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Differential Expression of RARβ Isoforms in the Mouse Striatum During Development: A Gradient of RARβ2 Expression Along the Rostrocaudal Axis

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The retinoic acid receptor RARβ is highly expressed in the striatum of the ventral telencephalon. We studied the expression pattern of different RARβ isoforms in the developing mouse striatum by *in situ* hybridization. We found a differential ontogeny of RARβ2 and RARβ1/3 in embryonic day (E) 13.5 lateral ganglionic eminence (striatal primordium). RARβ2 mRNA was detected primarily in the rostral and ventromedial domains, whereas RARβ1/3 mRNAs were enriched in the caudal and dorsolateral domains. Notably, by E16.5, a prominent decreasing gradient of RARβ2 mRNA was present in the developing striatum along the rostrocaudal axis, i.e., RARβ2 was expressed at higher levels in the rostral than the caudal striatum. No such gradient was found for RARβ1/3 and RARβ3 mRNAs. The rostrocaudal RARβ2 gradient gradually disappeared postnatally and was absent in the adult striatum. The differential expression pattern of RARβ isoforms in the developing striatum may provide an anatomical basis for differential gene regulation by RARβ signaling. *Developmental Dynamics 233:584–594, 2005.*

Key words: basal ganglia; lateral ganglionic eminence; nucleus accumbens; retinoic acid; telencephalon

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INTRODUCTION

Retinoic acid (RA) is a vitamin A derivative that has pleiotropic effects on development and homeostasis in vertebrates (Ross et al., 2000). The RA signaling is transduced by RA receptors (RARs) and retinoid X receptors (RXRs) that function as transcriptional regulators (Leid et al., 1992). Each family of RARs and RXRs consists of three receptor subtypes (RARα, RARβ, RARγ; RXRα, RXRβ, and RXRγ). The different subtypes of RARs and RXRs are encoded by different genes, and moreover, each subtype of RARs and RXRs has different isoforms that are derived from differential promoter usage and alternative splicing (Leid et al., 1992). The different isoforms are usually diversified in the N-terminus of proteins. For example, the *RAR*β gene contains four isoforms. RARβ1 and RARβ3 are transcribed by the first promoter, whereas RARβ2 and RARβ4 are transcribed by the second promoter (Leid et al., 1992). For RARβ1 and RARβ3, RARβ3 contains both exon1 and exon2, but RARβ1 contains only exon1 (Zelent et al., 1991). For RARβ2 and RARβ4, RARβ2 contains exon3, whereas RARβ4 contains a truncated exon 3 (Nagpal et al., 1992). It is notable that for each RARβ isoform, its N-terminus region is highly conserved between the mouse and human, suggesting potential physiological significance of RARβ isoforms (Leid et al., 1992).

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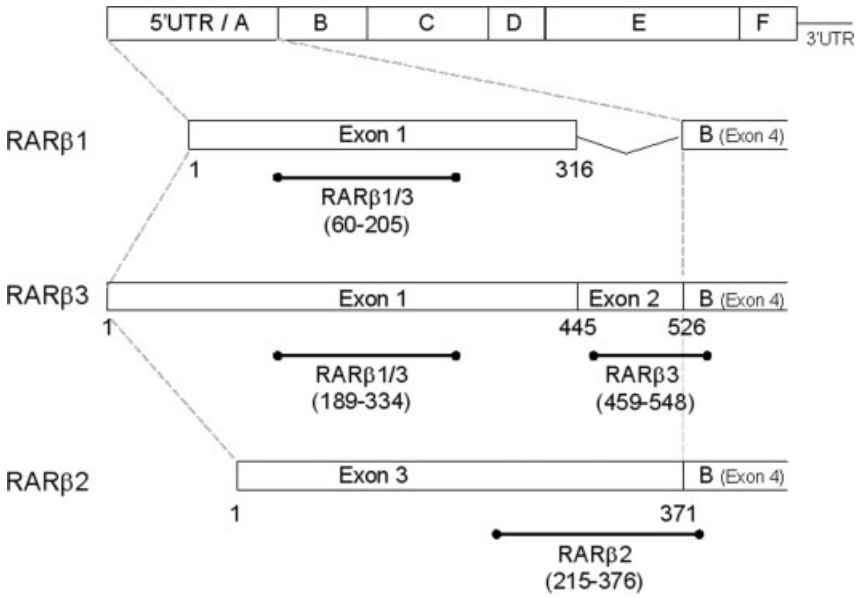

Fig. 1. Design of retinoic acid receptor-beta (RARβ) isoform-specific cRNA probes. The coding region of *RAR*β gene comprises the domains of A–F. Alternative splicing of exon 1, exon 2, and exon 3 from two promoters results in RARβ1, RARβ2, and RARβ3 isoforms, which differ at their 5′ untranslated region (5′ UTR) and the A domain. The corresponding nucleotide positions of the RARβ1/3, RARβ3, and RARβ2 probes in the *RAR*β gene are indicated. The GenBank accession numbers for RARβ1, RARβ2, and RARβ3 are X56569, AJ002942, and X56574, respectively.

It is of particular interest that RARβ are expressed at high levels in the striatum during development (Ruberte et al., 1993; Dolle et al., 1994). We and others have shown that exogenous RA can regulate expression of neurochemical molecules, including dopamine D1 and D2 receptors and DARPP-32, in striatal cell culture (Samad et al., 1997; Liu et al., 1998; Valdenaire et al., 1998; Wang et al., 1999; Toresson et al., 1999). RARβ, by virtue of its rich expression in striatal neurons, is likely to be involved in retinoid-mediated gene regulation. Indeed, genetic evidence shows that double null mutations of RARβ/RXRβ or RARβ/RXRγ results in decreases of dopamine D1 and D2 receptors expression in the striatum of null mutant mice (Krezel et al., 1998). Our recent mutant mice study also shows that null mutation of RARβ results in aberrant neurochemical compartments in the mutant striatum (Liao et al., 2003). Notably, in these mutant mice studies, all four RARβ isoforms were disrupted in the RARβ null mutant mice (Ghyselinck et al., 1997). It is yet unknown which RARβ isoform(s) is involved in regulating striatal gene expression.

As a first attempt to understand the potential contributions by different RARβ isoforms in regulating striatal development, we studied the expression pattern of different RARβ isoforms during striatal development by *in situ* hybridization. Using RARβ2, RARβ1/3, and RARβ3-specific probes, we found that different RARβ isoforms were enriched in different domains of developing striatum, which may provide an anatomical basis for differential gene control by RARβ signaling.

RESULTS

Ontogeny of Different RARβ Isoforms in the Developing Striatum

Embryonic day 11.5–13.5 striatal primordium.

The corresponding nucleotide positions of the RARβ1/3, RARβ2, and RARβ3 probes in the *RAR*β gene are shown in Figure 1. The expression patterns detected with these probes in the developing mouse striatum are summarized in Table 1. Low levels of RARβ2 mRNA were first detected in the lateral ganglionic eminence (LGE, striatal primordium) of mouse telencephalon at embryonic day (E) 11.5 with prolonged exposure time of X-ray film (Fig. 2C). Increasing levels of RARβ2 mRNA were detected in E12.5 LGE (Fig. 2D). In contrast, RARβ1/3 and RARβ3 mRNAs were not detected in E11.5 LGE. RARβ1/3 mRNA was barely detectable in E12.5 LGE (data not shown).

At E13.5, both RARβ2 and RARβ1/3 mRNAs were expressed in the differentiated mantle zone of the LGE (Fig. 3). However, their spatial distributions within the LGE were different. At the rostral level, only RARβ2 mRNA was detected (Fig. $3B_1$). In contrast, RARβ1/3 mRNA expression extended into the caudal ganglionic eminence (CGE), where at most, weak RARβ2 mRNA was detected (Fig. $3B_4$,C_4). At the middle level, where both RARβ2 and RARβ1/3 mRNAs appeared, RARβ2 mRNA was not only expressed in the dorsal LGE, but it also extended into the ventral LGE (Figs. $3B_2$, $4B_2$), whereas RARβ1/3 mRNA was mainly confined to the dorsal LGE (Figs. $3C_2$, $4C_2$). In addition to the ontogenic differences in the rostrocaudal and dorsoventral levels, RARβ2 and RARβ1/3 mRNAs also differed at the mediolateral level (Fig. 4). By comparing their expression patterns in parasagittal sections, RARβ1/3 mRNA was detected in the more lateral part of the LGE than RARβ2 mRNA (Fig. $4B_1$,C_1). While moving toward the medial plane, RARβ1/3 mRNA was gradually decreased whereas the expression of RARβ2 mRNA was gradually increased so that strong RARβ2 mRNA but weak RARβ1/3 mRNA were detected at the medial part of the LGE (Fig. $4B_3$,B_4,C_3,C_4). In contrast to RARβ2 and RARβ1/3, RARβ3 signals were not detectable in the developing telencephalon, even with prolonged exposure time of X-ray films (data not shown). These results suggested that the signals detected with the RARβ1/3 probe in E13.5 LGE might represent RARβ1 mRNA expression. However, due to the small size of the RARβ3 probe (Fig. 1), we could not rule out the possibility that the probe was too short to detect low levels of RARβ3 mRNA at this developmental stage. No signal was detected in the brain sections hybridized with the sense probes of RARβ2, RARβ1/3 and RARβ3 in any of the

TABLE 1. Summary of RARβ Isoform mRNA Expression in the Mouse Striatum at Different Developmental Stages[a]

Isoform/stage	E11.5	E12.5	E13.5	E16.5	P0	P7	Adult
RARβ2	+	++	++ (R/V/M)	+++ R-C gradient NA (shell)	+++ R-C gradient NA (shell)	++ R-C gradient NA (shell)	+
RARβ1/3	−	+/−	+ (C/D/L)	+++ (D/L)	+++	++	+
RARβ3	−	−	−*	++	++	+	+/−

[a]The asterisk indicates that the RARβ3 probe may fail to detect weak RARβ3 expression due to its small size (90 bp). See text for the details. RARβ, retinoic acid receptor-beta; E, embryonic day; P, postnatal day; R, rostral; C, caudal; V, ventral; D, dorsal; M, medial; L, lateral; NA, nucleus accumbens; +++, strong expression; ++, moderate expression; +, low expression; +/−, barely detectable.

brain tissues of different developmental stages (data not shown).

E16.5 developing striatum.

At this stage, in addition to RARβ2 and RARβ1/3, RARβ3 mRNA was also detected in the developing striatum (Fig. 5). For RARβ2 expression, it was of particular interest that there was a rostral to caudal expression gradient, i.e., a high level of RARβ2 mRNA was present in the rostral part of striatum with a decreasing gradient of expression levels toward the caudal part of striatum (Fig. $5B_{1-4}$), which was clearly demonstrated in the parasagittal sections (Fig. 5B). The quantitative densitometry showed the mRNA level at the caudal striatum was ca. 50% of that in the rostral striatum (data not shown). In contrast to RARβ2, no such rostrocaudal gradient was observed for RARβ1/3 and RARβ3 expression (Fig. $5C,C_{1-4},D,D_{1-4}$), which argued against the possibility that the gradient might have resulted from the uneven distribution of fiber bundles in the developing striatum along the rostrocaudal axis. Notably, RARβ1/3 and RARβ3 mRNAs were slightly enriched in the dorsolateral part of developing striatum (Fig. $5C_{1-4},D_{1-4}$). Despite the weak RARβ2 expression in the caudal striatum, a strip containing high levels of RARβ2 mRNA was present in the dorsomedial striatum at caudal levels (Fig. $5B_3,B_4$).

Postnatal day 0 striatum.

The prominent rostrocaudal gradient of RARβ2 mRNA expression was maintained in the newborn striatum (Fig. $6B,B_{1-3}$). The RARβ2 gradient not only occurred in the caudoputamen of dorsal striatum, but it also occurred in the ventral striatum, including the nucleus accumbens and the olfactory tubercle, where high levels of RARβ2 mRNA was present at the rostral level (Fig. $6B_{1-3}$). In contrast, RARβ1/3 and RARβ3 mRNAs appeared without gradient in the striatum (Fig. $6C,C_{1-3},D,D_{1-3}$).

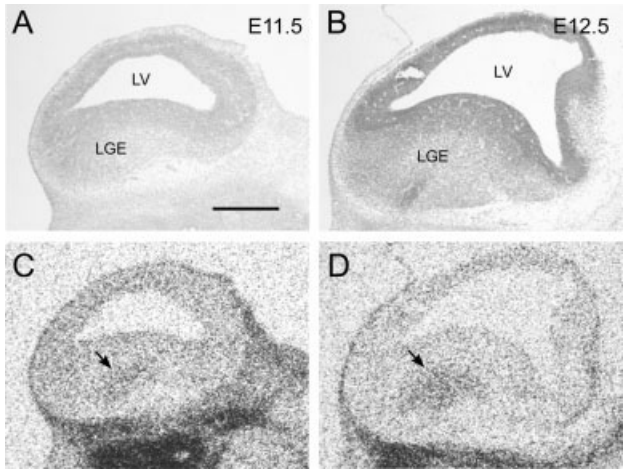

Fig. 2. Ontogenetic expression of retinoic acid receptor-beta (RARβ) 2 in the striatal primordium. **C:** Parasagittal sections showing that low levels of RARβ2 mRNA (arrow) were first detected in the LGE at embryonic day (E) 11.5. **D:** Increasing levels of RARβ2 mRNA were present in E12.5 LGE (arrow). Note that the X-ray film exposure time for the E11.5 brain shown in C is 168 hr, which is far longer than 96 hr for the E12.5 brain shown in D. **A,B:** The sections of C and D are counterstained with hematoxylin to illustrate the brain structure. LGE, lateral ganglionic eminence; LV, lateral ventricle. Scale bar = 500 μm in A (applies to A–D).

Postnatal day 7 striatum.

The rostrocaudal gradient of RARβ2 mRNA expression was still maintained in the postnatal day (P) 7 striatum, albeit with weaker signal intensity than that in the P0 striatum. Strong RARβ2 expression was maintained in the nucleus accumbens (Fig. $7B_1$). Note that RARβ2 but not RARβ1/3 and RARβ3 was detected in the nucleus accumbens at middle level (Fig. $7B_2$). Both RARβ1/3 and RARβ3 mRNAs remained detectable in the P7 striatum (Fig. $7C_{1-3},D_{1-3}$). It was notable that the expression levels of RARβ2, RARβ1/3, and RARβ3 mRNAs were decreased in the P7 striatum compared with the P0 striatum (Figs. 6, 7).

Adult striatum.

The expression of RARβ2, RARβ1/3, and RARβ3 mRNAs was further

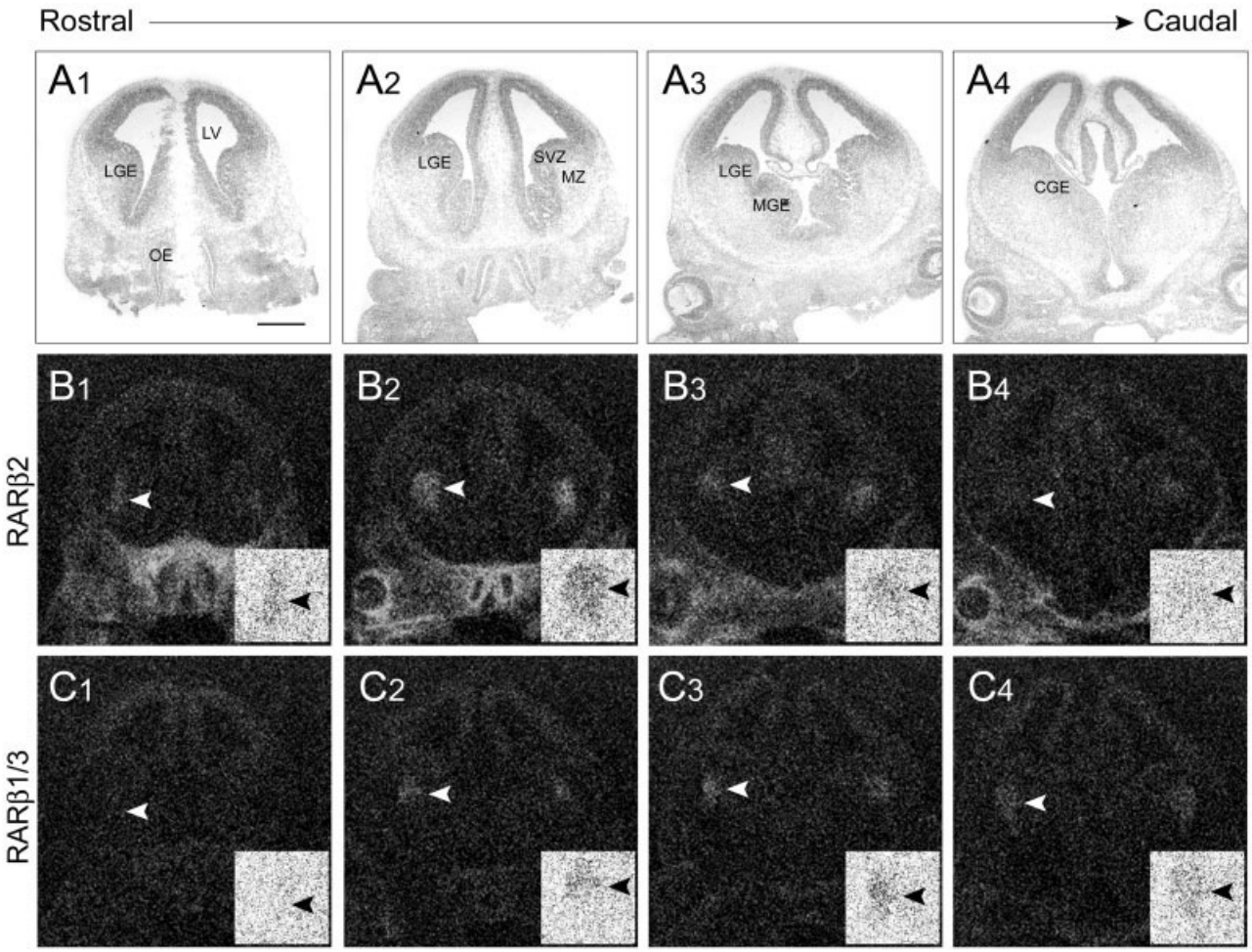

Fig. 3. **A_1–C_4:** Differential ontogeny of retinoic acid receptor-beta (RARβ) 2 and RARβ1/3 in E13.5 striatal primordium along the rostrocaudal axis. Representative coronal sections from the rostral to caudal levels are illustrated for each cRNA probe (B_1–B_4, C_1–C_4). To compare spatial distribution of different isoforms, adjacent sections in B and C are processed in parallel for hybridization with different cRNA probes. RARβ2 but not RARβ1/3 is expressed in the MZ of the LGE (B_1, arrowhead) at rostral levels (B_1, C_1). In contrast, RARβ1/3 extends into the CGE at caudal levels (B_4, C_4, arrowheads). The insets in B_1–B_4 and C_1–C_4 show the regions indicated by the arrowheads at high magnification with brightfield photomicroscopy. The sections of C_1–C_4 are counterstained with hematoxylin to illustrate the brain structure (A_1–A_4). CGE, caudal ganglionic eminence; LGE, lateral ganglionic eminence; LV, lateral ventricle; MGE, medial ganglionic eminence; MZ, mantle zone; OE, olfactory epithelium; SVZ, subventricular zone. Scale bar = 500 μm in A_1 (applies to A_1–C_4).

down-regulated in the adult striatum (Fig. 8). Despite the down-regulation, RARβ2 was still detectable and enriched in the adult striatum (Fig. $8B_{1–3}$). However, the rostrocaudal gradient of RARβ2 could not be clearly identified (Fig. $8B_{1–3}$). In the adult striatum, weak signals of RARβ1/3 were detected (Fig. $8C_{1–3}$), and RARβ3 signals were barely detectable (Fig. $8D_{1–3}$).

Expression of RARβ1/3 and RARβ2 mRNAs in Striatal Compartments

We studied the expression patterns of RARβ1/3 and RARβ2 mRNAs in relation to the striatal compartmentation of striosome (or patch) and matrix in the P0 striatum (Graybiel, 1990; Gerfen, 1992). We used digoxigenin-labeled probes to study gene expression at the cellular resolution level. Regardless the RARβ2 gradient, RARβ1/3-positive and RARβ2-positive cells appeared homogenously in the striatum (Fig. 9C,D). Using DARPP-32 as a marker for striosomal compartment (Fig. 9B; Foster et al., 1987) and Ebf-1 as a marker for the matrix compartment (Fig. 9A; Garel et al., 1999), we found that RARβ1/3 and RARβ2 mRNAs were expressed in both striosomal and matrix compartments in the P0 striatum (Fig. 9E–H). Moreover, the double *in situ* hybridization and immunofluorescent staining demonstrated that RARβ1/3 and RARβ2 were expressed in neurons of the developing striatum, as RARβ1/3 and RARβ2 mRNAs were colocalized with the neuronal marker of class III β-tubulin protein (TuJ1; Fig. 9I–L; Menezes and Luskin, 1994). As only a few glial fibrillary acidic protein-positive astrocytes were present in the P0 striatum, we did not assay whether RARβ1/3 and RARβ2 mRNAs were expressed by glial cells, although RARβ mRNA has been detected in human primary astrocytes *in vitro* (Chattopadhyay and Brown, 2001).

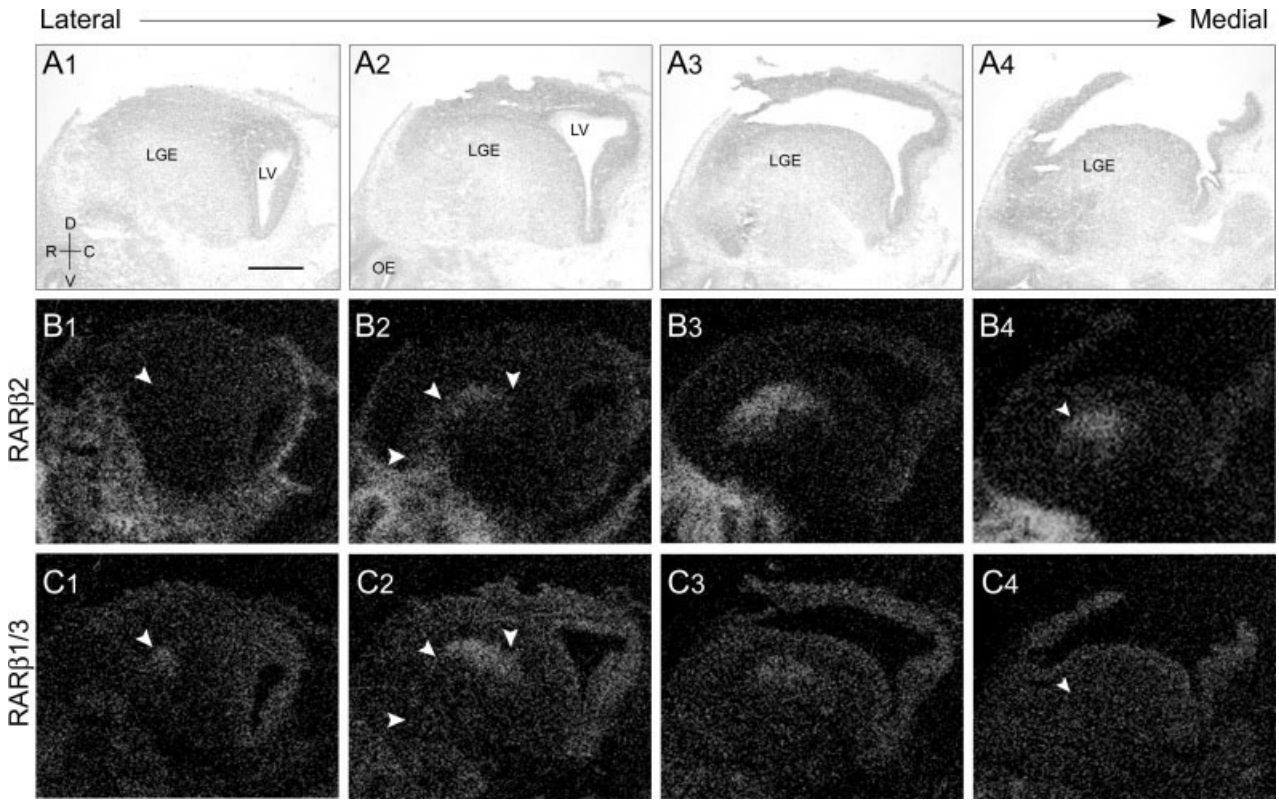

Fig. 4. **A_1–C_4:** Differential ontogeny of retinoic acid receptor-beta (RARβ) 2 and RARβ1/3 in E13.5 striatal primordium along the lateromedial axis. Representative parasagittal sections from the lateral to medial levels are illustrated for each cRNA probe (B_1–B_4, C_1–C_4). To compare spatial distribution of different isoforms, adjacent sections in B and C are processed in parallel for hybridization with different cRNA probes. RARβ1/3 is detected at the most lateral part of LGE (C_1, arrowhead), where RARβ2 is not detectable (B_1, arrowhead). At the next level of lateral LGE, RARβ1/3 is highly expressed (C_2), but RARβ2 is only weakly expressed (B_2). The trend is reversed in the medial level, where strong RARβ2 expression is dominant in the medial part of LGE (B_3, B_4) with low (C_3) and nondetectable level (C_4) of RARβ1/3 mRNA. Note that weak RARβ2 signals extend into the anteroventral part of LGE (B_2, arrowheads). The sections of C_1–C_4 are counterstained with hematoxylin to illustrate the brain structure (A_1–A_4). The arrowheads mark the corresponding regions in adjacent sections. LGE, lateral ganglionic eminence; LV, lateral ventricle; OE, olfactory epithelium; R, rostral; D, dorsal; C, caudal; V, ventral. Scale bar = 500 μm in A_1 (applies to A_1–C_4).

DISCUSSION

Differential Ontogeny of RARβ2 and RARβ1/3 in E13.5 Striatal Primordium

Our study indicated that RARβ2 mRNA was expressed in the LGE as early as E11.5, when the striatal primordium began to form, whereas very low levels of RARβ1/3 mRNA were first detected in the LGE at E12.5. At E13.5, RARβ2 and RARβ1/3 mRNAs were expressed in the LGE, and they shared an overlapping but domain-specific ontogenic pattern. The RARβ2 expression extended into the rostral and ventral domains of LGE, where very low level of RARβ1/3 was found. In contrast, the RARβ1/3 expression extended into the CGE at the caudal level. Moreover, RARβ2 and RARβ1/3 were differentially expressed in the medial and lateral domains of LGE. These differences constituted a domain-specific ontogenic pattern in E13.5 LGE, i.e., RARβ2 expression covered as far as the rostral and ventromedial domains, whereas RARβ1/3 expression was enriched in the caudal and dorsolateral domains. These expression patterns cannot be simply accounted for by a general neurogenesis gradient in the striatum which is from the ventrolateral to dorsomedial striatum (Bayer, 1984), suggesting a complex control for the ontogeny of RARβ isoforms.

Rostrocaudal Gradient of RARβ2 Expression in the Developing Striatum

Our finding of RARβ2 expression in the developing striatum is at good accord with the previous report that RARβ2/4 mRNAs are present in the developing striatum (Mollard et al., 2000). A major finding of our study was that there was a rostrocaudal gradient of RARβ2 expression in the developing striatum, which was not described in the previous report. The expression levels of striatal RARβ2 were gradually decreased from the rostral to caudal levels. The gradient was found not only in the caudoputamen of dorsal striatum, but it was also present in the nucleus accumbens and olfactory tubercle of ventral striatum. It is of particular interest that the RA-synthesizing enzyme retinaldehyde dehydrogenase-3 (Raldh3) is concentrated in the rostral part of developing striatum, including the nucleus accumbens (Li et al., 2000), which suggests that high levels of RA may be maintained in the rostral part of the striatum during development. Notably, RARβ2 is inducible by RA, as the promoter region of *RARβ2* gene contains a functional RA response element (Sucov

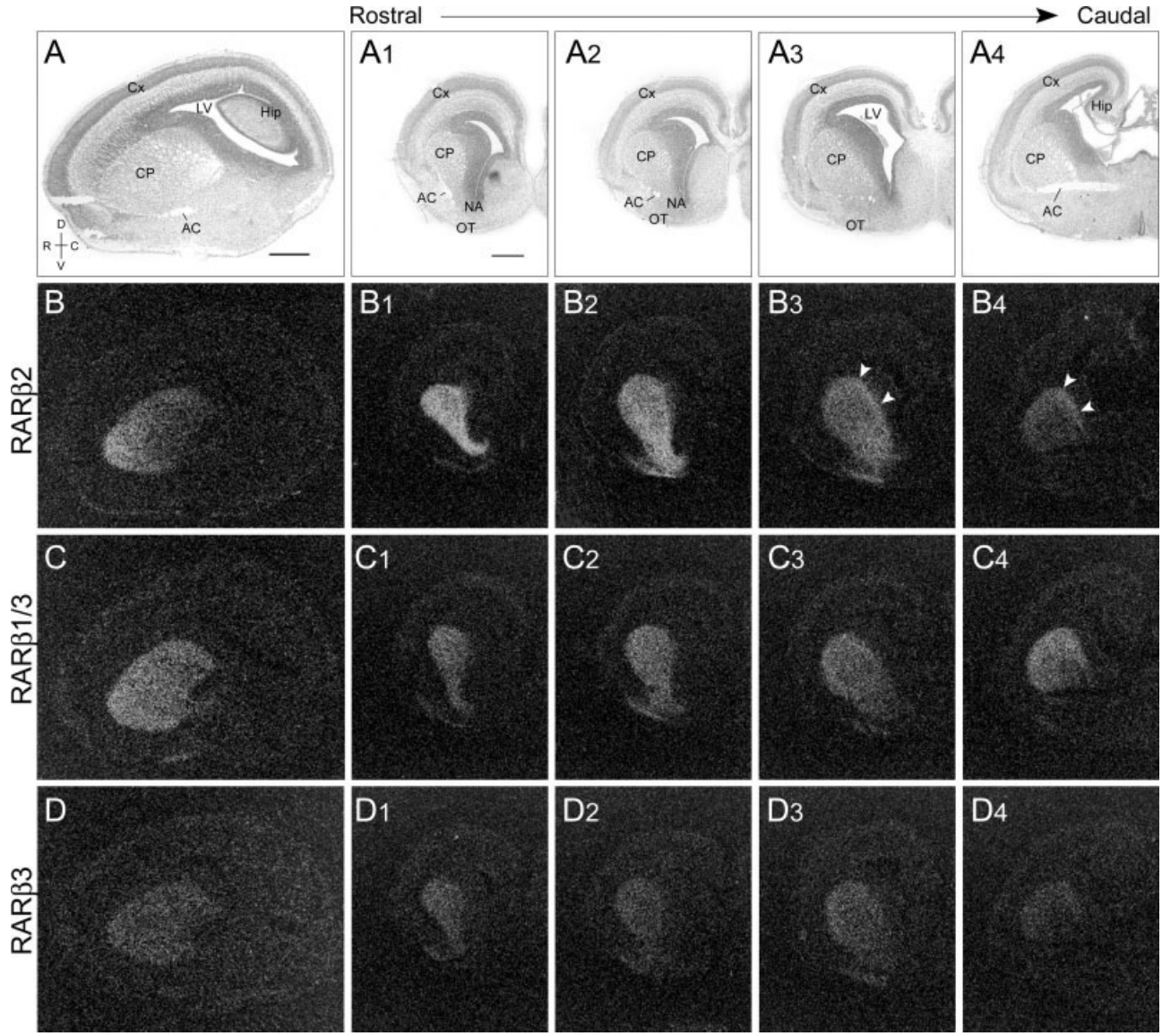

Fig. 5. A–D_4: Expression pattern of different retinoic acid receptor-beta (RARβ) isoforms in embryonic day (E) 16.5 striatum. Representative parasagittal sections (B–D) and coronal sections from the rostral to caudal levels (B_1–B_4, C_1–C_4, D_1–D_4) are illustrated for each cRNA probe. A prominent gradient of RARβ2 expression is present in the developing striatum, with decreasing signal intensity from the rostral to caudal levels (B, B_1–B_4). No such prominent rostrocaudal gradient is found for RARβ1/3 (C, C_1–C_4) and RARβ3 (D, D_1–D_4) mRNA expression. Note that a strip containing high levels of RARβ2 mRNA was present in the dorsomedial striatum at caudal levels (arrowheads in B_3, B_4). The sections in B, C, and D are serial adjacent sections. The sections in C and C_1–C_4 are counterstained with hematoxylin to illustrate the brain structure (A, A_1–A_4). AC, anterior commissure; CP, caudoputamen; Cx, cortical plate; Hipp, hippocampus; LV, lateral ventricle; NA, nucleus accumbens; OT, olfactory tubercle; R, rostral; D, dorsal; C, caudal; V, ventral. Scale bars = 500 μm in A (applies to A–D), in A_1 (applies to A_1–D_4).

et al., 1990). Taken together, it raises the possibility that the rostrocaudal gradient of RARβ2 expression may be set up by the RA gradient along the rostrocaudal axis in the striatum, i.e., the high levels of RARβ2 expression is induced by high concentrations of RA at rostral levels. Further evidence supporting this hypothesis is based on the fact that Raldh3 is down-regulated postnatally (Wagner et al., 2002), which is correlated with the gradual disappearance of the RARβ2 gradient during postnatal maturation. Unlike RARβ2, the promoter activity of RARβ1/3 is not inducible by RA, but RARβ1/3 is subject to RA regulation by an RA-dependent release of a block in RNA chain elongation (Mendelsohn et al., 1994). However, no RARβ1/3 gradient was found in the developing striatum, suggesting independent regulatory mechanisms underlying RARβ2 and RARβ1/3 expression.

The enrichment of RA and RARβ2 at rostral levels predicts that there may be a differential regulation of striatal development by RA along the rostrocaudal axis. Indeed, our recent genetic study indicates that null mutation of RARβ results in aberrant development of neurochemical compartments in the mutant striatum, and the aberrant phenotype of defective striosomal compartment is most evident in the rostral striatum (Liao et

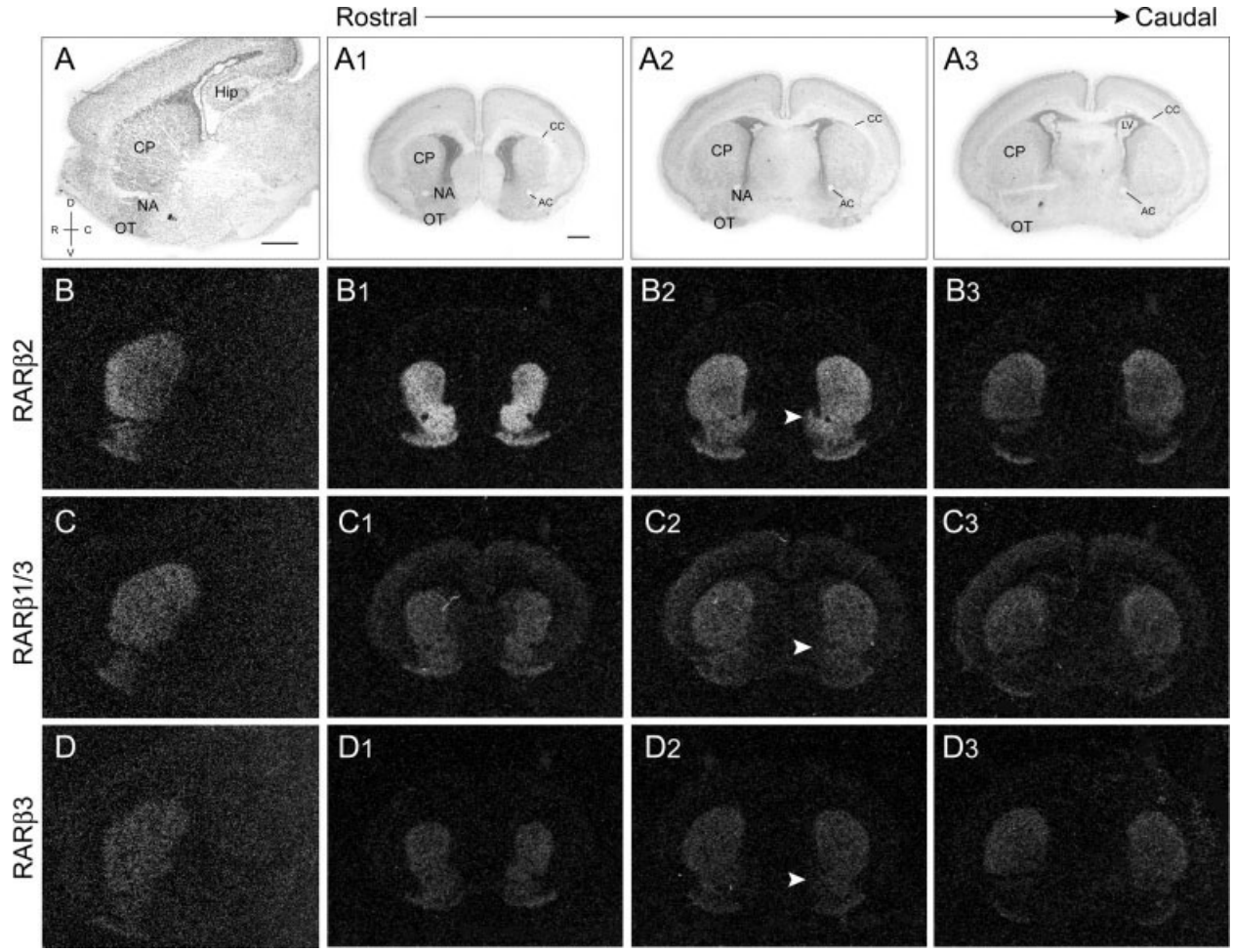

Fig. 6. A–D_3: Expression patterns of different retinoic acid receptor-beta (RARβ) isoforms in postnatal day 0 striatum. Representative parasagittal sections (B–D) and coronal sections from the rostral to caudal levels (B_1–B_3, C_1–C_3, D_1–D_3) are illustrated for each cRNA probe. The RARβ2 gradient appears in the striatum along the rostrocaudal levels (B, B_1–B_3). No such gradient is found for RARβ1/3 (C, C_1–C_3) and RARβ3 (D, D_1–D_3) mRNAs. Note that RARβ2 but not RARβ1/3 and RARβ3 is detected in the NA at the middle level (arrowheads in B_2, C_2, D_2). The sections in B, C, and D are serial adjacent sections. The sections of C and C_1–C_3 are counterstained with hematoxylin to illustrate the brain structure (A, A_1–A_3). AC, anterior commissure; CC, corpus callosum; CP, caudoputamen; Hipp, hippocampus; LV, lateral ventricle; NA, nucleus accumbens; OT, olfactory tubercle. Scale bars = 500 μm in A (applies to A–D), in A_1 (applies to A_1–D_3).

al., 2003). The RARβ null mutation disrupts all RARβ isoforms in the mutant mice; however, the relative contribution of each isoform to the aberrant phenotype is unclear. Notably, none of the RARβ isoforms we have examined in the present study has specificity of compartmental expression pattern. The RARβ null mutation also induces increases of calbindin expression in the shell of the nucleus accumbens, particularly at the rostral levels where high levels of RARβ2 occur (Liao et al., 2003).

Expression of RARβ1/3 Isoforms in the Developing Striatum

Unlike RARβ2, RARβ1/3 was expressed in the developing striatum without a distinct rostrocaudal gradient. Nonetheless, RARβ1/3 was expressed at a slightly higher level in the dorsolateral striatum at E13.5 and E16.5. Several neurochemical molecules, particularly the dopamine signaling molecules (including dopamine D1 receptor [D1R]) and DARPP-32, are expressed in the lateral striatum during prenatal development (Foster et al., 1987; Caille et al., 1995). RA signaling also is known to regulate D1R and DARPP-32 (Liu et al., 1998; Krezel et al., 1998; Wang et al., 1999; Toresson et al., 1999). Of interest, overexpression of RARβ3 in the LGE can enhance D1R expression, suggesting an involvement of RARβ3 in regulating D1R (W.-L. Liao and F.-C. Liu, unpublished observations). Moreover, ectopic expression of RARβ1 in developing cortical cells can induce DARPP-32 expression (Liao and Liu, 2002). Due to the fact that the size of the RARβ3 probe (90 bp) was smaller than the RARβ1/3 probe (146 bp), our attempt to delineate the expression pattern of RARβ1 by comparing the patterns of RARβ1/3 and RARβ3 was inconclusive at E11.5–E13.5, at which time no signal was detected with the RARβ3 probe. We could not rule out the possibility that the small probe might have failed to detect weak RARβ3 expression in the brain.

Developmental Regulation of RARβ Isoforms in the Striatum

For comparison of the signal intensity across different developmental stages,

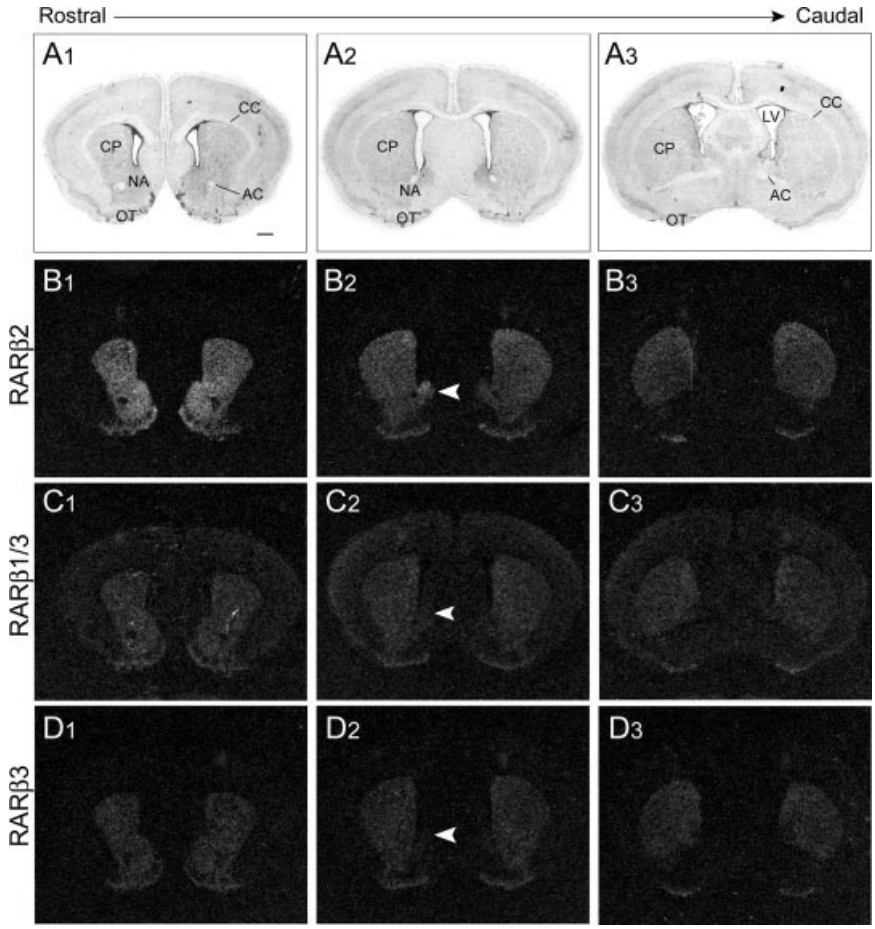

Fig. 7. **A_1–D_3:** Expression patterns of different retinoic acid receptor-beta (RARβ) isoforms in postnatal day (P) 7 striatum. Representative coronal sections from the rostral to caudal levels (B_1–B_3, C_1–C_3, D_1–D_3) are illustrated for each cRNA probe. The rostrocaudal RARβ2 gradient is still maintained in the striatum, albeit with weaker signal intensity than that in P0 striatum. Note that RARβ2 but not RARβ1/3 and RARβ3 is detected in the NA at the middle level (arrowheads in B_2, C_2, D_2). The sections in B, C, and D are serial adjacent sections. The sections of C_1–C_3 are counterstained with hematoxylin to illustrate the brain structure (A_1–A_3). AC, anterior commissure; CC, corpus callosum; CP, caudoputamen; LV, lateral ventricle; NA, nucleus accumbens; OT, olfactory tubercle. Scale bar = 500 μm in A_1 (applies to A_1–D_3).

a complete set of developmental brain sections simultaneously processed with each probe were packed in the same cassette for X-ray film autoradiography. The results showed that all the signals of RARβ2, RARβ1/3, and RARβ3 were decreased during postnatal development, suggesting a developmental down-regulation of RARβ mRNAs in the striatum. It is notable that the RARβ2 and RARβ1/3 probes contained similar numbers of ^{35}S-uridine but that the intensity of RARβ2 signals was higher than that of RARβ1/3 signals in all the developmental stages examined. This finding suggests that RARβ2 may be a major RARβ isoform expressed in the striatum during development. Due to lack of adequate unique DNA sequences to specifically distinguish RARβ4 from other isoforms, we were not able to examine the expression pattern of RARβ4.

EXPERIMENTAL PROCEDURES

Preparation of Brain Tissue

Embryos and pups from time-pregnant CD1 mice (National Yang-Ming University) were used for brain tissue harvesting. The day of the presence of vaginal plug was designated as E0.5, and the day of birth as P0. Prenatal tissues were obtained from pregnant mice deeply anesthetized with sodium pentobarbital. The embryonic brain tissues were obtained by immersion fixation of the heads of E11.5 (n = 4), E12.5 (n = 3), E13.5 (n = 8), and E16.5 (n = 6) embryos with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C for overnight. For postnatal brain tissues, P0 (n = 6) and P7 (n = 1) pups and adult mice (3–5 months, n = 4) were anesthetized by cooling on ice (P0, P7) or by intraperitoneal injection of sodium pentobarbital (adult), and they were then perfused transcardially with the same fixative followed by post-fixation at 4°C for overnight. The brains were cryoprotected at 4°C for 36–48 hr in 30% sucrose in 0.1 M PB. The embryonic heads were sectioned with a cryostat (Leica) at 20 μm in the coronal and parasagittal planes. The postnatal brains were sectioned at 20 μm in the coronal plane with a sliding microtome (Microm). The brain sections were stored in the anti-freezing solution containing 20% glycerol at −20°C before being processed for *in situ* hybridization. All efforts were made to minimize both the suffering and the number of animals used. The protocol of animal use conformed to the NIH Guide for the Care and Use of Laboratory Animals.

Cloning of RARβ-Specific Probes

The probes were generated by reverse transcription-polymerase chain reaction (RT-PCR) cloning. The 5′ primer and 3′ primer for each gene and the predicted sizes of the PCR product were as follows: RARβ2 (GenBank accession no. AJ002942): 5′-tgttctgtcagtgagtcc-3′, 5′-cgagacacagagtaccag-3′, 162 bp; RARβ3 (X56574), 5′-atgtcagaggacaactgg-3′, 5′-cgagacacagagtaccag-3′, 90 bp; RARβ1/3 [RARβ1 (X56569); RARβ3 (X56574)]: 5′-caccaccttctgtgtgattctg-3′, 5′-cagctgggtagtgagtcatgtg-3′, 146 bp. The PCR products were cloned into pCRII (Invitrogen) or pGEM-T-easy (Promega) vectors following the manufacturer's instruction and were confirmed by DNA sequencing.

In Situ Hybridization With Isotope-Labeled Probes

A modified procedure of *in situ* hybridization was performed (Simmons et al., 1989). The RARβ1/3-pGEM-T-easy was

linearized with *Sal*I and *Nco*I for generating antisense and sense probes, respectively. The RARβ2-pCRII and RARβ3-pCRII plasmids were linearized with *Bam*HI and *Xho*I for generating antisense and sense probes, respectively. The production of cRNA probes were carried out in the presence of ^{35}S-UTP (Amersham) with T7 or SP6 RNA polymerase by *in vitro* transcription. The percentages of uridines in the RARβ1/3, RARβ2, and RARβ3 cRNA probes were 21.23%, 19.63%, and 18.89%, respectively. Brain sections were treated with 10 μg/ml proteinase K at 37°C for 5 min, 10 min, and 20 min for embryonic, P0, and other postnatal sections, respectively, before hybridization. The RNA hybridization was carried out with ^{35}S-UTP-cRNA probes (10^7 cpm/ml) in the hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 1× Denhardt's solution, 0.01 M Tris (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA, pH 8.0), 0.5 μg/μl yeast tRNA, and 1 mM dithiothreitol (DTT) at 58°C for 16 hr. After several rinses in 4× standard saline citrate (SSC), the slides were treated with RNase A (10 μg/ml) and were washed with 2× SSC, 1× SSC, and 0.5× SSC at room temperature. The slides were then rinsed with 0.1× SSC at 50°C for 30 min followed by another 0.1× SSC wash at room temperature for 5 min. The sections were dehydrated and then exposed to X-ray film for autoradiography. Due to the small size of RARβ3 probe, its exposure time was doubled of that for RARβ1/3 and RARβ2 probes.

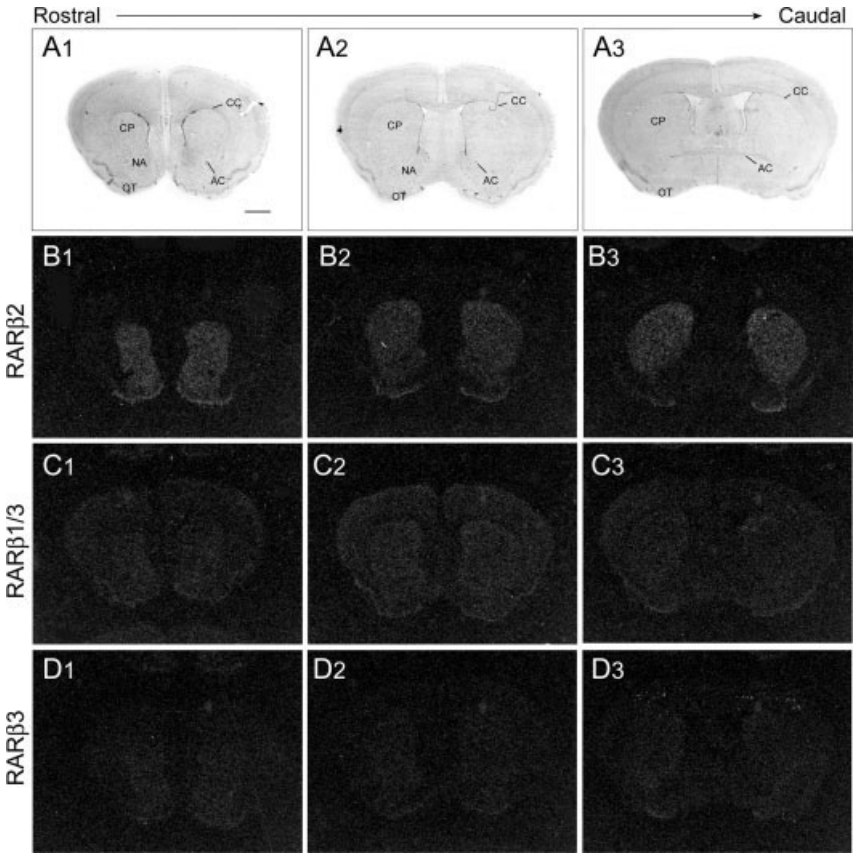

Fig. 8. **A_1–D_3:** Expression patterns of different retinoic acid receptor-beta (RARβ) isoforms in the adult striatum. Representative coronal sections from the rostral to caudal levels (B_1–B_3, C_1–C_3, D_1–D_3) are illustrated for each cRNA probe. RARβ2 mRNA is detected in the striatum without gradient; so are RARβ1/3 and RARβ3 mRNAs. The sections in B, C, and D are serial adjacent sections. The sections of C_1–C_3 are counterstained with hematoxylin to illustrate the brain structure (A_1–A_3). AC, anterior commissure; CC, corpus callosum; CP, caudoputamen; NA, nucleus accumbens; OT, olfactory tubercle. Scale bar = 1 mm in A_1 (applies to A_1–D_3).

In Situ Hybridization With Non–Isotope-Labeled Probes

The digoxigenin-labeled antisense cRNA probes for RARβ2, RARβ1/3, DARPP-32 (GenBank accession no. AF281662, nt 68-274) and Ebf1 (NM_053820, nt 1847-2214) were synthesized by *in vitro* transcription with T7 polymerase (Promega), RNA labeling mix (Roche) and cDNA plasmids. The probe hybridization and signal detection were performed as previously described with some modifications (Takahashi et al., 2003). Briefly, 30-μm sections of P0 brains were treated with 0.2 N HCl and proteinase K (1 μg/ml) and were then prehybridized with 50% formamide in 2× SSC for 90 min at 65°C. The sections were hybridized with digoxigenin-labeled cRNA probes in the hybridization buffer (10.6% dextran sulfate, 53% formamide, 1 mM EDTA, 10.6 mM Tris, 318 mM NaCl, 1.06 × Denhardt solution, 500 μg/ml tRNA and 10 mM DTT) for 16 hr at 65°C. The sections were washed with 2× SSC containing 50% formamide for 1 hr at 65°C, followed by RNase A (20 μg/ml) treatment in 10 mM Tris HCl (pH 8.0) and 500 mM NaCl for 30 min at 37°C. The sections were washed sequentially with 2× SSC and 0.2× SSC for 20 min. The sections were treated with 2% blocking reagent and 20% sheep serum for 1 hr at 30°C and then incubated with alkaline phosphatase-conjugated sheep anti-digoxigenin antibody (Roche) for 1 hr at room temperature. The signals were detected by colorimetric reaction using nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate as the substrate.

Double *In Situ* Hybridization and Immunofluorescent Staining

After *in situ* hybridization, some of the hybridized sections were processed for immunofluorescent staining as previously described (Liu and Graybiel, 1992). The sections were immunostained for neuronal class III β-tubulin with rabbit anti-class III β-tubulin antibody (Covance, 1:2,000), biotinylated goat anti-rabbit IgG (Vector, 1:500), and DTAF-conjugated streptavidin (Jackson IRL, Inc., 1:1,000) using Elite ABC kit (Vector).

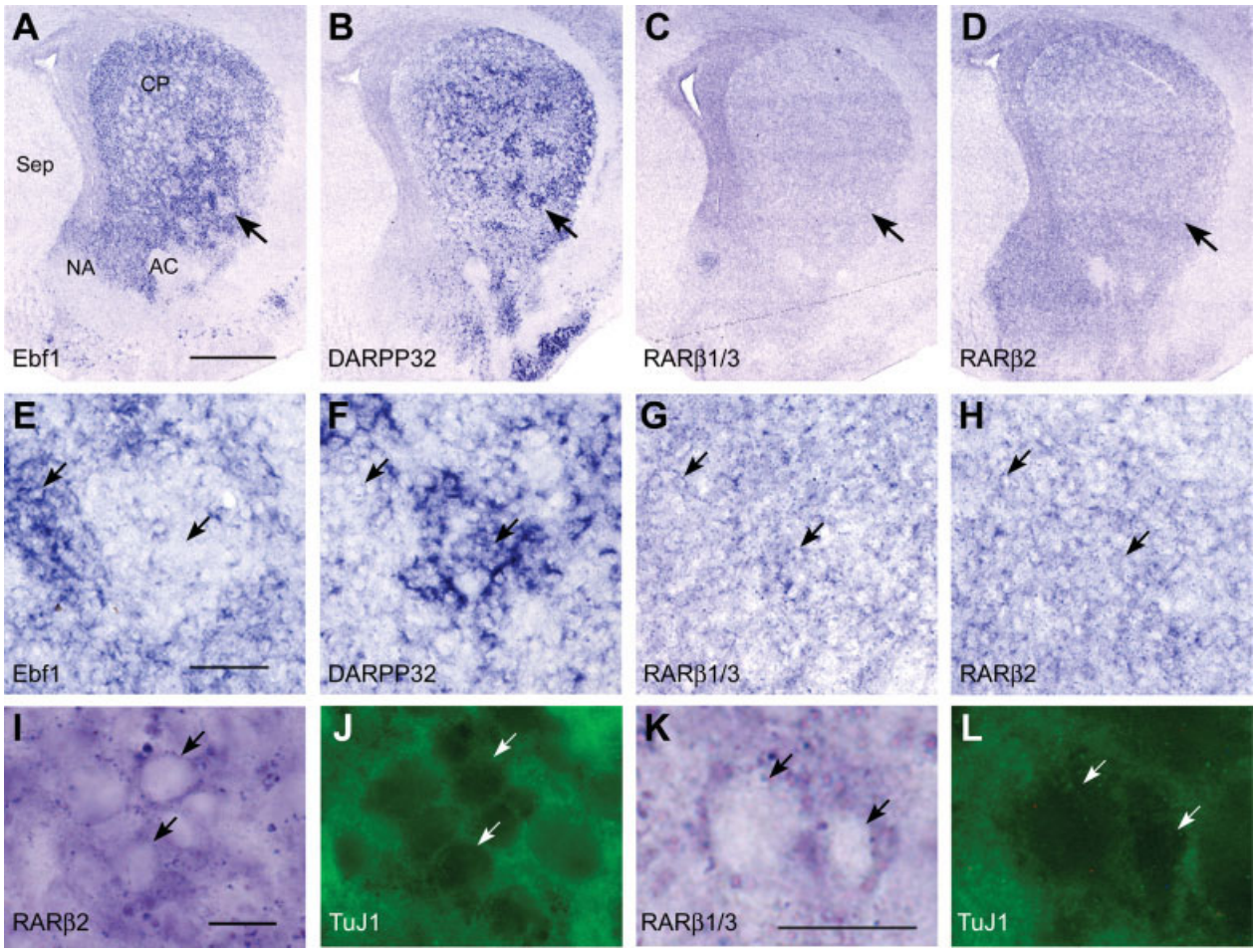

Fig. 9. Expression of retinoic acid receptor-beta (RARβ) 1/3 and RARβ2 mRNAs in both striosomal and matrix compartments. **A–D:** Digoxigenin-labeled antisense cRNA probes were hybridized with Ebf-1 (A), DARPP-32 (B), RARβ1/3 (C), and RARβ2 (D) mRNAs in the postnatal day 0 striatum. A–C are serial adjacent 30-μm sections, whereas D is 30 μm apart from C. DARPP-32–positive cell clusters mark the loci of striosomal compartment (B), whereas Ebf-1 is enriched in the matrix (A). An example of a DARPP-32–positive striosome in alignment with an Ebf-1–poor zone is illustrated by the arrows in B and A. The corresponding regions indicated by the arrows in A–D are shown at high magnification in E–H. **E–H:** RARβ1/3- and RARβ2-positive cells appear homogenously in the striatum (C,D; examples at arrows in G,H), and they are distributed in both DARPP-32–positive striosomes (F) and Ebf-1–enriched matrix (E) compartments. **I–L:** Double *in situ* hybridization and immunofluorescent staining shows that RARβ2 (I) and RARβ1/3 (K) mRNAs are colocalized with the neuronal marker of class III β-tubulin protein (TuJ1, examples at arrows in J,L). AC, anterior commissure; CP, caudoputamen; NA, nucleus accumbens; Sep, septum. Scale bars = 500 μm in A (applies to A–D), 50 μm in E (applies to E–H), 10 μm in I (applies to I,J), 10 μm in K (applies to K,L).

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