonic development, homeostasis, reproduction, and neurogenesis (Mangelsdorf et al., 1995). NRs are generally ligand-activated transcription factors, and those factors whose ligands are not yet identified, are designated as orphan nuclear receptors (Chawla et al., 2001).

In addition to the retinoic acid receptor (RAR)-related orphan receptor

(ROR α) (Steinmayr et al., 1998; Gold et al., 2003), several other NRs, such as testicular orphan receptor 4 (TR4) (Chen et al., 2005) and Rev-Erb α (Chomez et al., 2000), have been reported to play crucial roles during cerebellar development. However, the physiological roles of other NRs in this process are largely unknown. Under the auspices of the Nuclear Receptor Signaling Atlas (NURSA) project, we characterized many NR antibody preparations and found that some of them were suitable for immunostaining. In this study, the detailed expression patterns of 18 nuclear receptors were determined during cerebellar development. For this systemic analysis of temporal and spatial NR expression, the embryonic stage and three different postnatal days (P0, P7, and P21) were examined, given the distinct changes in the cerebellum that occur at each stage (Wang and Zoghbi, 2001). Of the 18 NRs examined, ten receptors were identified to be expressed in the P21 cerebellum, and their dynamic expression patterns imply that this subset of nuclear receptors is likely to play a role in the development or maintenance of cerebellar function in adulthood. In addition, we focused more on chicken ovalbumin upstream promoter transcription factor I (COUP-TFI) (Wang et al., 1989), chicken ovalbumin upstream promoter transcription factor II (COUP-TFII) (Wang et al., 1991), ROR α , estrogen receptor β (ER β) (Kuiper et al., 1996), and estrogen-related receptor γ (ERR γ) (Heard et al.,

2000), since these five NRs are consistently expressed throughout cerebellar development. By providing extensive expression pattern data, this study provides the rationale basis for future studies aimed at understanding the diverse roles NRs play in the cerebellum. Furthermore, this study clearly illustrates the value of comprehensive expression profiling of a regulatory gene family so that future dissection of the physiological role played by individual family members can be achieved.

RESULTS AND DISCUSSION

Antibody Specificity

To determine the specificity of each NR antibody, we performed immunostaining experiments employing either tissues from specific receptor knockout mice, cells with exogenously over-expression of a specific receptor of interest, or cells treated with RNAi to specifically knockdown a given receptor. As shown in Figure 1A, COUP-TFI antibody positively stained cells are observed in the P21 cerebellum sections of wild-type mice, while no immunoreactivity is detected in the null mutant (Fig. 1B). To assess the specificity of COUP-TFII antibody, we examined P0 cerebellum sections of COUP-TFII conditional knockout mice (generated by Cre recombination of a COUP-TFII gene flanked by Flox sites (F/F), with Cre expression driven by the *neuron specific enolase* (*NSE*) promoter, see COUP-TFII^{NSE-Cre}; F/F in Fig. 1D). As compared with the null mutant (Fig.

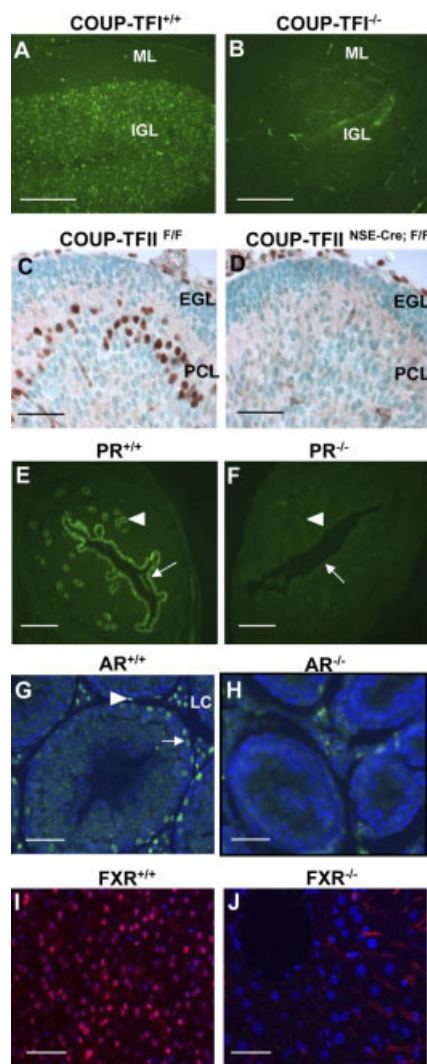


Fig. 1. Testing the specificity of NR antibodies in immunohistochemistry. COUP-TFI antibody (green) is used to stain P21 cerebellum sections of both wild-type control (A) and COUP-TFI knockout mice littermates (B). COUP-TFI knockout section does not show any specific signal in comparison with the control. COUP-TFII (brown) is clearly detected in the Purkinje cell layer of P0 wild-type control cerebellum (C), while no COUP-TFII immunoreactivity was detectable in the comparable section of COUP-TFII^{NSE-Cre; F/F} knockout mice (D). In contrast to the null mutant (F), PR (green) expression can be observed in luminal (arrow) and glandular epithelial cells (arrowhead) on the uterus section of wild-type mice (E). The wild-type and null mutant uterus are all from ovariectomized mice. AR (green) is expressed in the nuclei of Sertoli cells (arrow), peritubular myoid cells (arrowhead), and Leydig cells (LC) of wild-type testis (G), but undetectable in the null mutant (H). In contrast to the null mutant (J), FXR (red) is only detected in the nuclei of hepatocytes on the section of wild-type live tissue (I). DAPI is used for nuclear staining. ML, molecular layer; IGL, internal granule cell layer; EGL, external granule cell layer; PCL, Purkinje cell layer; LC, Leydig cells. Scale bars = 250 μ m (A, B, E, F); 50 μ m (C, D, G, H, I, J).

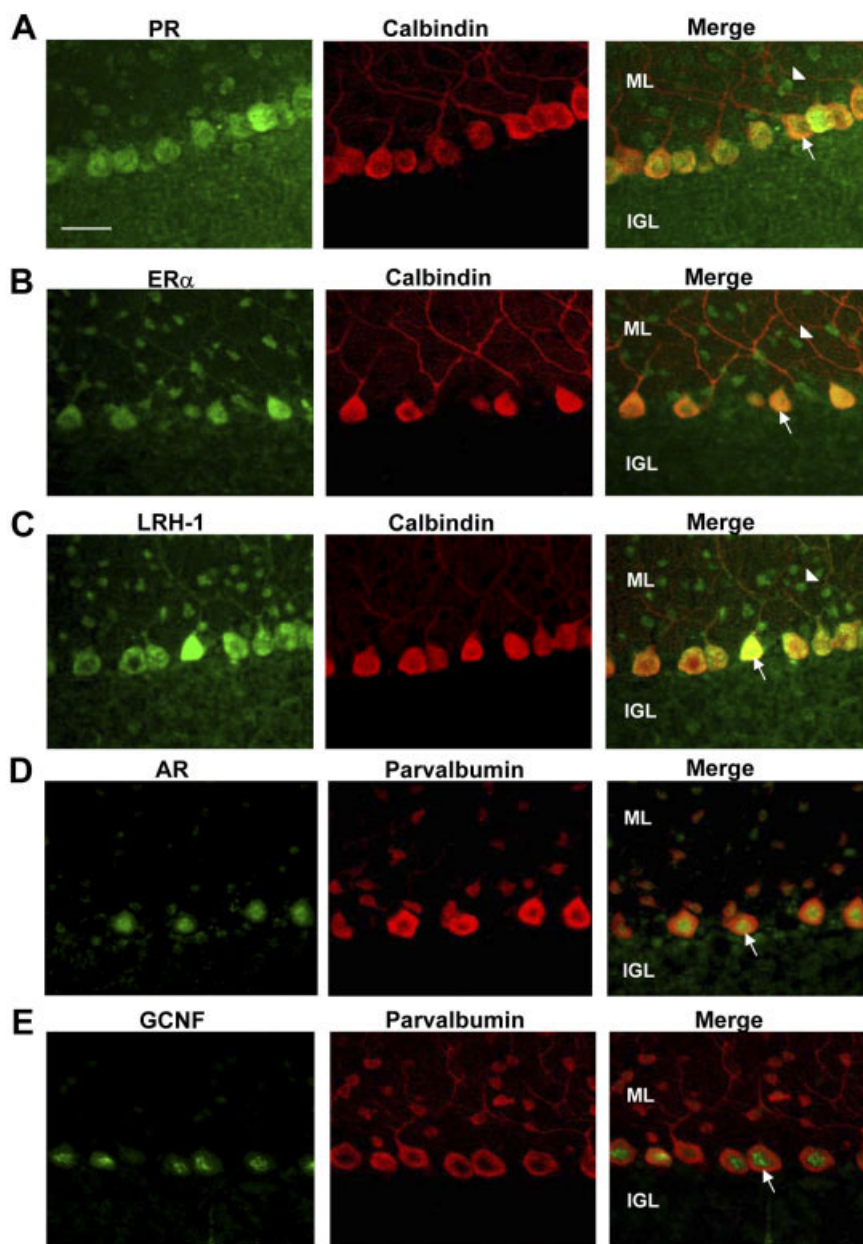


Fig. 2. Expression pattern of nuclear receptors in P21 cerebellum. Calbindin (Red), a Purkinje cells marker, was used for co-immunostaining with PR (A), ER α (B), and LRH-1 (C) on the middle-sagittal sections of P21 cerebellum. PR, ER α , and LRH-1 are expressed in the molecular layer (arrowheads) and Purkinje cells (arrows). AR (D) and GCNF (E) are detected in the nuclei of Purkinje cells (arrows), as indicated by the Basket/Stellate cell and Purkinje cell marker, Parvalbumin (Red). Scale bars = 30 μ m (A–E).

1D), only the section of a wild-type littermate (COUP-TFII^{F/F}) shows specific COUP-TFII signals localized in the Purkinje cell layer (PCL) (Fig. 1C). Uterine sections of progesterone receptor (PR) knockout mice were compared to that of wild-type mice to check the specificity of our PR antibody. Intense signals of PR were detected in the luminal and glandular epithelial cells of wild-type uterus (Fig. 1E), but no stain-

ing was observed in the PR null mutant (Fig. 1F). In order to validate the antibody for androgen receptor (AR), we examined the testis sections of AR^{+/+} (Fig. 1G) and AR^{-/-} (Fig. 1H) mice. As expected, AR was expressed only in the Sertoli cells, peritubular myoid cells, and Leydig cells (LC) in the wild-type AR section (Fig. 1G), consistent with previous reports (De Gendt et al., 2004). Finally, in comparison with wild-type

(Fig. 1I), no farnesoid X receptor (FXR) immunoreactivity was observed in the hepatocytes of adult liver tissue in the FXR null mice (Fig. 1J).

The specificities of ROR α , ERR γ , hepatic nuclear factor 4 α (HNF4 α), and RXR α antibodies were validated upon RNAi knockdown, and exogenous receptor over-expression demonstrated that the antibodies for vitamin D receptor (VDR), mineralocorticoid receptor (MR), RAR α , LXR α , and SF-1 are specific in immunostaining (data not shown, summarized in Table 1). Table 1 also cites several previous studies that tested the specificity of the estrogen receptor α (ER α), ER β , liver receptor homolog-1 (LRH-1), and germ cell nuclear factor 1 (GCNF) antibodies in immunostaining. In conclusion, all the NR antibodies utilized in this study are specific in immunostaining assays and are suitable for determining the expression pattern of these NRs during cerebellar development.

Nuclear Receptors Expression Patterns in the P21 Cerebellum

Among the 18 NRs examined, no signal was detected in P21 cerebellum for HNF4 α , FXR, RXR α , LXR α , VDR, MR, RAR α , or SF-1 proteins. However, in control experiments, they were detected in other tissues in which each was already known to be expressed (Sladek et al., 1990; Mangelsdorf and Evans, 1995; Zennaro et al., 1997; Zineb et al., 1998; Tulachan et al., 2003; Jeyasuria et al., 2004; Tanaka et al., 2006). These results suggest that they are not expressed or expressed at a very low level in the cerebellum at this stage of development.

All of the other ten NRs were detectable in P21 cerebellum. As a negative control, we performed immunohistochemistry without primary antibody incubation, and as expected, no signal was seen with primary antibody free slides (data not shown). To determine which cell types in the cerebellum expressed these ten NRs, we performed co-immunostaining using a Purkinje cell marker, Calbindin, or, instead, a Basket/Stellate and Purkinje cell marker, Parvalbumin. As shown in Figure 2A–E, PR, ER α , and LRH-1 are localized in both the cytoplasm

and nuclei of Purkinje cells and Basket/Stellate cells, while AR and GCNF are exclusively expressed in the nuclei of Purkinje cells. In addition, uniform expression of PR, AR, ER α , LRH-1, and GCNF are seen throughout the anterior-posterior axis (data not shown). Besides being expressed in P21, COUP-TFI, COUP-TFII, ROR α , ERR γ , and ER β are also expressed in the cerebellum throughout the other developmental stages examined (embryonic stage, P0, and P7), and their expression patterns are further described below. However, the expressions of PR, AR, ER α , LRH-1, and GCNF are undetectable in P7 cerebellum (data not shown).

The Expression of COUP-TFI and COUP-TFII in the Developing Cerebellum

COUP-TFI expression is detected in cerebellar anlage around embryonic day 13.5 (E13.5). As shown in Figure 3A, COUP-TFI is weakly expressed in the ventricular neuroepithelium, which will give rise to the different kinds of cerebellar precursor cells, but not granule cells. In addition, relatively strong signals can be observed in the rhombic lip from which granule cells arise. At P0, COUP-TFI is highly expressed in the EGL of the anterior compartment, as compared to that of the posterior part (Fig. 3B, top; arrows). After birth, cells in the EGL undergo extensive proliferation to generate a large pool of granule cell precursors (GCP). As new GCPs are generated, older cells exit the cell cycle, move inward from the pia surface to the pre-migratory zone, located underneath the proliferating zone, and then differentiate (Komuro and Yacubova, 2003). To address whether COUP-TFI is expressed in proliferating or post-mitotic cells, we performed co-immunostaining using a cell proliferation marker, Ki-67. In the EGL, the width of the COUP-TFI-positive cell zone is slightly thicker than that of the Ki-67-positive zone (Fig. 3B, bottom), suggesting that COUP-TFI is expressed in both mitotic and post-mitotic cells. At P7, different lobules and layers of cerebellum can be easily distinguished (Wang and Zoghbi, 2001). As seen in P0 cerebellum, COUP-TFI is more intensely ex-

pressed in the anterior than in the posterior portion of the EGL in P7 (Fig. 3C, top middle and right). At this stage, the intense immunoreactivity begins to be observed in the Purkinje cell layer. However, it is not co-localized with the Calbindin (Fig. 3C, top middle and right), indicating that COUP-TFI is not expressed in Purkinje cells. Given the localization of the positive cells, we then tested whether COUP-TFI might instead be localized in Bergmann glia cells by using a Bergmann glia cell marker, brain lipid binding protein (BLBP) in co-immunostaining assays. Clearly, COUP-TFI is localized in the cell body of Bergmann glia cells (Fig. 3C, bottom). In addition, co-immunostaining with a granule cell marker, Pax6, shows that COUP-TFI is also expressed in granule cells, besides Bergmann glia cells, in the P21 cerebellum (Fig. 3D). However, COUP-TFI is more strongly expressed in the Bergmann glia cells. Finally, we determined that the weakest COUP-TFI immunostaining in P21 cerebellum localizes to the Basket/Stellate cells, since they are co-localized with Parvalbumin in ML (Fig. 3D). In all cases, positive signals localized within nuclei.

For COUP-TFII, weak expression is detected at E13.5 (data not shown). As development proceeds, intense signals are observed in the cerebellar primordium at E18.5, and the majority of these positive cells are restricted to the middle part of the cerebellar cortex (Fig. 4A). At P0, compartmental expression of COUP-TFII is more obvious. As shown in Figure 4B, COUP-TFII expression expands to the anterior region of the cerebellum. Major COUP-TFII immunoreactivity is observed in the anterior half of the Purkinje cell layer, while it is hardly detectable in the posterior part. In P7 and P21 cerebellum, similar expression patterns are observed. In the lobule IX, not every Purkinje cell expresses COUP-TFII, and COUP-TFII is hardly detectable in the lobule X (Fig. 4C and D). In contrast, the expression level in the anterior region is robust, but it is still lower than that seen in the embryo stage. Finally, at P7 and P21, co-immunostaining with Calbindin further demonstrates that COUP-TFII is expressed only in the nuclei of Purkinje cells, but not in any

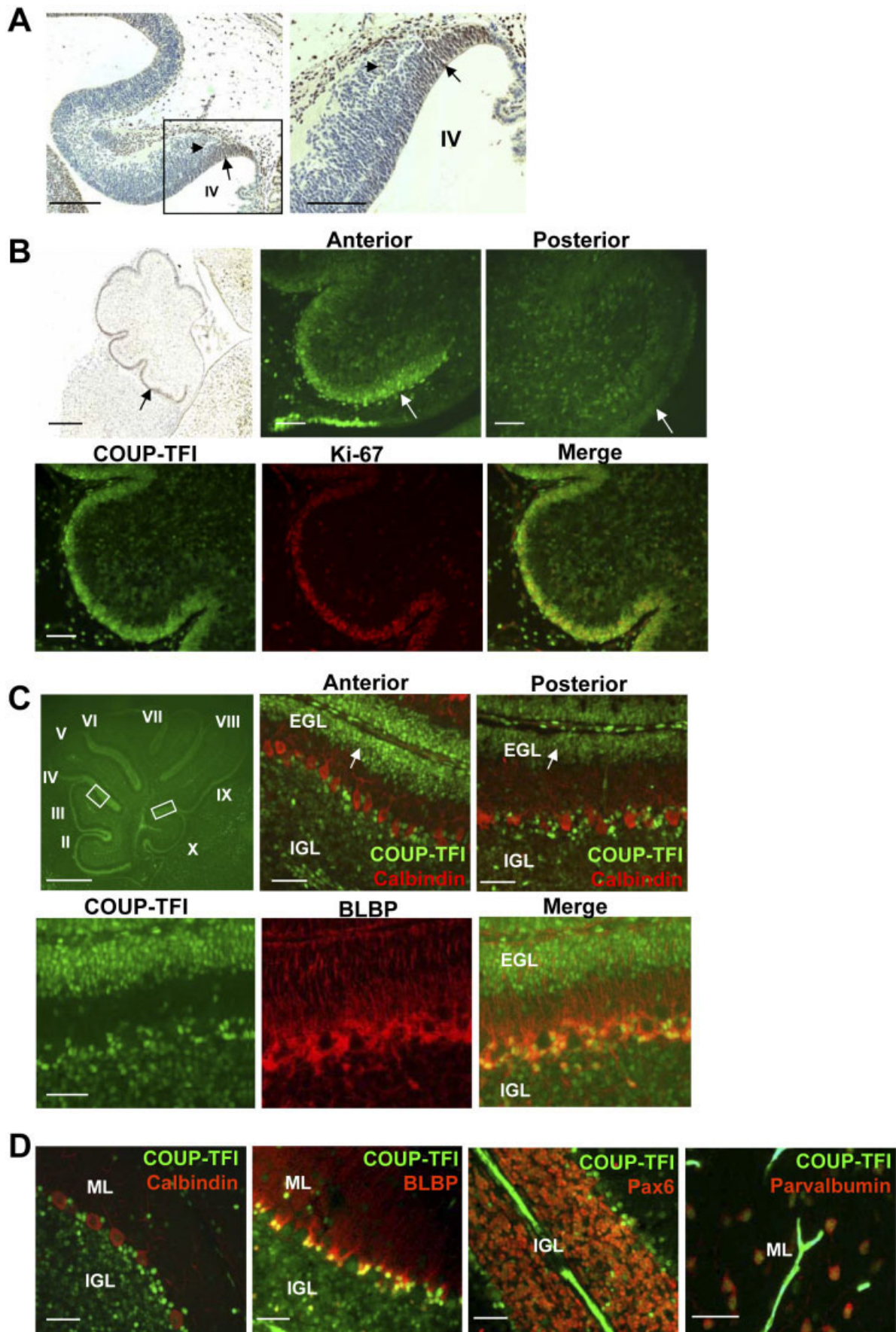


Fig. 3.

other cerebellar cell types (Fig. 4E and data not show).

The Expression Patterns of $ERR\gamma$, $ROR\alpha$, and $ER\beta$ in the Developing Cerebellum

$ERR\gamma$ expression can first be observed at E18.5 (Fig. 5A). At P0, strong signals are detected in deeper layers of the cortex (Fig. 5B), whereas no immunoreactivity is observed in the EGL. At P7, $ERR\gamma$ is weakly expressed in the ML, but intense immunostaining can be seen in the cells localized in the IGL (Fig. 5C, arrowheads). To further characterize the cell types where $ERR\gamma$ is expressed at this stage, Calbindin, Pax6, which is a marker for granule cells at the different developmental stages (Yamasaki et al., 2001), and Pax2, a marker of Basket/Stellate cell precursors and immature Golgi neurons at P7 (Maricich and Herrup, 1999), were used for co-immunostaining. As shown in Figure 5C, $ERR\gamma$ is expressed in Basket/Stellate cell precursors and immature

Golgi neurons (Fig. 5C, see arrowhead in right panel), but not in Purkinje cells (Fig. 5C, left panel) and granule cells (Fig. 5C, middle panel) such as pre-migratory granule cells and migrating granule cells (Fig. 5C, arrows). Furthermore, a similar expression pattern was still observed at P21. As shown in Figure 5D, $ERR\gamma$ is co-localized with Parvalbumin and the Golgi cell marker, Pax2, but not with BLBP. Therefore, $ERR\gamma$ is expressed in Basket/Stellate cells in the ML and Golgi cells in the granule cell layer (GL), but not in the Purkinje cells or Bergmann glia cells at P21. In addition, the immunoreactivity observed in Golgi cells is more intense than that seen in Basket/Stellate cells.

Mice with *staggerer* were found to carry a deletion within the $ROR\alpha$ gene that blocks Purkinje cells differentiation, resulting in congenital ataxia and cerebellar hypoplasia (Sidman et al., 1962; Dussault et al., 1998), and $ROR\alpha$ knockout mice show phenotypes similar to those seen with *staggerer* mice (Dussault et al., 1998; Steinmayr et al., 1998; Gold et al., 2003). Given these phenotypes, we next systematically analyzed the $ROR\alpha$ expression pattern in the cerebellum during different developmental stages. Weak $ROR\alpha$ expression begins at E12.5 (Fig. 6A, arrow) and expressing cells populate the presumptive cortex by E14.5 (Fig. 6B). As shown in Figure 6C, $ROR\alpha$ is uniformly expressed in the Purkinje cell layer at P0 along the anterior-posterior axis, which is different from COUP-TFII, which is expressed more in the anterior compartment. At P7, $ROR\alpha$ expression is not restricted to Purkinje cells, as positive staining is also seen in cells within the molecular layer (Fig. 6D, arrowhead). This expression is not co-localized with Pax6 (data not show), suggesting that the staining cells are not the migrating granule cells. At P21, all $ROR\alpha$ -positive cells are Parvalbumin-positive (Fig. 6E). In the granule cell layer, no $ROR\alpha$ -immunoreactive cells are observed, implying that granule cells, as well as Golgi cells, do not express $ROR\alpha$. Therefore, $ROR\alpha$ is expressed in the Basket/Stellate and Purkinje cells of the cerebellum.

At E12.5, a high level of $ER\beta$ expression is observed in the cerebellar

primordium. Surprisingly, almost all the cells in the cerebellar primordium are $ER\beta$ -positive (Fig. 7A, top left). At P0, strong $ER\beta$ immunoreactivity is seen in the EGL (Fig. 7A, top right). Furthermore, some scattered cells in the deeper layers of the cortex also express $ER\beta$. In rats, the generation of Basket cells peaks around P7, and afterwards Stellate cells are then created (Wang and Zoghbi, 2001). Given that the timetable of the cerebellar neurogenesis in rats is similar to that in mice, the majority of the $ER\beta$ -positive cells we observed should be migrating granule cell precursors, which is further confirmed by co-immunostaining with a granule cell marker, Pax6 (data not shown). Meanwhile, in the EGL, both proliferating and differentiating granule cell precursors are $ER\beta$ -positive (Fig. 7A, bottom). A similar pattern is observed in the EGL at P7 (Fig. 7B, bottom). At this stage, $ER\beta$ expression is co-localized with Pax6 in the EGL and IGL, indicating that this receptor is expressed in the granule cells (Fig. 7B, top). By P21, it is clear that $ER\beta$ is expressed in granule and Basket/Stellate cells, but not in Purkinje cells (Fig. 7C), consistent with a previous study (Perez et al., 2003).

Concluding Remarks

Table 2 summarizes the expression patterns of the ten positively staining NRs that were observed in this study, including localization to particular cell types and at different developmental stages.

From this study, the examined NRs can be divided into two functional categories. The first category includes AR, $ER\alpha$, $ER\beta$, and PR, which are known to be the classical endocrine receptors that mediate the actions of steroid hormones (Mangelsdorf et al., 1995). Except for $ER\beta$, they are expressed only at P21, implying that this subset of nuclear receptors may play roles in the maintenance of cerebellar function in adulthood after the cerebellum is fully differentiated. Consistent with this hypothesis, it has been reported that estrogen treatment decreases the levels of γ -amino butyric acid α (GABA α) receptors in the cerebellum (Bar-Ami et al., 1993), and that estrogens and anti-estrogens al-

Fig. 3. Expression pattern of COUP-TFI in the developing cerebellum. Immunohistochemistry demonstrates that COUP-TFI is expressed in the ventricular neuroepithelium (arrowhead) and rhombic lip (arrow) at E13.5 (A; right panel represents a higher magnification of the boxed area in the left panel), and it is mainly expressed in the EGL (arrow) at P0 (B, top left panel). Compared with its staining in the posterior part, COUP-TFI (green) is expressed at a higher level in the anterior part of EGL (B, top middle and right panels; arrow). Moreover, in the EGL, both the proliferative and post-mitotic granule cell precursors are COUP-TFI positive (green) at P0, as indicated by a cell proliferation marker, Ki-67 (red) (B, bottom panels). C: Left panel is low magnification of COUP-TFI expression at P7 and every lobule is indicated. High magnification indicates COUP-TFI (green) is strongly expressed in the anterior part of the EGL (middle and right panels; arrow). Furthermore, COUP-TFI is not co-localized with Calbindin (red) (middle and right panels), and the intense immunoreactivities in the Purkinje cell layer are co-localized with a Bergmann glia cell marker, BLBP (red) (bottom panels). At P21, COUP-TFI (green) is highly expressed in the Bergmann glia cells, moderately detectable in the granule cells, and weakly expressed in Basket/Stellate cells, as indicated by staining of the respective cell-specific markers (BLBP, Pax6, and Parvalbumin) (D). Position of the fourth ventricle (IV) is indicated. Scale bars = 200 μ m (A, left panel); 100 μ m (A, right panel); 400 μ m (B, top left panel); 50 μ m (B, top middle and right panels, bottom panels); 40 μ m (C, D).

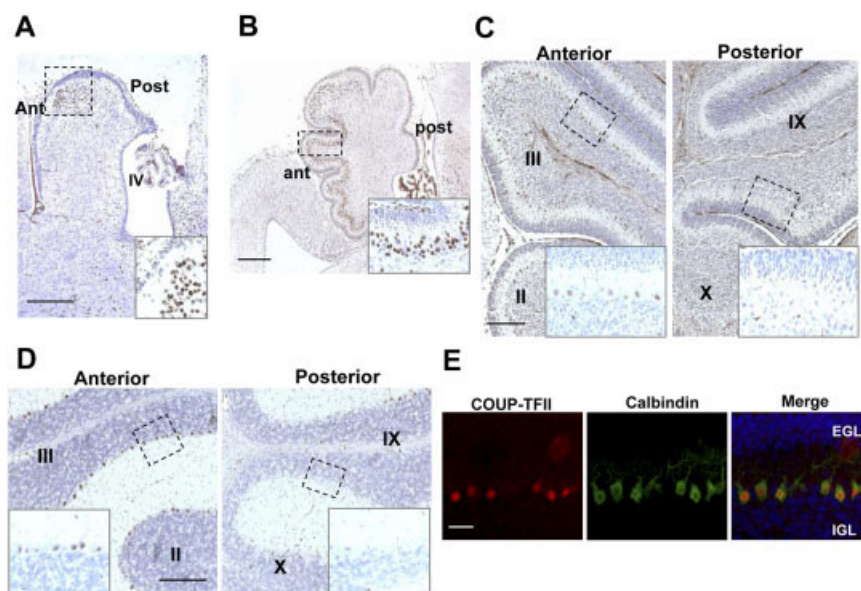


Fig. 4.

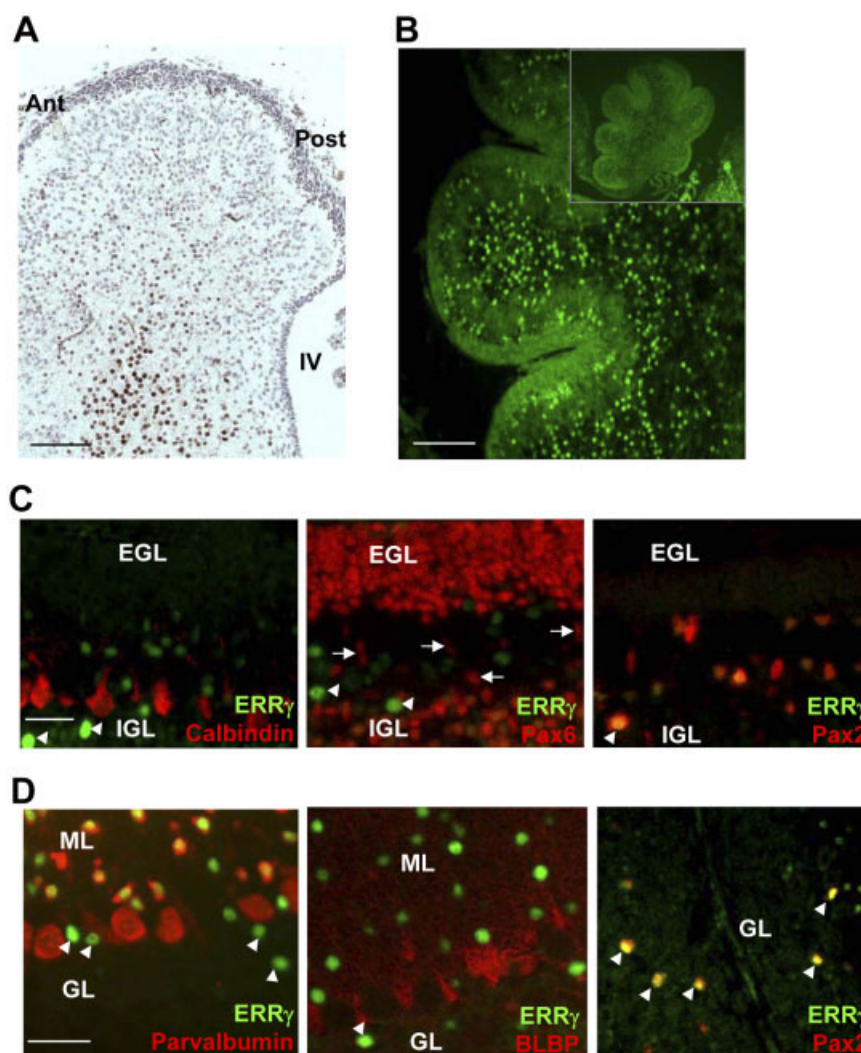


Fig. 5.

ter the response of Purkinje cells to glutamate (Smith et al., 1988; Smith, 1989). Together with our expression data, these studies suggest that estrogen receptors may play a role in the maintenance of cerebellar function.

The second category of nuclear receptors includes COUP-TFI, COUP-TFII, GCNF, $ERR\gamma$, LRH-1, and $ROR\alpha$, which are all classified as orphan receptors (Mangelsdorf et al., 1995). Their temporal expression patterns suggest they are more likely to be important in cerebellar development. The observed $ROR\alpha$ expression pattern at the different stages we examined was not surprising, since it was previously reported that this receptor plays a crucial role in the cerebellum development such as Purkinje cell differentiation and maturation (Dussault et al., 1998; Steinmayr et al., 1998; Gold et al., 2003). Except for $ROR\alpha$, the detailed expression pattern of COUP-TFI, COUP-TFII, GCNF, $ERR\gamma$, and LRH-1 in the cerebellum, however, was not known before this study. Among these five receptors, the

Fig. 4. Expression pattern of COUP-TFII in the developing cerebellum. **A:** The majority of COUP-TFII-positive cells (brown) are restricted to the middle part of cerebellar anlage at E18.5. **B:** At P0, intense immunoreactivity can be observed in the anterior region of the Purkinje cell layer. At P7 and P21, COUP-TFII is weakly expressed in Purkinje cells and the expression level decreases along the anterior-posterior axis (**C,D**). Every lobule is marked, and COUP-TFII-positive neurons indicated by dashed line are magnified as an inset (**A-D**). In addition, co-immunostaining with Calbindin (green) identifies that COUP-TFII (red) is expressed only in the Purkinje cells of P7 cerebellum (**E**). Scale bars = 200 μ m (**A, C, D**); 400 μ m (**B**); 40 μ m (**E**).

Fig. 5. Expression pattern of $ERR\gamma$ in the developing cerebellum. $ERR\gamma$ (brown) was expressed in the cerebellar primordium at E18.5 (**A**), and intense immunoreactivity was observed in the cells localized in deeper layers of the cortex at P0 (**B**). The inset in **B** is a lower magnification of the $ERR\gamma$ pattern at P0. **C:** Co-immunostaining with Pax2 (red; right) identified that $ERR\gamma$ (green) was expressed in Basket/Stellate cell precursors and immature Golgi neurons (arrowheads) of P7 cerebellum, while it was not co-localized with Calbindin (left) or Pax6 (middle). Migrating granule cells in the middle panel are indicated with arrows. **D:** $ERR\gamma$ is co-localized with Parvalbumin in the ML (left) and Pax2 in the IGL (right), but not expressed in the Bergmann glia cells that were stained by BLBP (middle). Golgi cells are indicated by arrowheads. Scale bars = 100 μ m (**A, B**); 30 μ m (**C, D**).

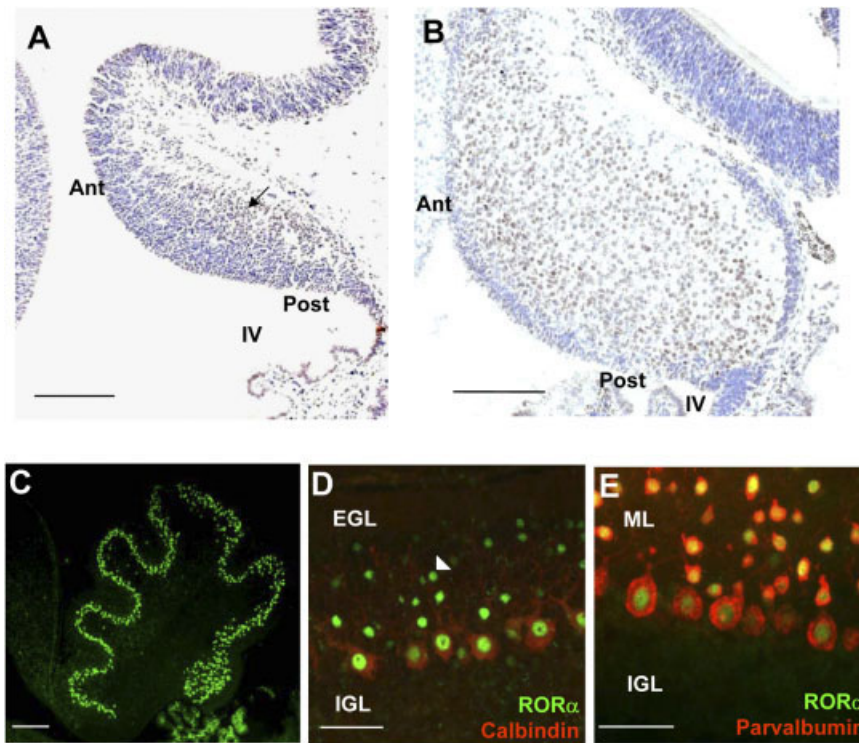


Fig. 6.

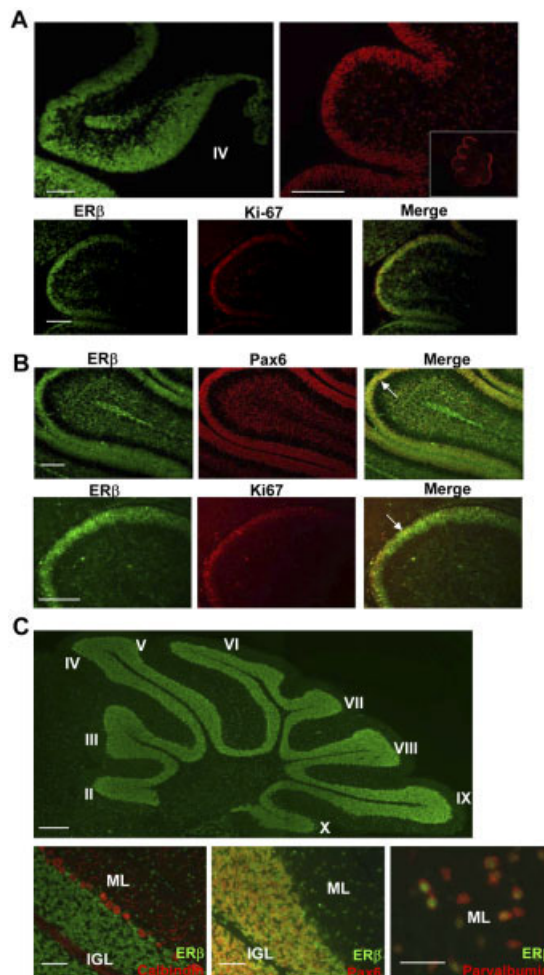


Fig. 7.

COUP-TFs are well-characterized orphan nuclear receptors. COUP-TFI and COUP-TFII show more than 97% amino acid identity in their DNA binding and putative ligand-binding domains (Qiu et al., 1994). However, COUP-TFI and COUP-TFII expression patterns are quite distinct from each other. In general, COUP-TFI is more highly expressed in the neuronal tissue of the central and peripheral nervous systems, whereas COUP-TFII is expressed in the mesenchyme of developing organs (Pereira et al., 1995). Loss of function assays indicate that COUP-TFI is important for neurogenesis and neural crest cell differentiation during embryonic development (Qiu et al., 1997; Zhou et al., 2001; Yamaguchi et al., 2004), while COUP-TFII acts as a major regulator of angiogenesis, vein identity, and organ development (Pereira et al., 1999; Takamoto et al., 2005; You et al., 2005). In the cerebellum, the expressions patterns of these two receptors are not co-localized. COUP-TFI is highly expressed in Bergmann glia cells with lower expression detected in granule and Basket/Stellate cells, while COUP-TFII is only expressed in Purkinje cells. Interestingly,

Fig. 6. Expression pattern of ROR α in the developing cerebellum. Weak ROR α signals are detected in the cerebellar primordium at E12.5 (A) and positive cells are localized in the cerebellar cortex at E14.5 (B). It is more obvious that ROR α is expressed in the Purkinje cell layer at P0 (C). At P7, except for the Purkinje cells, ROR α begins to be detectable in the ML (arrowhead) (D). In P21 section, all the ROR α -positive cells are co-localized with Parvalbumin (E). Scale bars = 200 μ m (A–C); 40 μ m (D, E).

Fig. 7. Expression pattern of ER β in the developing cerebellum. ER β (green) is strongly expressed in almost all the cells of cerebellar anlage at E12.5 (A, top left) and a high level of ER β (red) expression is detected in the EGL at P0 (A, top right). Furthermore, there are some positive cells localized in deeper layers of the cortex. The inset is a lower magnification of ER β expression at P0. At P0 and P7, ER β is expressed in both proliferating and post-mitotic granule precursor cells (as seen by Ki-67 marker staining) (A,B: bottom). At P7, ER β is co-localized with Pax6 (B, top). The EGL is indicated by arrows. In P21 cerebellum, ER β (green) is expressed in the granule and Basket/Stellate cells (see Pax6 and Parvalbumin staining), but not in the Purkinje cells (as seen by comparison to the Calbindin) (C). Scale bars = 100 μ m (A; B, bottom); 250 μ m (B, top); 1 mm (C, top); 50 μ m (C, bottom).

TABLE 2. NR Expression Pattern During Cerebellar Development^a

NR	Embryonic stage	P0	P7				P21		
			EGL	ML	PCL	IGL	ML	PCL	IGL
ROR α	+	+++	—	+++	+++	—	+++ (B/S)	+++ (PC)	—
COUP-TFI	++ (E13.5)	++ (EGL: Proliferating and post-mitotic cell)	+++ (Proliferating and post-mitotic cell)	+	+++ (BG)	+	+	+++ (BG)	++ (GC)
COUP-TFII	+	+++	—	—	+	—	—	+	—
ER α			—	—	—	—	+	+	—
ER α	+++ (E12.5)	+++ (EGL: Proliferating and post-mitotic cell)	+++ (Proliferating and post-mitotic cell)	+	—	++	+	—	++ (GC)
ERR α	++ (E18.5)	+++	—	+	—	+++ (Immature Golgi cell)	+++ (B/S)	—	+++ (Golgi cell)
PR			—	—	—	—	+	+	—
AR			—	—	—	—	—	++ (PC)	—
LRH-1			—	—	—	—	—	+	—
GCNF			—	—	—	—	—	++ (PC)	—

^aEGL, external granule cell layer; ML, molecular layer; PCL, Purkinje cell layer; IGL, internal granule cell layer; PC, Purkinje cell; BG, Bergmann glia cell; GC, granule cell; B/S, Basket/Stellate cells. —, absence; +, low intensity staining; ++, medium intensity staining; +++, high intensity staining.

they both show different expression patterns along the anterior-posterior axis in the EGL or in Purkinje cells. Unlike COUP-TFI, COUP-TFII is highly expressed only in the anterior half of the cerebellum in a manner similar to that of Sonic hedgehog (Shh), which is important for cerebellar foliation (Corrales et al., 2004, 2006). Since COUP-TFII is more likely to be a downstream target of Shh signaling (Krishnan et al., 1997; Takamoto et al., 2005), it is reasonable to expect that COUP-TFII may play an important role in cerebellar development. Additionally, when one considers the temporal expression pattern of COUP-TFI and ERR γ , it is probable that they may also function during cerebellar development.

In conclusion, this study provides a platform for future exploration of the critical function of individual nuclear receptors in cerebellar development. Future conditional ablation of the

NRs in the cerebellum will precisely reveal their individual physiological roles.

EXPERIMENTAL PROCEDURES

Mice (C57BL/6J) were purchased from the Jackson Laboratory. The day on which a vaginal plug was observed was considered embryonic day 0 (E0), and the day of birth was taken as postnatal day 0 (P0). The mouse embryos and the cerebellum at different developmental stages were fixed in 4% paraformaldehyde and embedded in paraffin. Samples were sectioned in a middle-sagittal manner with a 5–7- μ m thickness. Antibodies to HNF4 α , RXR α , FXR, VDR, MR, COUP-TFI, COUP-TFII, ERR γ , SF-1, RAR α , and LXR α were provided by PPMX, Perseus Proteomics, Inc., Tokyo, Japan. LRH-1 and GCNF antibodies were a gift of Austin J. Cooney.

AR, ER α , and ROR α were purchased from Santa Cruz Biotechnology, Santa Cruz, CA (sc-816, MC-20, sc-542). PR was obtained from Dako, Carpinteria, CA (A0098), and ER β was provided by Jan-Ake Gustafsson. Immunohistochemistry was performed using the ABC kit (Vector, Burlingame, CA) and sections were incubated with biotin-conjugated secondary antibodies and visualized under bright-field microscopy with a diaminobenzidine substrate (DAB) kit (Vector). For co-immunostaining, the TSA system (Invitrogen, Carlsbad, CA; nos. 22 and 25) was applied and processed as described by the manufacturer. The cerebellar cell markers were purchased from Chemicon (Temecula, CA; Calbindin D-28K, AB1778; BLBP, AB9558; Pax6, AB5409), Swant (Parvalbumin, PV-28), BD Pharmingen (Ki-67, 550609), and Covance (Pax2; PRB-276P). DAPI (Vector) was used for nuclear staining. siCONTROL and siR-

NAs against RXR α , MR, VDR, and HNF4 α were purchased from Ambion, while siRNAs to ROR α and ERR γ were from Dharmarcon.

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