RESEARCH ARTICLE

Retinoid Signaling Competence and RARβ-Mediated Gene Regulation in the Developing Mammalian Telencephalon

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To study retinoid signaling in the developing telencephalon, we transfected a retinoid reporter gene into different regions of developing telencephalon. We found that the ventral telencephalon was more competent to retinoid signaling than the dorsal telencephalon. Moreover, among all retinoic acid receptors (RARs) and retinoid X receptors (RXRs), RARβ was strongly induced by retinoic acid in the ventral telencephalon, suggesting that RARβ might be involved in retinoid signaling competence. The RT-PCR analysis indicated that RARβ was selectively expressed in the developing striatum of ventral telencephalon. We then demonstrated that null mutations of *RAR*β gene resulted in reduction of striatal-enriched tyrosine phosphatase (STEP) mRNA in the striatum of RARβ$^{-/-}$ mutant mice. Conversely, the gain-of-function study showed that ectopic expression of RARβ1 in the cerebral cortex enhanced STEP expression, and the effect was RARβ-isoform specific. Our study identified RARβ as an important molecule for transducing retinoid signals in developing ventral telencephalon. *Developmental Dynamics 232:887–900, 2005.*
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INTRODUCTION

Retinoids are important for development and homeostasis in vertebrates (Kastner et al., 1995; Ross et al., 2000). The powerful regulation of developmental process by retinoid signaling reflects that retinoid receptors are ligand-inducible transcriptional regulators (Leid et al., 1992; Mangelsdorf et al., 1995). Retinoid signals are transduced by binding to two families of receptors, retinoic acid (RA) receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ; Leid et al., 1992). Evidence suggests that RAR/RXR heterodimers mainly underlie transduction of retinoid signals *in vivo* (Kastner et al., 1997).

The developing telencephalon comprises the dorsal pallium and the ventral subpallium (Wilson and Rubenstein, 2000). The pallium includes the cerebral cortex (CTX), whereas the subpallium includes the lateral ganglionic eminence (LGE, striatal primordium) and the medial ganglionic eminence (MGE, primordium for the pallidum, basal forebrain, and telencephalic interneurons). Previous studies have shown that the RA-synthesizing enzymes, reti-

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naldehyde dehydrogenases (RALDHs), are expressed in the embryonic nervous system (McCaffery and Drager, 1994; Li et al., 2000; Mic et al., 2000; Suzuki et al., 2000; Smith et al., 2001; Niederreither et al., 2002). During prenatal development of telencephalon, RALDH3 is primarily expressed in the LGE and RALDH1 is expressed in the dopamine-containing mesostriatal system and meninges, whereas RALDH2 is present only in the meninges. Consistent with the presence of RALDH3 and RALDH1 in the LGE and its derivative striatum, high levels of retinoids are detected in the developing striatum (McCaffery and Drager, 1994; Zetterstrom et al., 1999; Toresson et al., 1999).

Given that retinoids are present in the developing telencephalon, a critical question is, which subtypes of RARs and RXRs and their associated transcriptional cofactors are involved in transduction of retinoid signaling in the developing telencephalon? Moreover, the molecular mechanisms of retinoid signal transduction by RARs and RXRs have not yet been characterized in developing telencephalic neurons, albeit the mechanisms are mostly studied in non-neuronal cell lines (Leid et al., 1992; Mangelsdorf et al., 1995; Chen and Evans, 1995; Kurokawa et al., 1995; Burke and Baniahmad, 2000; Glass and Rosenfeld, 2000).

In an attempt to understand the molecular mechanisms of retinoid-mediated gene regulation in the developing telencephalon, we carried out a series of experiments by first assaying retinoid signaling competence using an adapted retinoid reporter gene method. We then examined the expression profiles of RARs and RXRs and the endogenous levels of retinoids. We finally used the loss and gain of function approaches to explore RARβ-mediated gene regulation by studying RARβ$^{-/-}$ mutant mice and by transfecting different RARβ isoforms into CTX cells. Our study suggests that ligand-contingent de-repression of transcriptional activity underlies retinoid signal transduction in the telencephalon and that RARβ is important in transducing retinoid signals in developing ventral telencephalon.

RESULTS

Region-Dependent Retinoid Activity in Explant Cultures of Developing Telencephalon

To detect the endogenous retinoid activity in the developing telencephalon, we used the retinoid reporter construct β(RARE)$_4$–pTK-LUC in which four copies of the RA response element (RARE) from mouse *RAR*β gene were fused with a minimal thymidine kinase (TK) promoter upstream of the reporter gene of firefly luciferase (LUC; Saitou et al., 1994). The activity of the retinoid reporter gene was first tested by cotransfecting β(RARE)$_4$–pTK-LUC with the expression plasmids of RARα, RARβ1, or RXRγ into COS-1 cells. Robust induction of the reporter gene activity was detected in the transfected cells when the transfected cells were treated with all-*trans* RA (1 μM; data not shown). The β(RARE)$_4$–pTK-LUC or its control vector (pTK-LUC) were then cotransfected with pCMV–β-galactosidase into cells of the CTX, LGE, and MGE of embryonic day (E) 15 rat forebrain by electroporation, and the tissues were then cultured for 2 days *in vitro*.

When the β(RARE)$_4$–pTK-LUC vector was transfected into explant culture without the retinol treatment, its activity was 11.5 ± 2.2%, 43.2 ± 3.7%, and 39.5 ± 6.9% of the pTK-LUC activity in the CTX, LGE, and MGE, respectively (at least n = 5 for each group; Fig. 1A–C). These results suggested that binding of RAR/RXR to the RAREs were repressive for TK promoter activity in explant culture. Of interest, the RARE-mediated repressive activity was higher in the CTX of dorsal telencephalon than that in the LGE and the MGE of ventral telencephalon.

We then assayed retinoid reporter gene activity in the transfected cultures in the presence of the RA precursor retinol. With the treatment of all-*trans* retinol (1 μM), the repression of reporter gene was significantly relieved in the LGE (43.1 ± 3.7% vs. 111.9 ± 26.1%; Fig. 1B), whereas the repressive activity remained mainly unchanged in the CTX (11.5 ± 2.2% vs. 10.9 ± 2.5%; Fig. 1A) and in the MGE (39.5 ± 6.9% vs. 33.7 ± 7.6%; Fig. 1C). As RA synthesizing enzymes were required to convert retinol to RA, these results suggested that RALDH enzymes were present in the LGE, which was at good accord with the previous reports that RALDH3, RALDH1 and endogenous retinoids were present in the LGE (McCaffery and Drager, 1994; Toresson et al., 1999; Li et al., 2000; Smith et al., 2001).

The physiological concentration of RA has been estimated to be in the range of 0.1–10 nM (Blaner and Olson, 1994). If the low activity of retinoid reporter gene in the CTX and the MGE cultures were due to insufficient amounts of endogenous RA, increasing RA concentrations should relieve the repression of reporter gene in the CTX and the MGE. In line with this hypothesis, application of exogenous RA increased the β(RARE)$_4$–pTK-LUC activity in the CTX and the MGE cultures. With all-*trans* RA (1 μM) treatment, the repression of reporter gene was fully relieved in the LGE (from 21.6 ± 3.7% to 154.0 ± 28.1%; Fig. 1E) and the MGE (from 61.3 ± 15.5% to 402.3 ± 61.3%; Fig. 1F), but the repression was only partially relieved in the CTX (from 9.8 ± 1.2% to 24.1 ± 4.1%; Fig. 1D). These findings suggested that the LGE and the MGE of ventral telencephalon were more competent to RA signaling than the CTX of dorsal telencephalon.

To demonstrate the specificity of retinoid activity that was detected with the retinoid reporter gene assay, we transfected a dominant-negative mutant of RARs (pCMX–Epi-RAR–E) into explant culture. The dominant-negative mutant is impaired in ligand binding but with intact abilities of heterodimerization and DNA binding, and the mutant has been shown to be capable of inhibiting RARα, RARβ, and RARγ activity (Saitou et al., 1994). The transfection of pCMX–EpiRAR–E significantly reduced the RA-induced β(RARE)$_4$–pTK-LUC activity in all the three cultures of CTX, LGE, and MGE (Fig. 1G–I), which demonstrated the specificity of retinoid activity that was detected with the retinoid reporter gene assay.

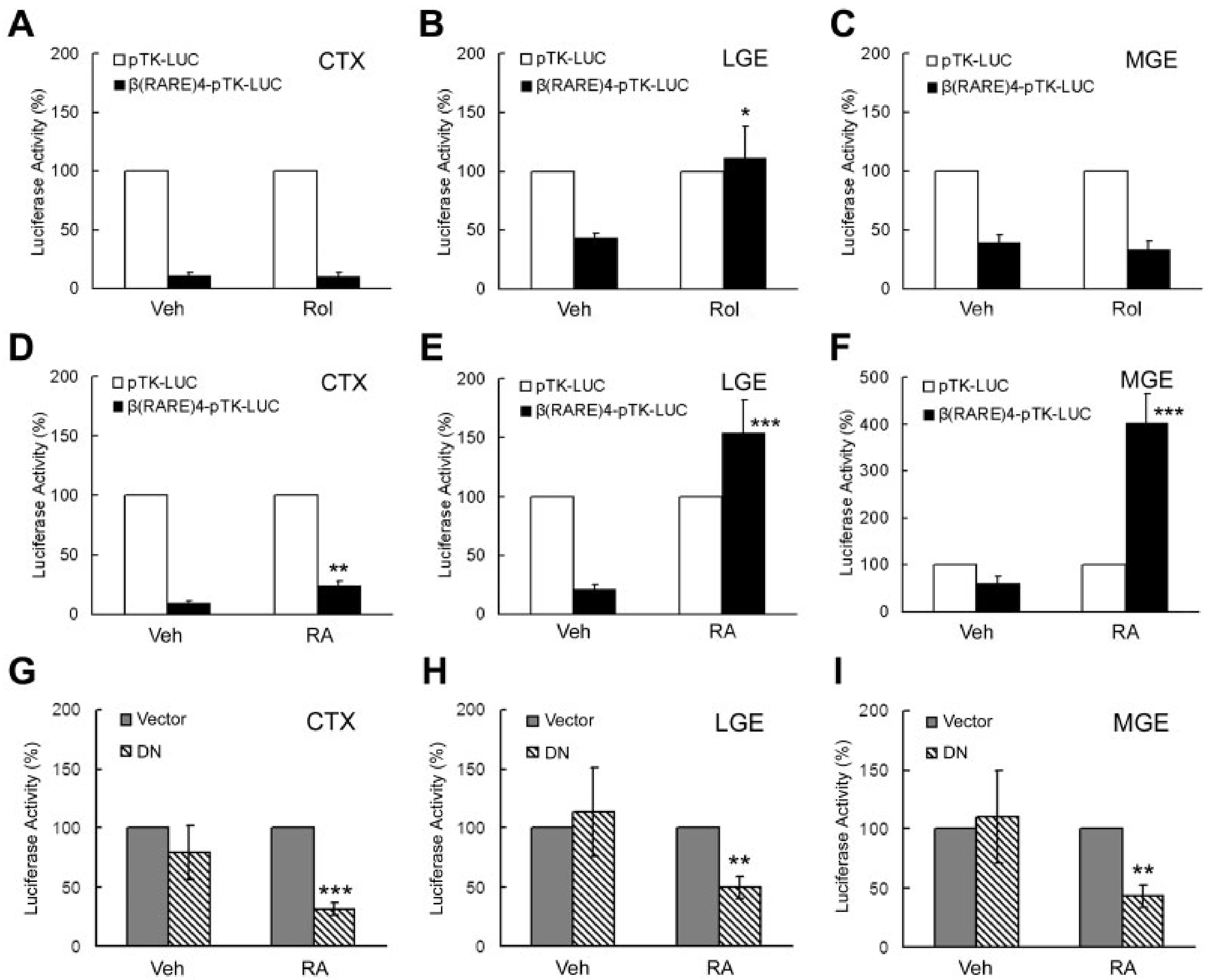

Fig. 1. Retinoid reporter gene activity in explant cultures of different regions of embryonic day 15 rat telencephalon. **A–C:** Without the retinol treatment (Veh), the activity of β(RARE)$_4$–pTK-LUC was significantly lower than that of pTK-LUC in all the CTX, LGE, and MGE cultures. B: With the all-*trans* retinol treatment (Rol, 1 μM), the activity of β(RARE)$_4$–pTK-LUC was selectively increased in the LGE culture. **D–F:** In the presence of all-*trans* RA (1 μM), the activity of β(RARE)$_4$–pTK-LUC was significantly increased in the CTX (D), LGE (E), and the MGE (F) cultures at different degrees with prominent increases in the ventral telencephalon of LGE and MGE. **G–I:** Cotransfection of the dominant-negative mutant (DN) of pCMX–EpiRAR–E with β(RARE)$_4$–pTK-LUC resulted in significant reduction of retinoid reporter gene activity in all the three cultures of CTX (G), LGE (H), and MGE (I). CTX, cerebral cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; RARE, retinoic acid response element; Vector, pCMX vector; Veh, vehicle of retinol or RA. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, comparing the groups with retinol and without retinol treatment, the groups with RA and without RA treatment, or the vector with the DN groups by Student's *t*-test, respectively. Data represent at least n = 5 for each set of experiment.

Expression Profiles of RARs, RXRs, and Their Associated Transcriptional Cofactors in the Developing Telencephalon

The activity of retinoid reporter gene in the transfected cells is presumably affected by at least two factors. One is the expression level of retinoid receptors and their associated transcription cofactors, and the other is the endogenous levels of retinoids in the tissue. We performed reverse transcription-polymerase chain reaction (RT-PCR) assay to determine the expression levels of RARs, RXRs and associated transcription corepressors and coactivators in different regions of the telencephalon during development. The PCR primers were designed to detect all isoforms of each subtype of RARs and RXRs genes (Leid et al., 1992). Differential expression pattern was found for RARβ, RXRγ, RARα, and RARγ mRNAs in E15 explant cultures (Fig. 2A). For explant cultures without any drug treatment, RARβ was selectively expressed in the LGE (Fig. 2A). Similarly to RARβ, RXRγ was preferentially expressed in the LGE. RXRγ was barley detectable in the MGE and was not detectable in the CTX (Fig. 2A). RARα was expressed at higher levels in the LGE and MGE than the CTX. RARγ was barely detectable in the CTX and was not detectable in the LGE and MGE. The expression of RXRα and RXRβ was found in all three cultures without a differential expression pattern (Fig. 2A).

We also assayed the expression levels of each subtype of RARs and RXRs in E15 explant tissues cultured for 2

days *in vitro* with RA treatment. The exogenous application of all-*trans* RA (1 μM) strongly increased RARβ expression in all three cultures of CTX, LGE, and MGE as well as in the hindbrain (HB) culture (Fig. 2A). The quantitative RT-PCR indicated that RA induced 12.75-fold increases of RARβ mRNA in the LGE (12.75 ± 1.66, $P < 0.001$, n = 3; Fig. 2C). For the CTX and MGE where endogenous RARβ was below detectable level, RA induced 1.65-fold and 6.59-fold increases of RARβ mRNA in the CTX and MGE, respectively, compared with that in vehicle-treated LGE (1.65 ± 0.08, $P < 0.05$, n = 3; 6.59 ± 0.96, $P < 0.01$, n = 3; Fig. 2C). RXRγ mRNA appeared to be induced by RA in the CTX (Fig. 2A). The treatment of all-*trans* RA did not significantly alter the expression levels of RARα, RARγ, RXRα, and RXRβ in the CTX, LGE, and MGE cultures except that a strong induction of RARα was found only in the HB culture (Fig. 2A). These results indicated that it was RARβ, not other RARs, that was inducible in the developing telencephalon.

As the expression patterns of some retinoid receptors and associated transcriptional cofactors have not been characterized in details in the developing telencephalon, we examined their expression profiles by RT-PCR assay. The nuclear receptor corepressor (N-CoR) and the silencing-mediator of retinoid and thyroid hormone receptors (SMRT; Chen and Evans, 1995; Kurokawa et al., 1995; Burke and Baniahmad, 2000; Glass and Rosenfeld, 2000) were expressed in E15 CTX, LGE, and MGE cultures without apparent regional variations (Fig. 2B). The coactivators steroid receptor coactivator-1 (SRC-1) and P300/CBP-associated factor (P/CAF; Glass and Rosenfeld, 2000) were also expressed in the three regions at comparable levels (Fig. 2B). The RA treatment did not appear to change the expression levels of these four cofactors in any of the CTX, LGE, and MGE cultures (Fig. 2B). To control the genomic DNA contamination, all RT-PCR reactions were run in parallel with controls without reverse transcriptase. No DNA band was detected in the control group (data not shown).

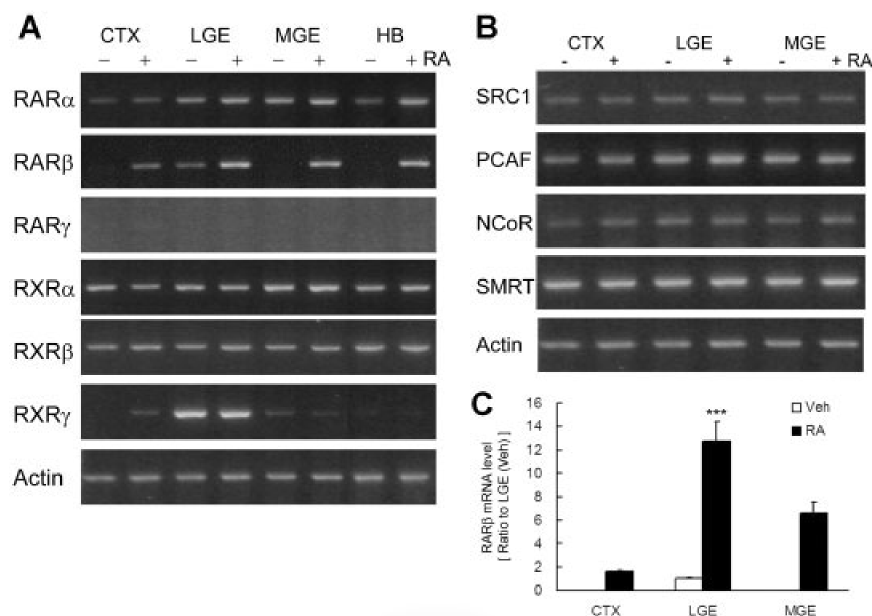

Fig. 2. Expression profiles of RARs, RXRs, and associated transcriptional cofactors in embryonic day 15 telencephalic explant cultures. **A:** The CTX, LGE, MGE, and hindbrain (HB) explant tissues were cultivated in the presence of all-*trans* RA (1 μM) for 2 days in vitro. Induction of RARβ mRNA is found in all three types of cultures, whereas RARα mRNA is only induced in the HB culture. RXRγ mRNA appears to be induced in the CTX. **B:** Expression profiles of retinoid receptors associated coactivators (SRC-1, P/CAF) and corepressors (N-CoR, SMRT). These four cofactors are ubiquitously expressed in the CTX, LGE, and MGE cultures, and the RA treatment does not appear to alter the expression levels of these cofactors. **C:** The quantitative reverse transcriptase-polymerase chain reaction indicates that RA induces 12.75-fold increases of RARβ mRNA in the LGE culture. For the CTX and MGE where endogenous RARβ was below detectable level, RA induces 1.65-fold and 6.59-fold increases of RARβ mRNA in the CTX and MGE, respectively, compared with that in vehicle-treated LGE. CTX, cerebral cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; Veh, vehicle. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, comparing the groups with RA and with vehicle treatments by Student's *t*-test. Data are representative of n = 3 for each set of experiment.

Detection of Retinoids in Explant Cultures of Developing Telencephalon

Another critical factor in determining the activity of retinoid reporter gene in the explant culture was the concentration of endogenous retinoids. We tested whether RA was present in our explant culture system with a retinoid reporter cell line. We used the Sil-15 retinoid reporter cell line that was derived from F9 teratocarcinoma cells. The Sil-15 cells were transfected with a reporter gene cassette containing a RARE from the human *RAR*β gene that was placed upstream of the *Escherichia coli lacZ* gene (Wagner et al., 1992). The Sil-15 cells express endogenous RARα, RARβ, and RXRγ, which renders the cells sensitive to retinoids, as the reporter gene will be activated in response to retinoid stimulation. We first cultured explant tissue alone in the membrane of culture inserts for 1 day *in vitro*. The inserts were then placed in culture dishes where Sil-15 cells were growing, and the coculture was carried out overnight. The detection of β-gal–positive Sil-15 cells in the presence of cocultured explants, thus, would indicate the release of retinoids from the cocultured explant tissue. As the RA precursor retinol was not synthesized in the brain tissue, all-*trans* retinol was omitted or added into the culture medium for comparing the effects of retinol. In the absence of retinol, all the three brain regions induced little β-gal response of Sil-15 cells without regional differences (Fig. 3A–C,I). In the presence of retinol, the E15 rat LGE induced a dramatic increase of β-gal–positive Sil-15 cells (Fig. 3E,I), whereas the responses of Sil-15 cells were much lower with cocultures of the MGE or the CTX (Fig. 3D,F,I). These findings suggested that the local concentration

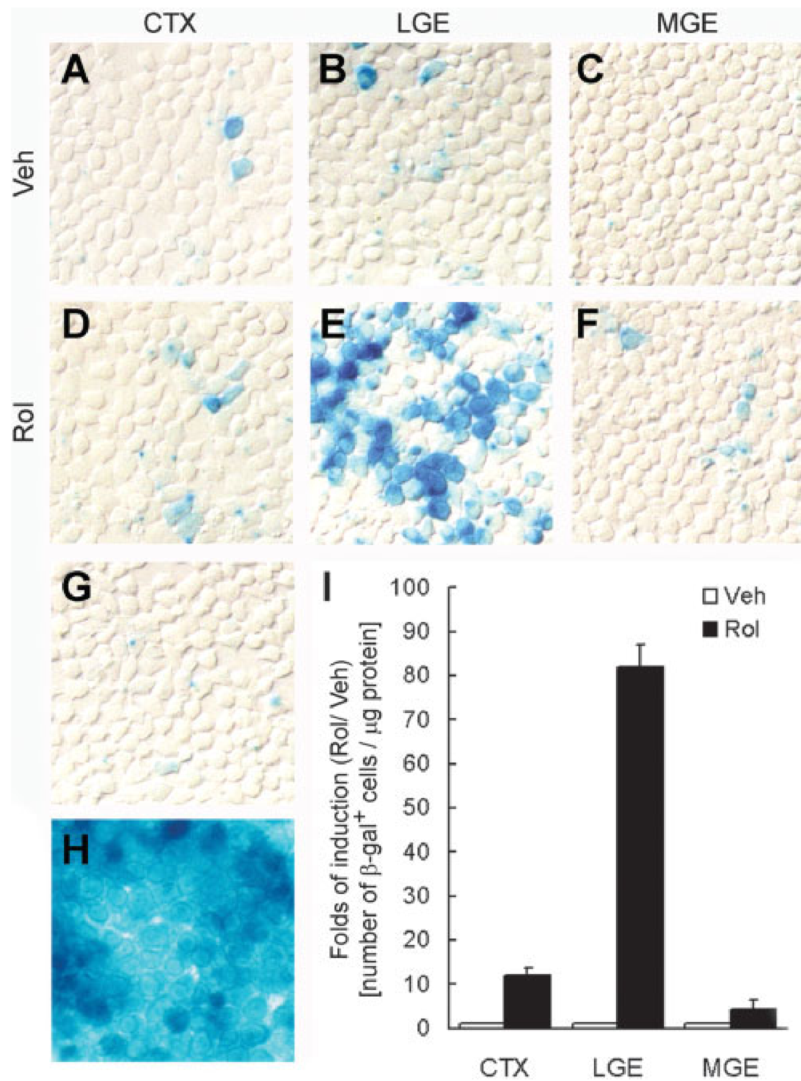

Fig. 3. Endogenous levels of retinoids in the developing telencephalon. The embryonic day 15 CTX, LGE, and MGE explants are cocultured with the Sil-15 retinoid reporter cells. After coculturing overnight, the Sil-15 cells are processed for X-gal staining of β-galactosidase reporter gene activity. **A–C:** Without the retinol treatment (Veh), few β-gal–positive cells are present, regardless of the tissue types of cocultured explants. **D–F:** With the retinol treatment (Rol), many β-gal–positive Sil-15 cells appear with the coculture of LGE (E), whereas the β-gal responses remain low in the Sil-15 cells that are cocultured either with the CTX (D) or with the MGE (F). **G:** Few β-gal–positive Sil-15 cells are detected without cocultured explant tissue even with retinol treatment. **H:** Positive control of Sil-15 cells in response to all-*trans* RA (1 μM) without coculture. **I:** The quantification of the β-gal responses of Sil-15 cells cocultured with different explant tissues. The β-gal response is most prominent in the Sil-15 cells that were cocultured with the LGE in the presence of retinol. CTX, cerebral cortex; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; Rol, retinol; Veh, vehicle of retinol. A–H: Data are representative of n = 3 for the experiments.

of retinoids was significantly higher in the LGE than that in the CTX and the MGE, which were in agreement with a previous study using different retinoid reporter cell lines (Toresson et al., 1999). Our study differed from the previous study in that we used the transmembrane culture system with a chemically defined culture medium. By manipulating the availability of retinol, we were able to demonstrate a regional difference of retinol-dependent retinoid activity, which presumably reflected the RA-synthesizing ability in each brain region. This retinol-dependent effect had not been clearly shown in the previous study. Because the retinoid reporter gene assay was carried out by electroporating a retinoid reporter gene construct into explant culture (see above, Fig. 1), we also tested whether the electroporation per se would affect retinoid activity in the transfected explant culture. The results showed that there was no statistical difference between the electroporated and nonelectroporated cultures in activation of β-gal responses of Sil-15 cells (data not shown).

Retinoid-Mediated Gene Regulation in the Developing Telencephalon

Regulation of RARβ Gene Expression by Retinoid Signaling.

To determine whether retinoids were capable of regulating endogenous gene expression in the developing telencephalon, we first assayed RA-mediated regulation of the RA-responsive gene *RARβ* in the developing telencephalon. The *RARβ* gene is known to be autoregulated by retinoids through RARE-dependent mechanism (Sucov et al., 1990; de The et al., 1990; Wu et al., 1992). The RAREs of the retinoid reporter construct $\beta(RARE)_4$–pTK-LUC were in fact derived from mouse *RARβ* gene. Indeed, the above RT-PCR analyses showed that exogenous RA could increase RARβ mRNA expression in the CTX, LGE, and MGE cultures (Fig. 2A,C), which was correlated with the parallel increases of retinoid reporter gene activity by RA (Fig. 1D–F). It appeared, however, to be paradoxical that, although exogenous RA did not fully reverse the suppression of $\beta(RARE)_4$–pTK-LUC activity back to the level of pTK-LUC in the CTX (Fig. 1D), RARβ mRNA was increased by RA in the CTX (Fig. 2A,C). Nonetheless, RA did increase $\beta(RARE)_4$–pTK-LUC activity in the CTX, indicating that a partial de-repressive activity by RA could lead to transcription of the reporter gene. Therefore, the increases of RARβ by RA in the CTX may be due to a partial de-repressive mechanism. However, one cannot exclude the possibility that *trans*-activation of the reporter gene and *RARβ* gene may be different, as *trans*-activation of gene expression may be influenced by different configurations of the synthetic and the natural promoters (Nagpal et al., 1992a).

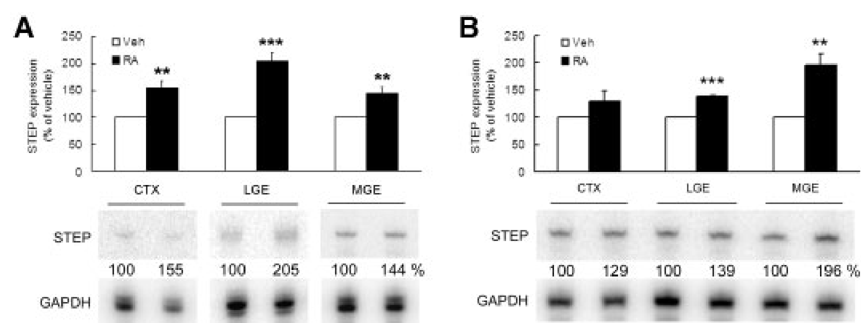

Fig. 4. Induction of striatal-enriched tyrosine phosphatase (STEP) mRNA expression by RA in telencephalic explant culture. **A:** All-*trans* RA (1 μM) increases STEP mRNA expression by 1.55-fold, 2.05-fold, and 1.44-fold, respectively, in the CTX, LGE, and MGE explant tissues cultivated for 1 day in vitro as measured with the ribonuclease protection assay. **B:** In the explant tissues cultivated for 2 days *in vitro*, all-*trans* RA (1 μM) increases STEP mRNA expression by 1.29-fold, 1.39-fold, and 1.96-fold in the CTX, LGE, and MGE cultures, respectively. Data are normalized with the internal control GAPDH. CTX, cerebral cortex; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LGE, lateral ganglionic eminence; MGE, medial ganglionic eminence; RA, retinoic acid; Veh, vehicle of RA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, comparing the groups with RA and with vehicle treatments by Student's *t*-test. Data are representative of at least n = 4 for (A) and n = 3 for (B).

Regulation of Striatal-Enriched Tyrosine Phosphatase Gene Expression by Retinoid Signaling.

In addition to *RAR*β gene, we also screened for genes that were responsive to RA stimulation in the developing telencephalon. We cultured LGE explant culture and measured the expression levels of several LGE/striatum-enriched genes in response to exogenous RA stimulation (Wang et al., 1999). One gene that we found to be regulated by RA was the striatal-enriched tyrosine phosphatase (STEP; Lombroso et al., 1991). The ribonuclease protection assay showed that all-*trans* RA (1 μM) significantly enhanced STEP mRNA expression by 2.05-fold in E15 LGE explants cultivated for 1 day *in vitro* (1 DIV; 2.05 ± 0.16, $P < 0.001$, n = 5; Fig. 4A). All-*trans* RA also increased STEP mRNA levels by 1.44-fold and 1.55-fold in the MGE and CTX cultures, respectively (1.44 ± 0.12, $P < 0.01$, n = 5; 1.55 ± 0.13, $P < 0.01$, n = 4; Fig. 4A). We also assayed the effects of RA on STEP mRNA expression in E15 explants cultivated for 2 DIV (Fig. 4B). In this case, all-*trans* RA (1 μM) increased STEP mRNA by 1.96-fold, 1.39-fold, and 1.29-fold in the MGE, LGE, and CTX cultures, respectively (1.96 ± 0.20, $P < 0.01$, n = 3; 1.39 ± 0.01, $P < 0.001$, n = 3; 1.29 ± 0.20, $P > 0.05$, n = 3; Fig. 4B). Therefore, RA could increase STEP mRNA in all three types of cultures with different kinetics, and the STEP induction by RA was higher in the LGE (1 DIV) and the MGE (2 DIV) than that in the CTX. We also assayed the mRNA of the neuronal cytoskeleton gene MAP-2. All-*trans* RA (1 μM) did not significantly alter MAP-2 mRNA levels in the LGE, MGE, and CTX cultures, which demonstrated the specificity of gene regulation by RA (data not shown).

Reduction of STEP Expression in the Striatum of RARβ Null Mutant Mice

The induction of STEP by RA in the LGE and the MGE cultures suggested that the *STEP* gene expression in the ventral telencephalon might be regulated by retinoid signaling. We then took a genetic approach to ask whether STEP mRNA expression was regulated by retinoids in vivo by studying the gene-targeting mice carrying null alleles of retinoid receptor. We studied the RARβ mutant mice, as RARβ was selectively expressed in the LGE of ventral telencephalon and RARβ was inducible by RA in the developing telencephalon (Fig. 2). As STEP mRNA was primarily expressed in the striatum (an LGE derivative) of the ventral telencephalon (Lombroso et al., 1991), we analyzed its expression level in the mutant striatum. The *in situ* hybridization showed that STEP mRNA was significantly reduced in the striatum of newborn $RAR\beta^{-/-}$ mutant mice (79.6 ± 6.3%, $P < 0.05$, n = 3; Fig. 5A,B,E). Consistently, the examination of adult $RAR\beta^{-/-}$ mutant mice also showed significant decreases of STEP expression in the caudoputamen of dorsal striatum (87.0 ± 3.7%, $P < 0.01$, n = 4) and the nucleus accumbens of ventral striatum (79.6 ± 4.4%, $P < 0.01$, n = 4; Fig. 5C,D,F). These results were in agreement with the explant culture experiment that RA was capable of regulating STEP expression in the developing striatum, and the regulatory mechanism might be maintained at adulthood. It further indicated that the retinoid signaling mediated by RARβ was involved in regulating striatal STEP expression.

Increases of STEP Expression by Ectopic Expression of RARβ1 in the CTX

The mutant mice study indicated that RARβ was involved in regulating STEP expression. The *RAR*β gene has four different isoforms that are generated by different uses of two promoters and alternative splicing, and the four isoforms are highly conserved among different species (Zelent et al., 1991; Leid et al., 1992; Nagpal et al., 1992b). Previous study using probes recognizing RARβ1/3 or RARβ2/4 has shown that RARβ1/3 and RARβ2/4 mRNAs are expressed in the developing brain (Mollard et al., 2000). All four isoforms were disrupted in the RARβ null mutant mice that were used in the present study (Ghyselinck et al., 1997). As RARβ was not detected in the CTX (Fig. 2A), we then asked whether ectopic expression of RARβ in CTX cells was sufficient to enhance STEP expression, and if so, whether there was an isoform-specific regulation. We electroporated each isoform of *RAR*β gene into E15 CTX, and the transfected CTX was then cultured for 2 days with or without all-*trans* RA (1 μM). The transfection of RARβ1 into the CTX enabled RA to induce 1.52-fold increases of STEP mRNA in transfected cells (1.52 ± 0.21, $P < 0.05$, n = 3; Fig. 6A). In

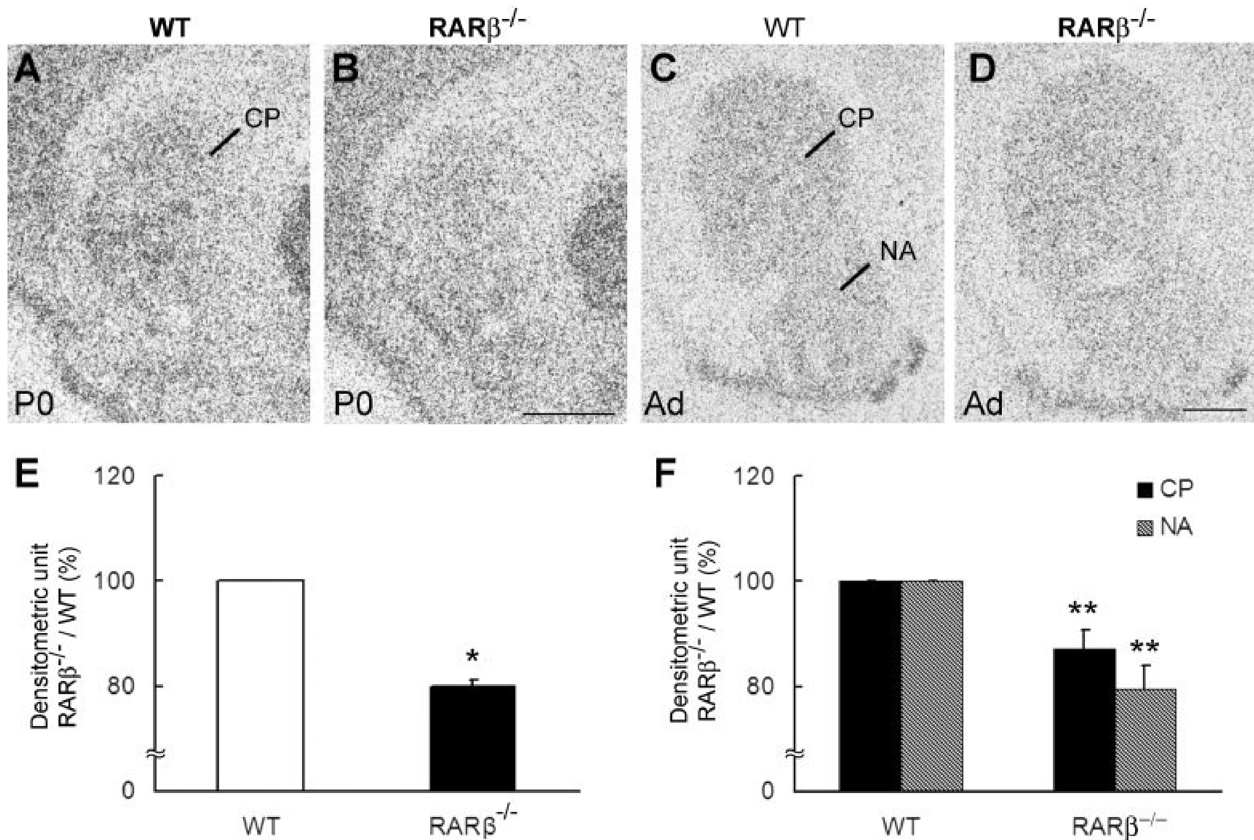

Fig. 5. Reduction of striatal striatal-enriched tyrosine phosphatase (STEP) mRNA expression in $RAR\beta^{-/-}$ mutant mice. The quantification of STEP mRNA expression is performed in the striatum of postnatal day 0 (P0; **A,B,E**) and adult (Ad; **C,D,F**) wild-type (WT) and $RAR\beta^{-/-}$ mutant mice. The adult striatum is further divided into the caudoputamen (CP) and the nucleus accumbens (NA) for quantification. The STEP mRNA is significantly reduced in both the newborn (E) and adult (F) $RAR\beta^{-/-}$ mutant striatum. $^{**}P < 0.01$, comparing the wild-type mice with the $RAR\beta^{-/-}$ mutant mice by Student's *t*-test. Data are representative of n = 3 (P0) and n = 4 (adult) in each genotype of mice. RAR, retinoic acid receptors. Scale bar = 500 μm in B (applies to A,B), 500 μm in D (applies to C,D).

contrast, transfection of RARβ4 significantly reduced STEP mRNA in transfected cells treated with RA (0.58 ± 0.05, $P < 0.001$, n = 4; Fig. 6D). The transfection of either RARβ2 or RARβ3 did not significantly affect STEP mRNA in transfected cells treated with RA (0.95 ± 0.08, $P > 0.05$, n = 4, Fig. 6B; 0.87 ± 0.17, $P > 0.05$, n = 4, Fig. 6C; respectively). No significant changes of MAP-2 mRNA expression were observed in the CTX cultures transfected with any of the four RARβ isoforms, which indicated the specificity of gene regulation (data not shown).

Increases of Dopamine D1 Receptor Expression by Ectopic Expression of RARβ1 in the CTX

Our previous genetic screening study also identified dopamine D1 receptor (D1R) as a RA-responsive gene in the developing telencephalon (Wang et al., 1999). Similar to the STEP expression, D1R is highly expressed in the LGE/striatum (Schambra et al., 1994; Caille et al., 1995). We previously found that exogenous all-*trans* RA increased D1R mRNA in E15 LGE explant culture, and moreover, the same RA treatment induced D1R mRNA from nondetectable level to high level in the MGE explant culture, but the RA treatment failed to induce D1R in the CTX culture (Wang et al., 1999; Wang and Liu, 2001). Consistent with these findings, it has been reported that double null mutations of RARβ/RXRγ, RARβ/RXRβ, or RXRβ/RXRγ resulted in a 40% reduction of D1R mRNA in the adult mouse striatum, although a single null mutation of RARβ or RXRγ did not alter striatal D1R expression (Krezel et al., 1998).

To further test whether D1R was also inducible by RARβ in the CTX as that of STEP, we measured D1R mRNA expression levels after ectopic expression of different RARβ isoforms in E15 CTX explant culture. Transfecting RARβ1 into the CTX enabled all-*trans* RA (1 μM) to induce 1.60-fold increases of D1R mRNA in transfected cells (1.60 ± 0.11, $P < 0.01$, n = 3; Fig. 7A). In contrast, transfection of either RARβ2 or RARβ4 reduced D1R mRNA in transfected cells treated with RA (0.69 ± 0.10, $P < 0.01$, n = 4, Fig. 7B; 0.78 ± 0.06; $P < 0.01$, n = 3, Fig. 7D; respectively). Transfection of RARβ3 did not affect D1R mRNA in transfected cells treated with RA (0.97 ± 0.09, $P > 0.05$, n = 3, Fig. 7C). Thus, the regulation of D1R by ectopic expression of different RARβ was similar to that of STEP except that ectopic RARβ2 down-regulated D1R without affecting STEP expression.

DISCUSSION

In the present study, we carried out a comprehensive study of retinoid signaling in the developing telencephalon. We used a novel approach of electroporating a retinoid reporter gene into developing telencephalon, which permitted us to explore retinoid signaling competence in different regions of developing telencephalon. The retinoid reporter gene that we used contained four copies of a direct repeat 5 (DR5) of RARE, which is more frequently found in the RA-regulated target genes than DR2 and DR1 RAREs (Leid et al., 1992). As different retinoid-responsive genes have different configurations of promoters, the pattern of the reporter gene activity may not fully represent the endogenous retinoid-mediated gene regulation due to the promoter context-dependent *trans*-activation (Nagpal et al., 1992a). Nonetheless, our study provides the first molecular evidence in the developing telencephalon that the ligand-contingent property of retinoid receptors can repress gene activity in retinoid-poor regions, but conversely, it can promote gene expression in retinoid-rich regions. The ligand-dependent dual mechanisms of retinoid receptors may enable retinoid signaling to sharpen its target gene expression pattern in the developing telencephalon. Our study also provides evidence that RARβ is important in transducing retinoid signals in developing ventral telencephalon, which is more competent to retinoid signaling than dorsal telencephalon, and that different RARβ isoforms may differentially regulate gene expression in developing telencephalic neurons.

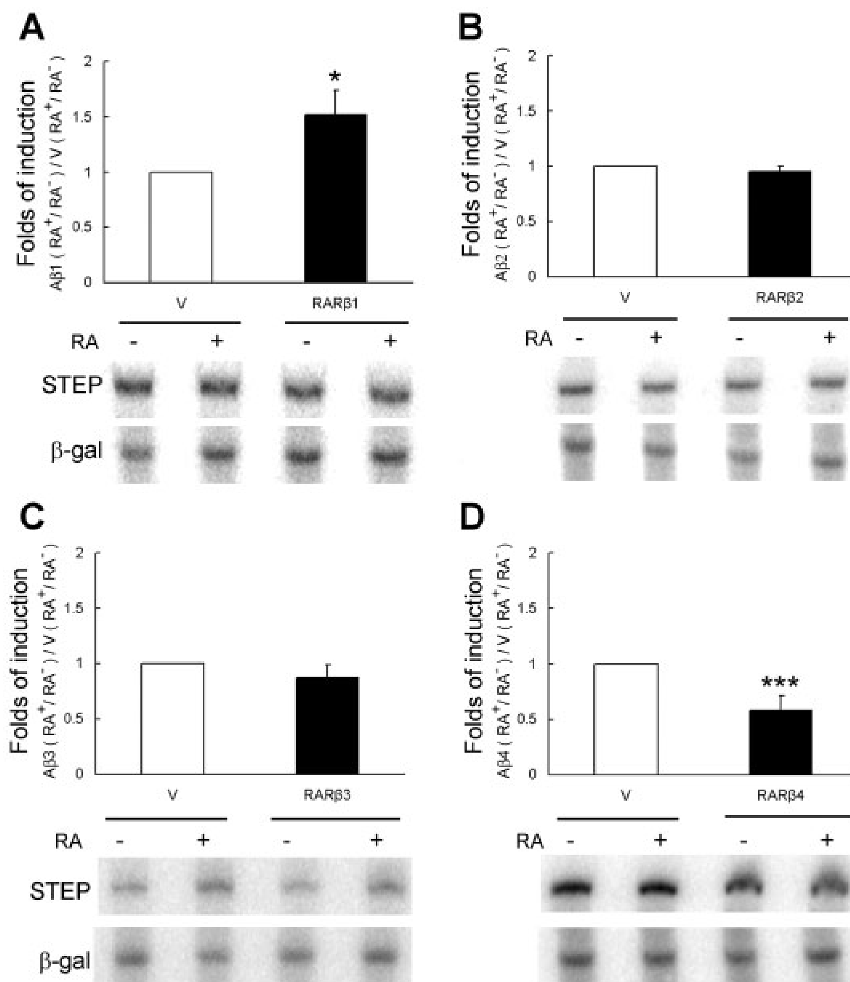

Fig. 6. Differential regulation of striatal-enriched tyrosine phosphatase (STEP) mRNA by different RARβ isoforms. The expression plasmids of RARβ1, RARβ2, RARβ3, or RARβ4 were electroporated into embryonic day 15 cerebral cortex (CTX) cells, which were cultured for 2 days with (RA^+) or without (RA^-) all-*trans* RA (1 μM) treatment. **A:** The ribonuclease protection assay shows that transfection of RARβ1 enhances STEP mRNA in transfected cells treated with RA. **D:** In contrast, transfection of RARβ4 suppresses STEP mRNA in transfected cells treated with RA. **B,C:** The transfection of either RARβ2 (B) or RARβ3 (C) into CTX cells does not significantly alter STEP mRNA in transfected cells treated with RA. The intensity of the protected fragment was quantified and normalized with the cotransfected β-gal gene. The relative mRNA levels among transfected groups are determined by using the mRNA level in the normalized vector group [vector (RA^+/RA^-)] as 1. The data are representative of at least n = 3 for each set of experiment. *$P < 0.05$, ***$P < 0.001$, Student's *t*-test. β-gal, β-galactosidase; RAR, retinoic acid receptor; V, pSG5 vector without cDNA insert.

Retinoid Signaling Competence in the Developing Telencephalon

Our retinoid reporter gene study showed that, in addition to the LGE, the CTX and MGE were also competent to exogenous RA stimulation. As endogenous RARα, RXRα, and RXRβ were expressed in the CTX and MGE and RARβ was induced by RA in these two regions, RAR/RXR heterodimers of these retinoid receptors may mediate the RA competence. However, although RARα and RARβ have similar binding affinity for all-*trans* RA, the EC_{50} of RARα for *trans*-activation of a RARE–TK-LUC reporter gene is at least 18-fold higher than that of RARβ, suggesting that RARβ is more effective in transducing retinoid signals (Idres et al., 2002). Notably, despite that previous cell line studies have shown that all RAR subtypes can be induced by RA (Sucov et al., 1990; de The et al., 1990; Wu et al., 1992), our study provides the first evidence that RARβ is the only RAR subtype that is inducible by RA in the developing telencephalon. Exogenous RA significantly increased RARβ expression in the CTX, LGE, and MGE cultures, which was correlated with the increases of $\beta(RARE)_4$–pTK-LUC reporter gene activity. Importantly, the expression levels of RARβ appear to be correlated with the RA signaling competence in each region, i.e., the retinoid reporter gene response and the STEP induction were more limited in the CTX of dorsal telencephalon where RA-induced RARβ expression was low, whereas the reporter gene

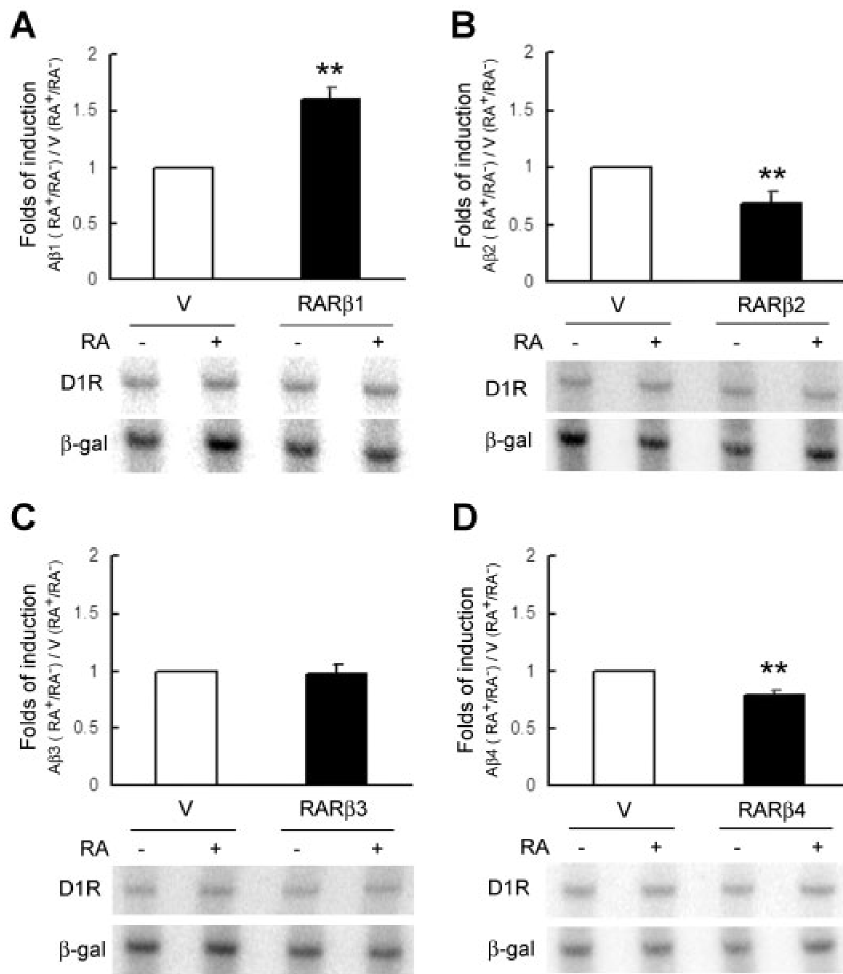

Fig. 7. Differential regulation of D1R mRNA by different RARβ isoforms. The expression plasmids of RARβ1, RARβ2, RARβ3, or RARβ4 were electroporated into embryonic day 15 CTX cells, which were cultured for 2 days with (RA^+) or without (RA^-) all-*trans* RA (1 μM) treatment. **A:** The ribonuclease protection assay shows that transfection of RARβ1 enhances D1R mRNA in transfected cells treated with RA. **B,D:** In contrast, transfection of RARβ2 or RARβ4 suppresses D1R mRNA in transfected cells treated with RA. **C:** The transfection of RARβ3 into CTX cells does not significantly alter D1R mRNA in transfected cells treated with RA. The intensity of the protected fragment was quantified and normalized with the cotransfected β-gal gene. The relative mRNA levels among transfected groups are determined by using the mRNA level in the normalized vector group [vector (RA^+/RA^-)] as 1. The data are representative of at least n = 3 for each set of experiment. $^*P < 0.05$, $^{**}P < 0.01$, Student's *t*-test. CTX, cerebral cortex; β-gal, β-galactosidase; RA, retinoic acid; RAR, RA receptor; V, pSG5 vector without cDNA insert.

response and the STEP induction were stronger in the LGE and MGE of ventral telencephalon where RA-induced RARβ expression was high, suggesting that RARβ may mediate the RA signaling competence. In agreement with this hypothesis, ectopic expression of RARβ1 resulted in enhanced STEP expression. All the four RARβ isoforms are inducible by RA at the transcriptional level (Zelent et al., 1991; Nagpal et al., 1992b; Mendelsohn et al., 1994). Previous *in situ* hybridization studies have shown that RARβ1/3 and RARβ2/4 mRNAs are present in the mouse LGE (Mollard et al., 2000) and that RARβ1/3, RARβ2, and RARβ3 mRNAs also are expressed in the mouse LGE (Liao et al., 2005). Due to lack of adequate unique DNA sequences to specifically distinguish RARβ1 and RARβ4 from other isoforms, we were not able to directly determine the expression patterns of these two isoforms in the developing telencephalon. Nonetheless, our findings suggest that RARβ may play a key role in mediating retinoid signaling competence in developing telencephalon.

Potential Distribution of Retinoids in the Developing Telencephalon *In Vivo*

The diffusion capability of retinoids in the developing telencephalon in vivo is yet unknown. Nonetheless, the sensitivity of RARβ induction to RA stimulation in the telencephalon, in fact, may provide hints for the extent of retinoids distribution in the telencephalon. The CTX and MGE regions are deficient of RA synthesis, as shown by the RA reporter cell line assay. If significant amounts of retinoids had been present in the CTX and MGE regions *in vivo*, the retinoids should have diffused into these two regions from other retinoid-rich sources, and accordingly, the retinoids should have induced significant RARβ expression in the CTX and MGE regions. However, the CTX and MGE regions have nondetectable level of endogenous RARβ expression *in vivo* as shown by our RT-PCR analysis of E17 CTX and MGE regions and also by other studies using *in situ* hybridization (Ruberte et al., 1993; Dolle et al., 1994; Mollard et al., 2000; Liao et al., 2005). These findings suggest that the CTX and MGE regions may contain limited amounts of retinoids *in vivo*.

Differential Repressor Activity of Retinoid Receptors in the Developing Telencephalon

Cell line-based studies have shown that unliganded retinoid receptors function as repressors by recruiting corepressors. Upon binding of retinoid ligands, retinoid receptors, instead of corepressors, recruit coactivators to *trans*-activate gene expression (Xu et al., 1999; Burke and Baniahmad, 2000; Glass and Rosenfeld, 2000). The molecular mechanisms of retinoid-mediated gene regulation are mostly characterized in non-neuronal cell lines and previously have not been demonstrated to occur in the developing mammalian brain. Our reporter gene study provides the first evidence that similar mechanisms may occur in developing neurons of telencephalon. We showed that several retinoid receptor-associated transcriptional corepressors and coactivators, including N-CoR, SMRT, SRC-1, and P/CAF,

were ubiquitously expressed in the developing telencephalon. Therefore, in the absence of retinol, the tissue may fail to synthesize RA, which renders the majority of retinoid receptors to function as repressors in all three brain regions. In the presence of retinol, the repressor activity is selectively relieved in the LGE where RA is locally synthesized. For the CTX and MGE, as they are deficient of RALDH enzymes for synthesizing RA, the repressor activity remains mainly unchanged.

It is notable that, in the absence of retinol, the repressor activity was higher in the CTX than that in the LGE and MGE, i.e., the extent of repression [100% − (β(RARE)$_4$–pTK-LUC/pTK-LUC × 100%)] was higher in the CTX (88.5%) than in the LGE (56.9%) and MGE (60.5%). Without retinol, the endogenous retinoid levels in the CTX, LGE, and MGE cultures were all very low as indicated by the Sil-15 reporter cell assay. It suggests that some factors other than ligand concentrations may affect the repressor activity of retinoid receptors in the CTX. Alternatively, the LGE and MGE may contain slightly higher levels of retinoids than the CTX, but Sil-15 cells may fail to detect the small differences of RA at very low concentration.

Regulation of the RA-Responsive Gene STEP in the Developing Telencephalon

Consistent with the increases of STEP by RA in the LGE, STEP mRNA was reduced in the LGE derivative striatum of RARβ$^{-/-}$ mutant mice, suggesting that RARβ signaling is involved in induction and maintenance of STEP expression in the striatum. The STEP reduction, however, was partial in the newborn and adult striatum, which may be due to functional redundancy by other members of retinoid receptors. Indeed, reduction of striatal dopamine D1 and D2 receptors is only observed in double null mutants of retinoid receptors and not in single null mutant mice (Krezel et al., 1998). Our genomic sequence analysis indicates that a putative RARE (DR2, AGGTCCtgAGTTCA) and two putative RAREs (DR5, AGGTTAtaaatAAGTCA; DR2, AGCTCAcgAAGTCA) are present in the 5′ flanking regions of rat and mouse *STEP* gene, respectively (W.-L. Liao and F.-C. Liu, unpublished observations). It is unclear, however, whether the STEP induction by RA is directly mediated through the RARE(s). As STEP was expressed in the explant cultures of CTX, LGE, and MGE without RA or retinol treatments, other signaling molecules may also regulate STEP gene expression.

STEP has been shown to be a downstream signaling molecule in the dopamine signal transduction pathway (Paul et al., 2000). In addition to STEP, striatal dopamine signaling molecules, including D1 and D2 receptors, G_{olf}, adenylyl cyclase type V and DARPP-32 are also regulated by retinoid signals, suggesting that the retinoid signaling may coordinately control developmental expression of dopamine signaling molecules in the striatum (Samad et al., 1997; Krezel et al., 1998; Valdenaire et al., 1998; Wang et al., 1999; Toresson et al., 1999).

RARβ Isoform-Specific Gene Regulation

The possibility that RARβ plays a role in mediating retinoid signaling competence in the developing telencephalon is further supported by our finding that ectopic expression of RARβ1 in the CTX could enhance RA-mediated increases of STEP expression. It is of interest that the effects were isoform-specific. In contrast to RARβ1, the transfection of RARβ4 resulted in a decrease of STEP expression in transfected cells treated with RA. Similar RARβ isoform-specific regulation also applies to D1R, another RA-responsive gene, suggesting that it may be a common mechanism underlying RARβ signaling. RARβ4 differs from the other three RARβ isoforms at the N-terminus, in which it contains only four amino acids in the A region of the protein. Notably, the RARβ4 transcript can produce a truncated RARβ4 protein that may function as a negative *trans*-dominant regulator due to its inability of binding to DNA but capable of dimerization with RXRs (Nagpal et al., 1992b). Thus, a truncated RARβ4 protein may dominant-negatively regulate STEP expression in the transfected cells. Collectively, our study provides the first evidence that different RARβ isoforms may differentially regulate gene expression in developing telencephalic neurons.

EXPERIMENTAL PROCEDURES

Electroporation

Time-pregnant rats (Sprague–Dawley, National Yang-Ming University, Taipei, Taiwan) at gestational day 15 were deeply anesthetized with 0.3% sodium pentobarbital. The day of sperm positivity was defined as E1. Embryos were placed in ice-cold phosphate-buffered saline (PBSA, pH 7.2) and rinsed four times with PBSA. The embryonic head was dissected from the embryo and placed in a concave glass slide filled with PBSA. Two microliters of β(RARE)$_4$–pTK-LUC or pTK-LUC plasmids were coinjected with pCMV–β-galactosidase plasmid (2:1; 5 μg/μl) into the lateral ventricle of forebrain. In some brains, the plasmids of pCMX–EpiRAR–E, β(RARE)$_4$–pTK-LUC, and pCMV–β-galactosidase (8:2:1) were coinjected. In another set of experiments, pSG5-RARβ1, pSG5-RARβ2, pSG5-RARβ3, pSG5-RARβ4, or pSG5 plasmids were coinjected with pCMV–β-galactosidase plasmids (4:1). The head was then directionally placed into an electroporation cuvette with either the dorsal or ventral side of the head against the anode of electrodes. The electroporation was performed using an electroporator (Gene Pulser, Bio-Rad, Hercules, CA) with 250 V and 960 μF capacitance. After electroporation, the tissues were cultured as described below. The protocol of animal use conformed to NIH guide for the care and use of laboratory animals. All efforts were made to minimize both the suffering and the number of animals used.

Explant Culture

The electroporated tissues were cultured on top of 0.4-μm microporous transparent biopore membranes in 30-mm culture plate inserts (Millicell-CM, Millipore, Bedford, MA), which were placed in six-well culture dishes. Each well was supplied with 1 ml of SF21 medium (Segal et al., 1992). The

cultures were incubated in a humidified incubator at 37°C with 5% CO_2/95% air. All-*trans* RA (1 μM; Sigma) or all-*trans* retinol (1 μM; Sigma) was applied to the culture medium 1–2 hr after the culturing. The explants were cultivated for 2 days *in vitro* before the tissues were lysed for assaying luciferase and β-galactosidase activities. For culturing explant tissues without electroporation, the MGE, LGE, and the overlying CTX were dissected from the E15 forebrain and were cultivated as described above. The tissues were cultivated for 1–2 days *in vitro* before the extraction of RNA for RT-PCR and ribonuclease protection assay.

Culture of Retinoid Reporter Cells and Coculture With Explant Tissue

The cultivation of Sil-15 retinoid reporter cells and X-gal staining were carried out as previously described (Wagner et al., 1992). For coculturing explants and Sil-15 cells, the explant tissues were first cultured on top of Millicell-CM membrane inserts (12 mm) for 1 day *in vitro*. The inserts were then placed into 24-well plates. For each well of the 24-well plates, it contained a gelatin-coated coverslip in which ca. 90% confluence of Sil-15 cells were growing. The coculture was carried out with or without retinol for over night. The total protein of the explant tissue was quantified by using the BCA protein assay kit according to the manufacture's instruction (Pierce, Rockford, IL). For quantification of β-gal responses of Sil-15 cells, the numbers of strongly stained β-gal–positive cells on the coverslips were counted. The numbers of β-gal–positive cells were normalized with the amounts of total proteins of cocultured explant tissues.

Assays of Luciferase and β-Galactosidase Activities

The tissue of transfected explant cultures were lysed, sonicated, and spun down to collect the supernatants. Aliquots of the supernatants were assayed for luciferase and β-galactosidase activities using the luciferase and β-galactosidase kits (Promega) according to the manufacturer's instructions. The luciferase activity was measured with a Luminometer (Promega) or with a Wallac multilevel counter (Victor II, Perkin-Elmer). The luciferase activity was normalized with the β-galactosidase activity for controlling the transfection efficiency.

RT-PCR

Total RNA was prepared from the explant tissues according to the single-step method of guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi, 1987) and was then reversely transcribed to cDNA. For PCR reaction of each gene, the GenBank accession number, the sense and antisense primers (the corresponding nucleotide positions in the gene), annealing temperature, number of reaction cycles, and the size of PCR product were as follows: RARα (U15211): sense 5′-TTTCT CTGCC TTCCT ACTGG-3′ (nucleotide [nt] 1660–1679), antisense 5′-GGCAT CTTCT GTCAC CATCC-3′ (nt 1978–1997), 50°C, 30, 338 bp; RARβ (AJ002942), sense 5′-TGTCA GGAAT GACAG GAACA AG-3′ (nt 647–668), antisense 5′-AGCAC TGGAA TTCGT GGTGT AT-3′ (nt 797–818), 60°C, 40, 172 bp; RARγ (X15848): sense 5′-AACCC TACAT GTTCC CAAGG-3′ (nt 1430–1449), antisense 5′-GAAAT GGTCA GTCTG CTGCC-3′ (nt 1720–1739), 50°C, 30, 310 bp; RXRα (L06482): sense 5′-GCGTG ACACC TACCC AGCCC-3′ (nt 1761–1780), antisense 5′-TTGAG GTGGT CCCAG AATCC-3′ (nt 2083–2102), 60°C, 26, 342 bp; RXRβ (M81766): sense 5′-CATGA GGATG GACAA GACGG-3′ (nt 1074–1093), antisense 5′-CTGTT AAGTG TTGCC AGACC-3′ (nt 1495–1514), 60°C, 35, 441 bp; RXRγ (AJ223083): sense 5′-CAATA TATGC CACGC TGCGG-3′ (nt 566–585), antisense 5′-AAGAG TCTCC ACCTC CGAGG-3′ (nt 925–944), 58°C, 35, 379 bp; N-CoR (U35312): sense 5′-CCCTC TTCAA CAGGT TCTAC TC-3′ (nt 7278–7299), antisense 5′-CACAG CTCAG TCGTC ACTAT CA-3′ (nt 7463–7484), 60°C, 40, 207 bp; SMRT (AF113001): sense 5′-GCCAT TATTA GAAAG GCACT-3′ (nt 7521–7540), antisense 5′-CACAC ACGGT TGGTG A-3′ (nt 7828–7843), 60°C, 35, 323 bp; SRC-1 (U64828): sense 5′-TGTTC AGTCA AGCTG TCC-3′ (nt 4527–4544), antisense 5′-AGCTG GTTGC AGTAG AGG-3′ (nt 4738–4755), 60°C, 40, 229 bp; P/CAF (AF254442): sense 5′-TGGAG TTCGG CAGAT TCC-3′ (nt 2022–2039), antisense 5′-TGAGG CGTTC ACTCA TGG-3′ (nt 2258–2275), 60°C, 35, 237 bp; actin (V01217): sense 5′-TCATG AAGTG TGACG TTGAC ATCC-3′ (nt 2727–2750), antisense 5′-CCTAG AAGCA TTTGC GGTGC ACGAT G-3′ (nt 3110–3135), 58°C, 15, 285 bp. The PCR primers were designed to detect all isoforms of each subtype of RARs and RXRs genes (Leid et al., 1992). The amplification of cDNAs was performed in a thermal cycler (Biometra, Germany) with each tube containing, at a final volume of 10 μl, 1 μl of cDNA, 1 μl of 10× PCR buffer, 0.1 μl of *Taq* DNA polymerase (5 U/μl; Qiagen, Germany), 0.1 μl of 0.25 mM dNTP, and 1 μl of 20 ng/μl primers. For PCR of RARα, RARβ, RARγ, RXRα, and N-CoR, the Q solution (Qiagen) was included in the PCR reaction mixture. The PCR reaction was run at the following condition: 94°C for 30 sec, respective annealing temperature for 30 sec and 72°C for 40 sec. Ten microliters of each PCR sample were resolved on 2% gel, and the PCR products were visualized with ethidium bromide staining and ultraviolet illumination. The controls for genomic DNA contamination were the RT-PCR reactions without reverse transcriptase. No DNA band was detected under this condition.

Quantitative RT-PCR Assay

The quantitative RT-PCR assay was performed using a one-step RT-PCR kit (Titanium, Clontech, Palo Alto, CA) with the fluorescent DNA dye SYBR Green I (BioWhittaker Molecular Applications, Rockland, ME) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia) according to the manufacturers' instructions. The primers for RARβ (AJ002942) were as described above. The first-strand cDNA was synthesized at 50°C for 60 min followed by denaturation at 95°C for 10 min. PCR reactions were then perfomed for 40 cycles at 95°C for 30 sec, 60°C for 30 sec, and 68°C for 60 sec. A melting curve was created at the end of PCR cycles to confirm that a single product was amplified. Independent RT-PCRs were processed using the same RNAs for both the inter-

ested gene and the reference gene (glyceraldehyde-3-phosphate dehydrogenase, GAPDH). Negative controls containing no RNA template were included in each experiment. Data were analyzed by Rotor-Gene 3000 operating software version 4.6.94 (Corbett Research) to determine the threshold cycle (C_T) above the background for each reaction. The relative transcript amounts of the target genes in each sample were calculated according to the standard curves and then normalized with that of GAPDH. The relative mRNA levels among groups were determined by using the mRNA level in vehicle-treated LGE as 1. The statistics was performed by Student's *t*-test.

Ribonuclease Protection Assay

The ribonuclease protection assay was performed as previously described (Yau et al., 2003). Total RNA was prepared by the single-step method of guanidinium thiocyanate-phenol-chloroform extraction. Plasmids containing cDNAs of STEP gene (GenBank accession no. S49400, nt 2088–2491), D1R gene (M35077, nt 1409–1743), or MAP-2 gene (X53455, nt 2236–2657) were linearized with *Bam*HI restriction enzyme. The antisense RNA probes were labeled with [^{32}P]α-UTP (Dupont, NEN Life Science, Boston, MA) by in vitro transcription. The ^{32}P-labeled antisense probes were hybridized to RNA and were then processed with RNase to digest single-strand RNA. Protected fragments were separated in 6% sequencing gel. The radioactivity of protected bands was quantified by scanning the protected fragments on a Phosphoimager 400S (Molecular Dynamic, Sunnyvale, CA). The results were analyzed with the ImageQuant software. To quantify the signal intensities of the protected fragments, the signal of each protected fragment was first obtained by subtracting the background level that was taken from the corresponding size of area below the protected fragment. The signal intensities of STEP and D1R mRNA were then normalized with that of *gapdh* gene or of the transfected β-galactosidase gene β-gal in the same lane. The effects of transfection of RARβ isoforms on the expression of STEP and D1R mRNA levels in the presence of RA were determined as the ratio between transfection of RARβ isoforms and mock transfection of the vector [RARβ (RA/vehicle)/vector (RA/vehicle)]. At least three independent experiments were performed for each study. Statistical analysis was performed by Student's *t*-test.

Genotyping of RARβ Mutant Mice

The RARβ mutant mice were generated as previously described (Ghyselinck et al., 1997). The RARβ heterozygous mice were maintained in C57/Bl6 background and were intercrossed to produce homozygous mice. The PCR genotyping of mouse tail DNA was performed with a combination of three primers: UD96 (5′-CCAGG CTCCT TTTTC TTCTA CCATA-3′), UD97 (5′-CTGTT TCTGT GTCAT CCATT TCCAA-3′), and UD98 (5′-AGGCC TACCC GCTTC CATTG CTCAG-3′). The PCR amplification was first carried out at 94°C for 3 min followed by 4 cycles at 94°C for 1 min, 55°C for 30 sec, and 72°C for 30 sec, and then 34 cycles at 92°C for 15 sec, 55°C for 15 sec, and 72°C for 15 sec. An additional process at 72°C for 5 min was run at the end of the PCR reaction. The PCR with the three primers gave rise to PCR products with different sizes with the wild-type allele of 275 bp and the mutant allele of 300 bp.

Preparation of the Brain Tissue of Mutant Mice

Littermates of 1.5- to 6-month-old wild-type (n = 4) and RARβ$^{-/-}$ mice (n = 4) and littermates of postnatal day 0 (P0) wild-type (n = 3) and RARβ$^{-/-}$ mice (n = 3) were used for the experiments. The mice were anesthetized by intraperitoneal overdose injection of sodium pentobarbital (adult) or by hypothermia (P0) and were then perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4). The brains were post-fixed in the same fixative for 6 hr and then cryoprotected at 4°C for at least 24 hr in 20% glycerol in 0.1 M PB. The brains were cut at a freezing microtome (Microtom, Germany) at 20 μm (adult) or 30 μm (P0) in the coronal plane, and the brain sections were stored in antifreezing solution containing 20% glycerol before processing for *in situ* hybridization.

In Situ Hybridization

The *in situ* hybridization was performed as previously described (Simmons et al., 1989). The brain sections of wild-type and RARβ$^{-/-}$ littermate mice were mounted on the same slides to ensure that they were processed under the same condition throughout the procedure. The pGEM-T-easy-STEP plasmid contained a PCR product of 243-bp fragment of the mouse STEP gene (GenBank accession no. S80329, nt 1210–1452). The ^{35}S-UTP (Dupont, NEN Life Science) -labeled antisense STEP riboprobes were synthesized by using T7 RNA polymerase-mediated *in vitro* transcription (Promega) with *Bam*HI-linearlized pGEM-T-easy-STEP plasmids as templates. The RNA hybridization was carried out in a mixer containing 1×10^6 cpm ^{35}S-UTP-cRNA hybridization buffer for 16 hr at 58°C. After several washes with standard saline citrate, the sections were exposed to X-ray film with different times to detect the autoradiographic signals. The autoradiographic brain images in X-ray film were digitalized and up-loaded into the Scion Image software program (Scion Incorporation) for densitometric measurement of autoradiogram. The mean density of the autoradiogram was measured from the dorsal striatum (caudoputamen, CP) and the ventral striatum (nucleus accumbens, NA) of adult brains at the rostral level. For P0 brains, the measurement was taken from the entire striatum. The background level taken from the corpus callosum overlying the striatum was subtracted from the measured intensity of autoradiogram to obtain the signal intensity. The sig-

nal intensities from RARβ null mutant brains were averaged, and were then expressed as ratio relative to that of wild-type brains. Statistical analysis was performed by Student's *t*-test.

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