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Retinoids are produced by glia in the lateral ganglionic eminence and regulate striatal neuron differentiation

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SUMMARY

In order to identify molecular mechanisms involved in striatal development, we employed a subtraction cloning strategy to enrich for genes expressed in the lateral versus the medial ganglionic eminence. Using this approach, the homeobox gene *Meis2* was found highly expressed in the lateral ganglionic eminence and developing striatum. Since *Meis2* has recently been shown to be upregulated by retinoic acid in P19 EC cells (Oulad-Abdelghani, M., Chazaud, C., Bouillet, P., Sapin, V., Chambon, P. and Dollé, P. (1997) *Dev. Dyn.* 210, 173-183), we examined a potential role for retinoids in striatal development. Our results demonstrate that the lateral ganglionic eminence, unlike its medial counterpart or the adjacent cerebral cortex, is a localized source of retinoids. Interestingly, glia (likely radial glia) in the lateral ganglionic eminence appear to be a major source of retinoids. Thus, as lateral ganglionic eminence cells migrate along radial glial fibers into the developing striatum, retinoids from these glial cells could exert an effect on striatal neuron differentiation. Indeed, the treatment of lateral ganglionic eminence cells with retinoic acid or agonists for the retinoic acid receptors or retinoid X receptors, specifically enhances their striatal neuron characteristics. These findings, therefore, strongly support the notion that local retinoid signalling within the lateral ganglionic eminence regulates striatal neuron differentiation.

Key words: CRBP I, DARPP-32, *Meis2*, Radial glia, RAR, RC2, Retinoic acid, RXR, Striatum

INTRODUCTION

The developmental mechanisms regulating the formation of forebrain structures (including both the telencephalon and diencephalon) is currently a subject of considerable interest (for reviews, see Fishell, 1997; Rubenstein and Shimamura, 1997). The telencephalon derives from the most anterior region of the neural plate and gives rise to two major structures: the cerebral cortex and the corpus striatum (including both the striatum and the pallidum). While the cortex develops largely from the dorsal telencephalic neuroepithelium (Bayer and Altman, 1991), the corpus striatum arises from the ganglionic eminences located in the floor of the telencephalic vesicle (Smart and Sturrock, 1979). Recent studies have shown that the lateral ganglionic eminence (LGE) is the principal source of striatal neurons generating both the GABAergic projection neurons (Pakzaban et al., 1993; Olsson et al., 1995, 1998) as well as a population of striatal interneurons colocalizing GABA and somatostatin (Olsson et al., 1998). The LGE also appears to give rise to certain populations of cortical neurons (de Carlos et al., 1996; Tamamaki et al., 1997; Anderson et al., 1997a). The medial ganglionic eminence (MGE), in contrast, is responsible for generating neurons of the pallidum and basal forebrain, such as the cholinergic neurons (Olsson et al., 1998). Although the cellular contributions of each of the ganglionic eminences to the neuronal subtypes present in the telencephalon are reasonably well understood, little is known about the signals and molecular mechanisms controlling their generation.

While morphological structures/boundaries, such as the LGE, provide indicators of regional differentiation within the developing brain, spatially and temporally restricted expression of developmental control genes is, in most cases, evident prior to these morphological distinctions. Although no genes known to date are localized exclusively to the LGE, *Gsh2* (Hsieh-Li et al., 1995) and different members of the *Dlx* gene family (Liu et al., 1997) are known to be expressed in both the MGE and LGE. In mice where *Gsh2* has been inactivated, the LGE fails to develop normally as evidenced by a reduction in size and the lack of *Dlx2* expression (Szucsik et al., 1997). Furthermore, mice with mutations in both the *Dlx1* and *Dlx2* genes show abnormal differentiation of cells in the

subventricular zone (SVZ) of the LGE accompanied by a defect in migration of late born striatal neurons (Anderson et al., 1997b).

As an approach to further understand the molecular control of striatal differentiation, we have taken a subtraction cloning strategy to identify genes enriched in the LGE and thus putative regulators of striatal development. We show here that the homeobox gene *Meis2* is highly enriched in the LGE with respect to the MGE and marks striatal progenitors/neurons from their earliest stage into adulthood. Since *Meis2* was recently shown to be induced by retinoic acid (RA) in P19 EC cells (Oulad-Abdelghani et al., 1997), we investigated a role for retinoids in striatal development. The results presented here show that retinoids are produced within the LGE by glial cells and that they enhance striatal neuron differentiation.

MATERIALS AND METHODS

Subtraction cloning of LGE enriched genes

Differential dissection of the MGE and LGE from E12.5 mouse embryos was performed as shown in Fig. 1 (see also Olsson et al., 1995). MGEs and LGEs from 25 embryos were collected and immediately thereafter frozen at −80°C. mRNA was extracted using Dynal's *mRNA Direct* kit. Extracted RNA was re-extracted to obtain highly enriched poly(A)+ RNA. Tester (LGE) and driver (MGE) cDNA was synthesized from approximately 600 ng of mRNA. Synthesis of cDNA and suppression subtraction hybridization was performed using Clontech's *PCR-Select* kit following the manufacturers instructions (for reference, see Diatchenko et al., 1996). Following subtractive hybridization and PCR-amplification, PCR products were cloned into the PCR-Script vector (Stratagene) and inserts were sequenced using the *Thermosequenase* kit (Amersham) and compared to the sequences in GenBank using the BLAST search program (Altshul et al., 1990) located at the NCBI website (www.ncbi.nlm.nih.gov).

In situ hybridization histochemistry (ISHH)

All embryos used for ISHH and immunohistochemistry were fixed in 4% paraformaldehyde (PFA) at 4°C overnight, subsequently sunk in PBS containing 30% sucrose and sectioned at 12-14 μm thickness on a cryostat. Adult mouse brains were removed fresh and rapidly frozen in dry ice before sectioning.

Non-radioactive ISHH

A subcloned fragment of *Meis2* cDNA (corresponding to nt 179-1412, Nakamura et al., 1996) in pBluescript (Stratagene) was used as a template to generate an antisense digoxigenin (DIG)-labelled cRNA probe as previously described (Campbell et al., 1995). The hybridization solution contained 50% formamide (deionized), 10% dextran sulphate, 1% Denhardt's, 1% sarcosyl, 0.3 M NaCl, 10 mM Na_2HPO_4 pH 7.2, 20 mM Tris-HCl pH 8.0, 5 mM EDTA, 250 μg/ml yeast tRNA and approximately 1 μg of DIG-labeled probe/ml. Hybridization was carried out overnight at 55°C in a sealed humid box. Following hybridization, slides were washed in 2× SSC/50% formamide at 65°C for 30 minutes followed by RNase treatment (20 μg/ml) at 37°C for 30 minutes. Slides were washed again in 2× SSC/50% formamide, twice for 20 minutes each, at 65°C; then in 2× SSC and 0.1× SSC for 15 minutes each at 37°C. Final wash was in PBT (PBS + 0.1% Tween-20) for 15 minutes and the colour reaction was carried out essentially as described (Campbell et al., 1995) except that BM Purple (Boehringer Mannheim) was used in place of NBT and BCIP.

Radioactive ISHH

Oligonucleotide sequences used were complementary to nt 652-701 of rat DARPP-32 (Ehrlich et al., 1990); nt 1264-1308 of *Meis2a* and *Meis2c* (Oulad-Abdelghani et al., 1997); nt 1243-1290 of *Meis2b* and *Meis2d* (Oulad-Abdelghani et al., 1997); nt 1030-1079 of mouse RARα (Pratt et al., 1990); nt 1346-1395 of mouse RARβ (Heiermann et al., 1993). Labelling of the oligonucleotides with $[^{35}S]$dATP and ISHH was as previously described (Campbell et al., 1995). For the *Meis2* oligo in situ, a mixture of the two oligonucleotides was used in order to detect all isoforms.

Immunohistochemistry

Immunohistochemistry was carried out as previously described by Olsson et al. (1997). Antibodies used were: mouse anti-β-III-tubulin (1:333, Sigma), mouse anti-DARPP-32 (1:20 000, provided by Dr P. Greengard), rabbit anti-distal-less (i.e. DLX, 1:200, provided by Dr G. Panganiban), rabbit anti-CRBP I (1:400, provided by Dr U. Eriksson) and mouse anti-RC2 (1:4, generated by Dr M. Yamamoto and obtained from the Developmental Studies Hybridomas Bank, University of Iowa). Double immunoflourescent staining for CRBP I and RC2 was carried out by incubating the two primary antibodies together overnight and thereafter followed by incubation in Cy3-conjugated donkey anti-rabbit (1:200, Jackson) and biotinylated goat anti-mouse IgM (1:200, Vector labs). A final incubation was performed using FITC-conjugated avidin (Vector labs).

Retinoid detection assay

LGE, MGE and cortex were separately dissected from E12.5 mouse embryos in L-15 medium (Gibco) as indicated in Fig. 1. The cortical pieces were incubated in 1% dispase (Gibco) in L-15 for 5 minutes at room temperature to remove the meninges. In previous studies using E13.5 mouse embryos, we have found that each of the dissected LGE, MGE and cortical pieces contain roughly similar numbers of cells (M. Olsson and K. C., unpublished results).

Retinoid production was assayed as previously described (Perlmann and Jansson, 1995; Zetterström et al., 1999). Briefly, human chorion carcinoma JEG-3 cells, maintained in Minimal Essential Medium (MEM; Gibco) supplemented with 10% bovine calf serum, 1% penicillin/streptomycin and 1% L-glutamine, were transfected with a plasmid containing the upstream activating sequence (UAS) upstream of the HSV TK promoter driving the luciferase gene and a plasmid with either the GAL4-RAR or GAL4-RXR construct, containing the DNA-binding domain of the yeast transcription factor GAL4 fused in frame with the ligand-binding domain of the human RARα or RXRα. After rinsing the transfected cells with PBS, 12 explants of either LGE, MGE or cortex were then added per well (LGE, 8 wells; MGE, 9 wells; cortex, 5 wells), incubated in the above medium (containing charcoal-stripped calf serum) with the transfected cells for 24 hours and assayed for luciferase activity as described (Zetterström et al., 1999). In the case of the conditioned medium from glial cultures (described below), 300 μl of condtioned medium was added to the transfected cells in each well (n=3 for each condition) and grown for 24 hours. As a control, unconditioned medium (the same that was used to collect from the glial cultures) was assayed.

Glial cultures

MGEs or LGEs were dissected from E13.5 embryos in L15 medium as described above and dissociated in 0.1% trypsin and 0.05% DNase in DMEM for 15-20 minutes at 37°C before mechanical dissociation and plating at high density in tissue culture treated flasks. Cells were grown in DMEM with 10% fetal calf serum (FCS), glutamine (2 mM), N2-supplement (Gibco), EGF (20 ng/ml) and antibiotics**.** Both neurons and glia were present in the initial cultures; however, by the 4th passage (P.4) the cultures were devoid of cells possessing neuronal morphologies or expressing neuronal markers (i.e. β-III-tubulin). These cultures were highly enriched in cells expressing glial phenotypes (i.e. RC2 and GFAP).

To test for retinoid production, flasks containing P.4 LGE glia, P.4

MGE glia or P.9 LGE glia were grown to near confluence in the above medium and subsequently changed to DMEM containing 10% FCS, glutamine (2 mM) and antibiotics, and grown for 3 days. Conditioned medium was collected every 24 hours over the 3 days and replaced with fresh medium. The conditioned medium from P.4 LGE glia was also collected over 3 days from cells grown in a serum-free medium (DMEM containing N2-supplement, glutamine (2 mM) and antibiotics) in order to exclude the retinol in the FCS. The conditioned medium collected from the glial cultures on each day was immediately frozen and stored at −80°C until assayed for retinoids as described above.

Neuron cultures

LGEs or MGEs were selectively dissected from E13.5 mouse embryos and dissociated as above, before plating as described by Nakao et al. (1994). Cells were plated at a density of 2×10^5 cells/cm^2 in 4- or 8-well chamber slides (Nalge Nunc Int.), precoated with poly-D-lysine (Sigma) and cultured under serum-free conditions for 5 days. This plating density was chosen since it has previously been shown to maximize both the number and survival of DARPP-32-expressing neurons in the LGE cultures (Nakao et al., 1996).

RA (all-*trans*, Sigma) dissolved in dimethyl sulfoxide (DMSO, Sigma) was added to the medium at concentrations of 10 nM or 1 μM. Fresh RA/DMSO was added every 24 hours of culture and a final dose was given 6 hours prior to fixation. Selective agonists of the RAR (TTNPB) or RXR (SR11237) subtypes of retinoid receptors (Perlmann and Jansson, 1995) were added to the cultures at 100 nM concentration on the first day of culture and also when the medium was changed to serum-free conditions on the second day (Nakao et al. 1994). Equal amounts of DMSO were added to control, TTNPB- and SR11237-treated cultures daily. Cultures were fixed for 20 minutes at room temperature in 4% PFA, rinsed and processed for DARPP-32 or β-III-tubulin immunohistochemistry, as described above. In order to compare the staining in control versus RA-treated cultures, DAB reactions were carried out for exactly the same time in each case. All cell counts were made in a non-biased manner using stereological counting methods (Gundersen, 1986). Approximately 300 cells were counted per culture.

RESULTS

Meis2 is expressed in the LGE and striatum

In an attempt to identify genes involved in striatal development, we performed a PCR-based suppressive subtraction hybridization (Diatchenko et al., 1996) to obtain cDNAs showing enriched expression within the E12.5 mouse LGE as compared to the MGE. One of the cDNA fragments obtained using this approach was identical to a 587 base pair stretch in the 3′ UTR of the recently cloned *Meis2* homeobox gene (Nakamura et al., 1996; Oulad-Abdelghani et al., 1997; Cecconi et al., 1997). *Meis2* is indeed expressed at a high level specifically within the E12.5 subventricular zone (SVZ) and mantle layer of the LGE (Fig. 2A; for orientation see also Fig. 1). Weak to moderate levels of expression are also seen within the ventricular zone (VZ) of the entire telencephalon with the exception of the most dorsomedial and ventromedial aspects (Fig. 2A). In contrast to *Meis2*, cells expressing DLX proteins (detected by an antibody generated against their *Drosophila* homologue, Distal-less) are observed throughout both the LGE and MGE (Fig. 2B) consistent with the expression of the *Dlx* gene family (Liu et al., 1997). High levels of *Meis2* expression continue at E16.5 (Fig. 2C) and E18.5 (not shown) within the SVZ and developing striatal complex (caudate/putamen,

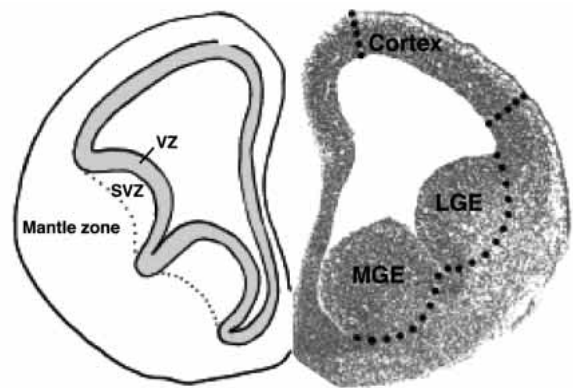

Fig. 1. Coronal section through the E12.5 mouse telencephalon. Nissl-stained section on the right shows the relative positions of the medial ganglionic eminence (MGE), lateral ganglionic eminence (LGE) and the developing cerebral cortex. Dotted lines approximate the regions dissected in the present study. Schematic diagram on the left shows the position of the ventricular zone (VZ), subventricular zone (SVZ) and the mantle zone.

nucleus accumbens and olfactory tubercle). In addition, *Meis2* expression is also seen in the cortical plate (Fig. 2C) and regions of the developing amygdala (data not shown). Even at these later stages, derivatives of the MGE (e.g. globus pallidus) do not express *Meis2* (asterisk in Fig. 2C). *Meis2* expression remains in the adult striatum, albeit at much lower levels. The pattern of *Meis2* expression in the mature striatum (Fig. 2D) is very similar to that of the *dopamine and cAMP-regulated phosphoprotein* (*DARPP-32*) (Fig. 2E), which is expressed in the GABAergic projection neurons that comprise the vast majority of striatal neurons (Anderson and Reiner, 1991).

Markers of retinoid synthesis and signalling in the LGE and developing striatum

The fact that *Meis2* is rapidly induced by RA in P19 EC cells (Oulad-Abdelghani et al., 1997) prompted us to investigate a role for retinoids in the differentiation of the striatum. As a first attempt to address this, we examined the expression of Cellular Retinol Binding Protein I (CRBP I) which is known to bind retinol and retinaldehyde in cells synthesizing RA (Napoli, 1996). We show here that within the telencephalon CRBP I protein is present at a high level in the LGE (particularly in the VZ) at both E12.5 (Fig. 3A) and E16.5 (Fig. 3B). Although the highest levels of CRBP I expression are found in the LGE VZ, weak levels are seen in the MGE and scattered cells with neuronal morphologies are found in the mantle regions of the telencephalon. These observations are largely consistent with an earlier study (Ruberte et al., 1993) showing that *CRBP I* mRNA is enriched in the corpus striatum.

Ruberte et al. (1993) have previously shown that *RARα* and *RARβ* are enriched in the corpus striatum. We have reassessed the expression of these two receptors and shown that they are expressed largely in different subregions of the developing striatum, with *RARα* being highest expressed in the SVZ (Fig. 3C,D) and *RARβ* in the differentiating striatum (Fig. 3F). At E12.5, we could not detect high levels of *RARβ* in the telencephalon (Fig. 3E) unlike that previously described by Ruberte et al. (1993); however, we used oligonucleotide probes that may be less sensitive than the cRNA probes used by these

authors. In addition to *RAR* gene expression, *RXRγ* is also expressed in the developing striatum in a similar pattern to that of *RARβ* (data not shown, Dollé et al., 1994).

Localized production of retinoids in the LGE and developing striatum

To obtain definitive evidence that the LGE is a source of retinoids, we made use of a cell-based reporter assay to detect retinoid signalling (Perlmann and Jansson, 1995; Zetterström et al., 1999). In this system, cells are co-transfected with GAL4-RAR or GAL4-RXR constructs and a UAS-luciferase construct so that only in the presence of retinoids can the RAR or the RXR activate *luciferase* expression. We dissected the E12.5 LGE, MGE and cerebral cortex (see Fig. 1) and grew them as explants in co-culture with the transfected reporter cells. The LGE explants produced a dramatic 29-fold increase in RAR signalling (measured by luciferase activity) as compared with control (Fig. 4, $P<0.001$, one-way ANOVA). In contrast to the LGE explants, neither MGE nor cortical explants significantly altered RAR signalling when compared with control (Fig. 4). Although the LGE explants were efficient at activating the UAS-luciferase construct through the GAL4-RAR construct, no significant activation was detected with the GAL4-RXR construct (data not shown). These results clearly demonstrate that, at E12.5, the LGE represents a localized source of retinoids.

CRBP I is expressed in radial glia of the LGE

Having demonstrated that the LGE is a localized source of retinoids in the embryonic brain, we were interested to determine what cell type(s) are responsible for this production. The strong correlation between CRBP I expression and retinoid signalling seen in the explant experiments stimulated us to first examine what cell types in the LGE express CRBP I. As mentioned above, the highest expression of CRBP I is seen in the VZ of the LGE (see Fig. 3A,B); however, higher power analysis shows processes extending through the LGE from the VZ to the pial surface (Fig. 5A). This morphology is very reminiscent of that seen in radial glia, which have their cell bodies in or near the VZ and send processes terminating in end feet at both the ventricular and pial surfaces (Rakic, 1995). Confocal microscopy of double stains for CRBP I and the radial glial marker, RC2 (Misson et al., 1988; Fig. 5B) at E12.5 confirmed that this retinoid marker is indeed expressed by radial glia (Fig. 5C).

Glial cultures from the LGE produce high levels of retinoids

To determine whether LGE glia do produce retinoids, we generated glial cultures from the E13.5 LGE. Analyses of these cultures at passage (P.)4 showed that many cells express CRBP I (Fig. 6A,D). Moreover, these cultures are devoid of neurons (as detected by β-III-tubulin) and highly enriched in glial phenotypes, such as RC2- (Fig. 6B,E) and GFAP- (data not shown) expressing cells. As was the case in vivo, extensive CRBP I and RC2 co-localization was also observed in the P.4 LGE glial cultures (Fig. 6C,F) suggesting that, even in culture, CRBP I is expressed by cells bearing phenotypical markers of radial glia. Interestingly, the expression of CRBP I appears to

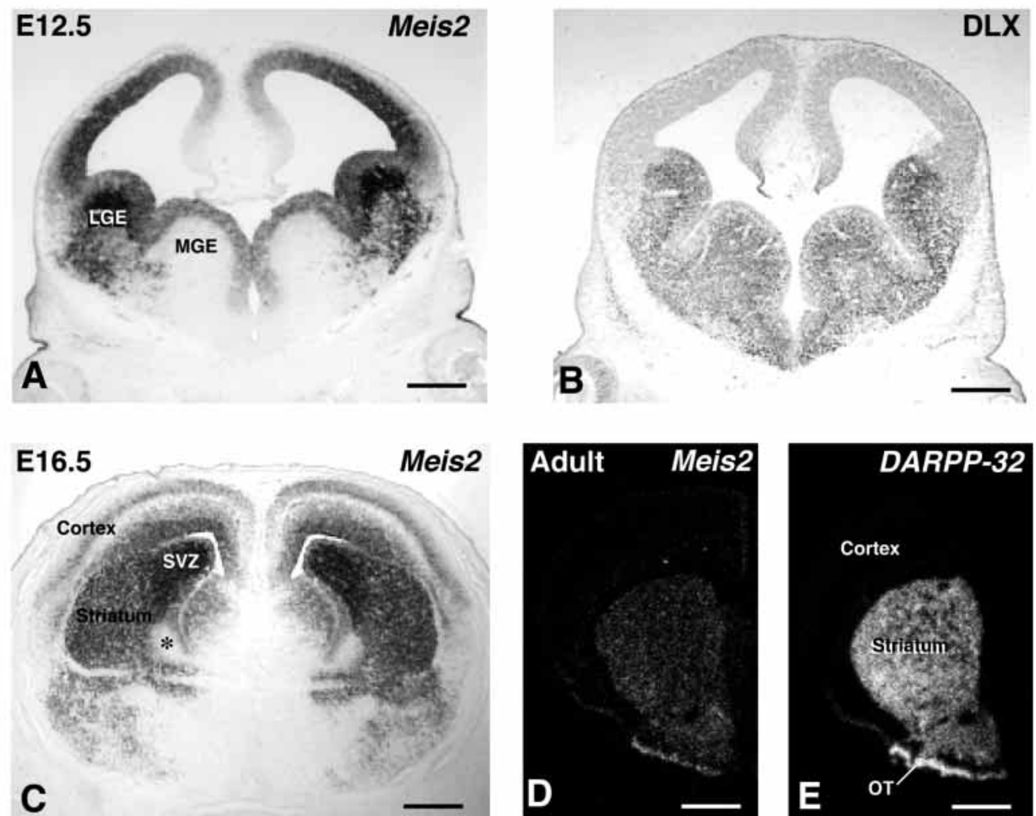

Fig. 2. Expression of *Meis2* in the developing telencephalon. (A) *Meis2* is expressed at high levels in the E12.5 LGE SVZ and underlying mantle zone while the MGE SVZ and mantle remains unlabeled. Low to moderate levels are also detected in the VZ of the telencephalon except for the most dorsomedial and ventromedial aspects. (B) Immunohistochemistry for proteins of the DLX family using an antibody directed against *Drosophila* distal-less labels cells of both the MGE and LGE at E12.5. (C) *Meis2* transcript distribution at E16.5 is essentially the same as at E12.5 with the SVZ and maturing striatum showing the highest level of expression and leaving the developing globus pallidus (asterisk) unlabeled. At this stage, cells in the cortical plate are also seen to express *Meis2*. (D) In the adult telencephalon, *Meis2* transcripts remain highly specific for the striatum, which in the adult is also marked by high levels of *DARPP-32* expression (E). *Meis2* expression is clearly down-regulated in the adult telencephalon as compared to prenatal levels. OP, olfactory pit; OT, olfactory tubercle. Scale bars in A,B, 370 µm; C, 500 µm; D,E, 1.3 mm.

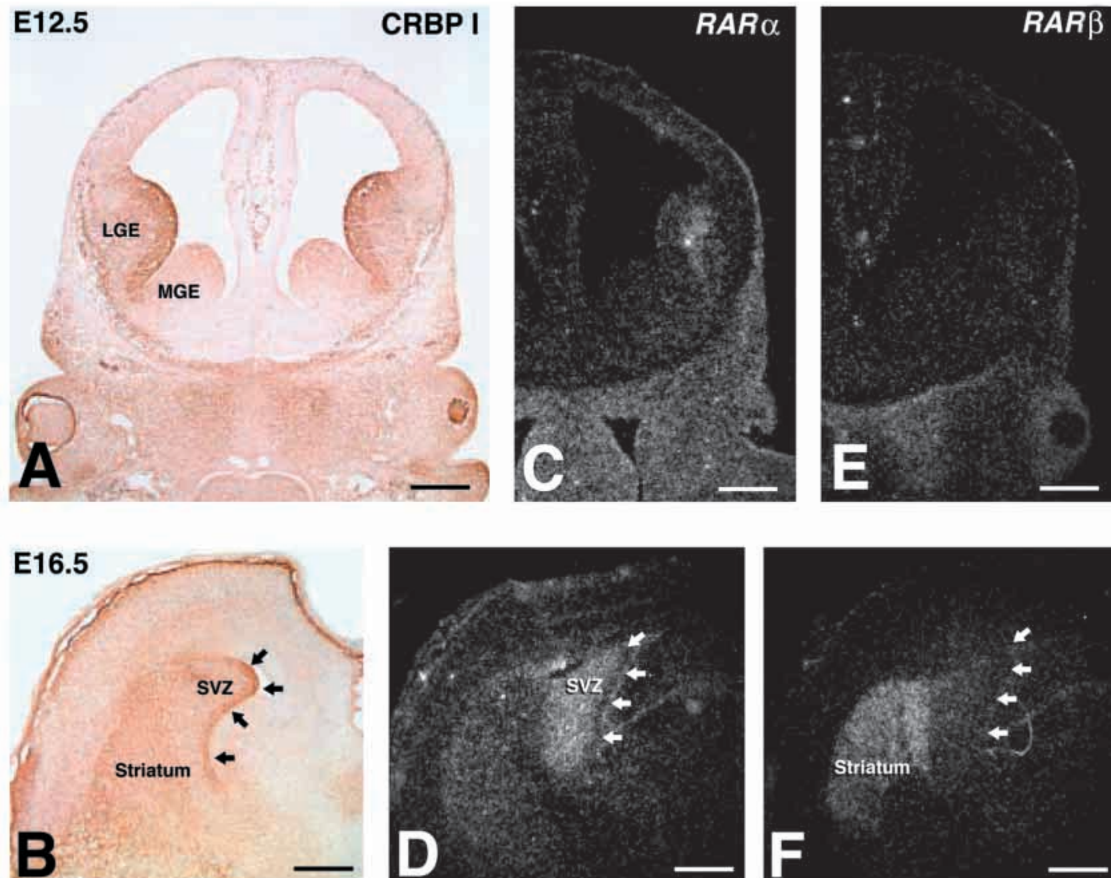

Fig. 3. Expression of retinoid-related markers in the LGE and developing striatum. (A,B) Immunohistochemistry for CRBP I shows that at E12.5 this protein is enriched within the LGE as compared to the adjacent MGE or cortex. (A) The highest level of CRBP I appears to be within the VZ. (B) At E16.5, CRBP I expression remains high in the striatal VZ zone (arrows in B) and developing striatum. (C,D) *RARα* expression is highly enriched in the LGE SVZ both at E12.5 (C) and E16.5 (D). *RARα* is also expressed in the developing striatum but at a lower level. Note in D that the VZ (outlined by arrows) is only expressing low levels of *RARα*. (E and F) Transcripts of *RARβ* could not be detected in the E12.5 telencephalon using our oligonucleotides (E). However, at E16.5 the developing striatum was shown to specifically express high levels of *RARβ* transcripts (F). Arrows in F are at the same positions as in D and indicate the lack of notable *RARβ* expression in the SVZ. Scale bars in A,C,E, 330 μm; B,D,F, 230 μm.

be dependent on the number of passages that these cells are subjected to since it was nearly absent in the P.9 LGE glial cultures.

Conditioned medium from near confluent cultures of P.4 LGE glia was collected over 3 consecutive days and assayed for retinoid production as described above. P.4 LGE glial conditioned medium induced a strong activation of the UAS-luciferase construct through the GAL4-RAR construct but not through the GAL4-RXR construct (Fig. 7A). The level of activation increased from the first day at approximately 77-fold over control ($P<0.001$, one-way ANOVA) to 122-fold over control on the third day ($P<0.001$, one-way ANOVA; Fig. 7A). The increase over the 3 days is likely due to the fact that the cultures continue to grow and thus the retinoid producing cells would increase in numbers. This effect was dependent on serum (which contains retinol/vitamin A) in the medium since serum-free conditions progressively abolished RAR signalling over the 3 days of collection (Fig. 7B). Unlike the P.4 LGE glia, the late passage (P.9) LGE glia were deficient in retinoid production (Fig. 7C). This is interesting in light of the low percentage of CRBP I-expressing cells in these late passage cultures. Moreover, the RA production from the P.4 LGE glia was specific for these glial cultures since conditioned medium from P.4 MGE glial cultures failed to produce notable activation neither with the GAL4-RAR nor GAL4-RXR constructs (Fig. 7C). These findings demonstrate that LGE glia, likely of the radial glial subtype, produce high levels of retinoids.

RA enhances striatal neuron differentiation

In order to determine whether locally produced retinoids could regulate striatal neuron differentiation, we cultured LGE cells in serum-free conditions and assessed the effect of RA on the differentiation of DARPP-32-expressing neurons. Neurons expressing DARPP-32 constituted on average about 6% of the total cell population in control cultures of E13.5 mouse LGE (Fig. 8A). This value is very similar to that reported by Nakao et al. (1996) using the same plating density. When these cells were grown for 5 days with either 10 nM or 1 μM RA added to the medium, the proportion of DARPP-32-positive neurons rose to 181±5% ($P<0.01$, n=3 independent experiments) and 205±17% ($P<0.01$, n=3, Fig. 8B) of that seen in control

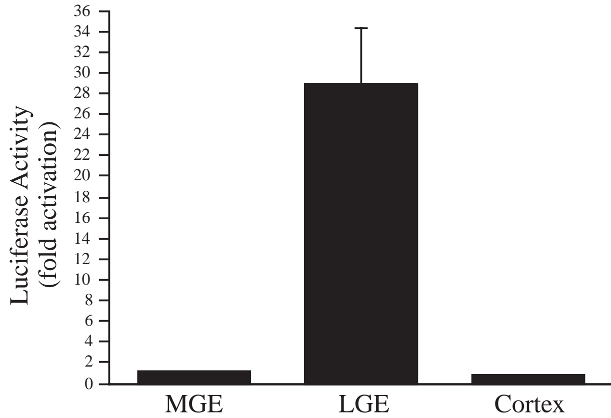

Fig. 4. Localized production of retinoids in the E12.5 LGE. Bar graph showing the retinoid induced luciferase activity through the GAL4-RAR contruct from the MGE (n=9 wells), LGE (n=8 wells) and cortex (n=5 wells). Control luciferase activity was determined by incubating cells in the absence of tissue pieces.

cultures, respectively (Fig. 8C). Although the 1 μM treatment tended to give a higher proportion of DARPP-32-expressing neurons than 10 nM, the difference between the two conditions was not significant. The general neuronal marker β-III-tubulin was expressed by 76±6% of the cells in the control cultures and by 79±8% of cells in the 1 μM RA-treated cultures, a non-significant 3% increase from control (n=3). Thus the RA-induced increase in DARPP-32-expressing neurons is specific and not simply due to a general enhancement of neuronal differentiation. Furthermore, the increase in the proportion of DARPP-32-expressing neurons in these cultures was not due to an effect of RA enhancing cell numbers (data not shown). In addition to the increase in DARPP-32-positive cell numbers after either 10 nM or 1 μM RA treatment, the level of DARPP-32 protein detected per cell was considerably higher (compare Fig. 8A and B). Again this difference in staining intensity was not evident in the β-III-tubulin-stained cultures (data not shown). Finally, the RA effect was specific to the LGE cultures since no induction of DARPP-32 was seen in MGE cultures (data not shown). These results demonstrate that LGE cells are indeed responsive to RA and that this signal specifically enhances striatal neuron characteristics.

Since RA can signal through both RARs and RXRs, and receptors of both types are present in the LGE and developing striatum (Fig. 3C-F, Ruberte et al., 1993; Dollé et al., 1994), we wanted to examine their respective contributions to the RA effect in our LGE culture system. Using agonists specific for either RARs (TTNPB) or RXRs (SR11237) at a concentration of 100 nM, we found that both signalling pathways can mediate the retinoid-induced increase in DARPP-32-expressing neurons equally well. The RAR agonist TTNPB increased the proportion of DARPP-32 neurons by 207±37% of control (P<0.05, n=3) while the RXR agonist SR11237 increased the DARPP-32 proportion to 224±47% of control (P<0.05, n=3) (Fig. 8D). The slightly higher proportion in the SR11237-treated cultures was not significantly different from the TTNPB-treated cultures. When both TTNPB and SR11237 were added to cultures together the proportion of DARPP-32 neurons was 210±28% of control (P<0.05, n=3) which was not significantly different from when they were added separately (Fig 8D). Once again, the total number of neurons in control and agonist-treated cultures was not significantly different (data not shown). These findings indicate that both RAR and RXR signalling can positively regulate striatal neuron differentiation.

DISCUSSION

Meis2 as a marker of striatal progenitors and neurons

Using a subtraction cloning strategy, we have identified the homeobox gene *Meis2*, to our knowledge the earliest known marker of striatal precursors/neurons. *Meis2* has recently been shown to be expressed at many sites in the developing embryo including a number of regions in the brain, developing ganglia, face, limbs as well as in the female genital tract (Oulad-Abdelghani et al., 1997; Cecconi et al., 1997). However, this is the first description of *Meis2* expression within the LGE and striatum. From the earliest stages of striatogenesis, *Meis2*-positive cells are found in the LGE SVZ and at later stages also in large numbers within the developing striatal complex. Since the SVZ of the ganglionic eminences is known to remain proliferative throughout gestation (Smart and Sturrock, 1979), *Meis2* is likely to mark proliferating precursors, many of which will ultimately differentiate into striatal neurons. In the mature striatum, the expression of *Meis2* is very similar to that of the

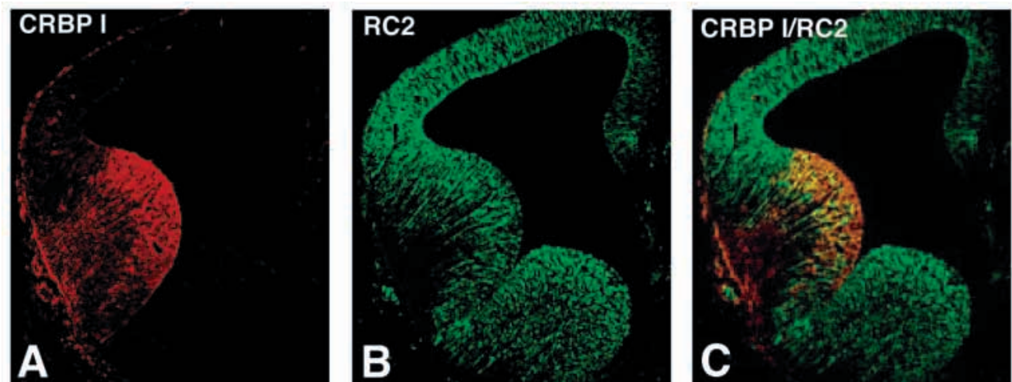

Fig. 5. CRBP I is expressed in radial glia of the LGE. (A) CRBP I expression in the E12.5 LGE. In addition to the strong expression in the VZ, labelled processes extend through the LGE with high levels of expression at the ventricular and pial surfaces. This staining pattern is reminiscent of the morphology of radial glia with their cell bodies in the VZ and processes extending through the mantle zone terminating in end feet on both the pial and ventricular surfaces. (B) Expression of the radial glial marker, RC2 showing expression in radial glial structures throughout the telencephalon. (C) Merged confocal image showing the RC2-positive elements co-expressing CRBP I (yellow) specifically in the LGE.

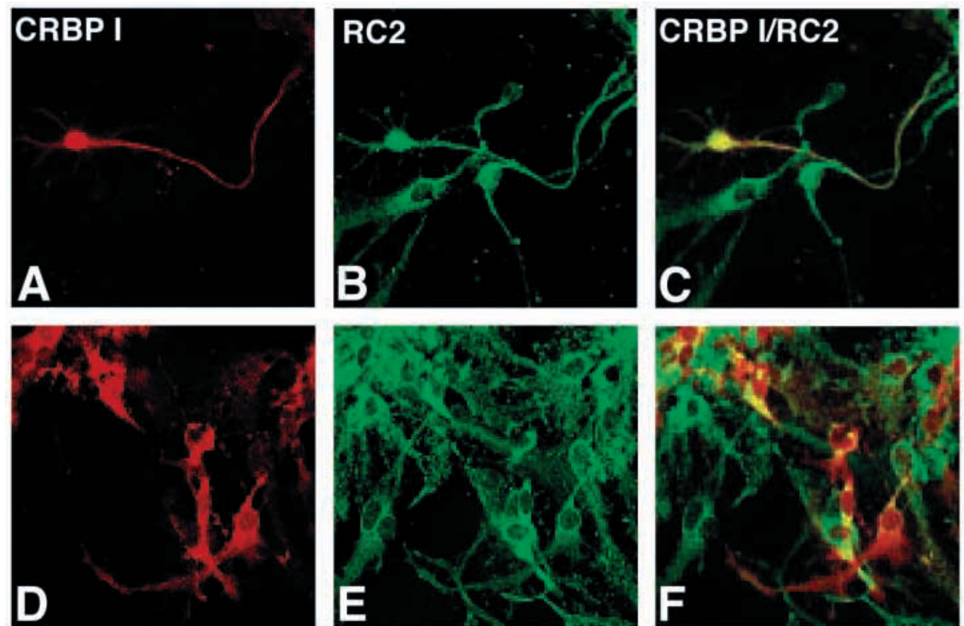

Fig. 6. Cultured glia from the LGE maintain CRBP I and RC2 expression. (A,D) CRBP I is expressed in P.4 LGE glia. (A) Some CRBP I-expressing glial cells were bipolar and possessed long processes while many others exhibited more typical glial morphologies (D). (B,E) RC2 is expressed by many cells in these cultures. (C,F) Merged confocal images of CRBP I and RC2 show glial cells which co-express these two markers similar to that seen in vivo.

striatal projection neuron marker, DARPP-32 (Anderson and Reiner, 1991) suggesting that many of these neurons express this gene. While further studies will be needed to determine the role of retinoids in telencephalic *Meis2* expression as well as the requirement of this gene in the normal differentiation of the striatum, *Meis2* already represents a valuable and needed marker of the LGE and/or striatal phenotypes.

The LGE represents a localized source of retinoids

The signals regulating forebrain development are as yet poorly understood, particularly those regulating patterning and differentiation in the ganglionic eminences. *Sonic hedgehog* (*Shh*), which is well known for its signalling properties during development, is expressed within the MGE (Shimamura et al., 1995; Platt et al., 1997) in a complementary pattern to *Meis2* (unpublished results). In fact, SHH does regulate the expression of the MGE marker, TTF-1/Nkx2.1 (Ericson et al., 1995) which is clearly required for the normal development of the ventromedial telencephalon (Kimura et al., 1996). To date, however, no signals regulating the development of precursors in the LGE have been suggested.

The fact that the LGE-enriched *Meis2* gene is rapidly upregulated in P19 EC cells following administration of RA (Oulad-Abdelghani et al., 1997) stimulated us to examine whether retinoids could represent such a signal. In fact, markers of retinoid synthesis and signalling are highly enriched within the LGE and developing striatum (Ruberte et al., 1993; Dollé et al., 1994; present results). Although localized retinoid production within the nervous system has previously been demonstrated at limb levels in the developing spinal cord (McCaffery and Dräger, 1994), regional production of RA in the developing brain has not been shown. In this study, we

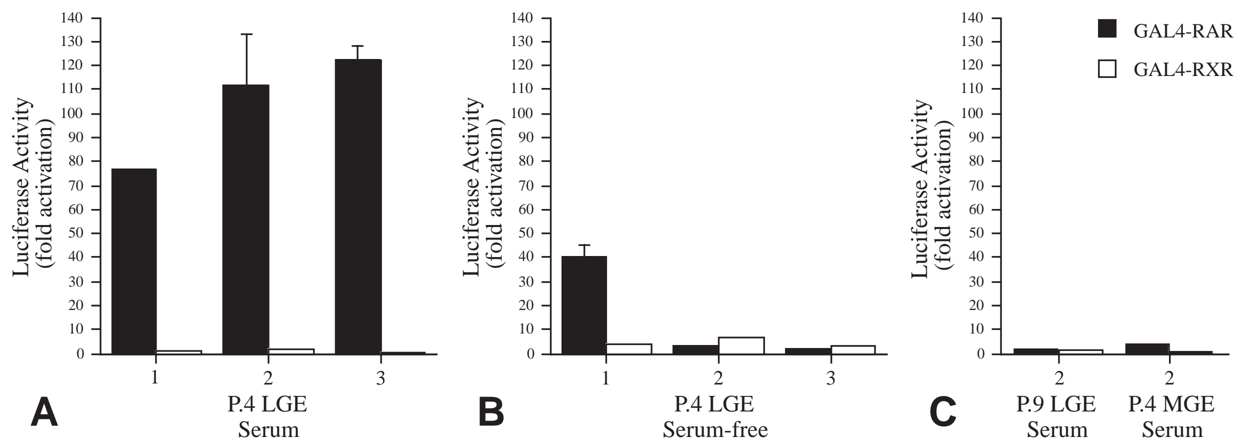

Fig. 7. LGE glia produce high levels of retinoids. (A) Conditioned medium from P.4 LGE glia dramatically activates luciferase activity through the GAL4-RAR construct but not through the GAL4-RXR construct. The levels of RAR activation increases from the first day of collection (1) to the third day (3). (B) P.4 LGE glia cultured under serum-free conditions progressively abolished the activation through the GAL4-RAR construct over the 3 days of collection, likely due to the lack of retinol (Vitamin A) in the medium. (C) Neither P.9 LGE glia nor P.4 MGE glia produced high levels of activation through either the GAL4-RAR or GAL4-RXR constructs. Only results from the second day of collection (2) are shown, however, the levels of activation seen on the first or last day of collection were not different.

provide evidence that the LGE, unlike the adjacent MGE or cortex, represents a unique source of RA within the developing telencephalon. Recent results have also shown that the early postnatal striatum continues to generate high levels of RA (Zetterström et al., 1999). Thus the LGE and developing striatum represent a significant source of RA which is capable of regulating striatal neuron differentiation (see below) but may also contribute to developmental processes in adjacent telencephalic regions (e.g. MGE or cortex).

LGE glia produce high levels of retinoids

A recent study has indicated that retinoids present at limb levels of the embryonic spinal cord are produced by motor neurons (Sockanathan and Jessell, 1998). However, our explant experiments argue against a neuronal source in the LGE, since these explants are likely to contain few neurons and are largely composed of precursor cells and radial glia. Considering that CRBP I expression correlated so well with the production of retinoids in the LGE explant experiments, we first examined what cells express this retinoid marker. Interestingly, CRBP I appears to be expressed specifically in radial glia of the LGE. Moreover, many cells in early passage glial cultures from the LGE were observed to express both CRBP I and the radial glial marker, RC2 (Misson et al., 1988). These cultures were also found to produce high levels of retinoids that activate the RAR but not the RXR pathway. Therefore, the facts that the major glial subtype present in the embryonic telencephalon is radial glia (Rakic, 1995) and that CRBP I was found localized in radial glia of the LGE argue strongly that radial glial cells in the LGE are a major source of retinoids. Unlike the early passage LGE glia, late passage LGE glia did not produce notable amounts of RA and were particularly deficient in CRBP I expression. Thus these cells may mature through many passages and perhaps lose their capacity to produce retinoids. In support of this, recent experiments have shown that glial cultures generated from the postnatal striatum do not produce retinoids (D. F. Castro and T. P., unpublished observations).

Although we cannot rule out the possibility that other cell types in the LGE also contribute to RA production, our results open up the intriguing possibility that radial glia, long known to serve an important function in the migration of newly born neurons (Rakic, 1995), might also have an instructive role. Indeed, this would represent a very efficient way to provide the migrating neuron with high levels of differentiating factors, such as RA, since the neuron and radial glial fibre are in intimate contact throughout the migration process.

Retinoid signalling regulates striatal neuron differentiation

To study whether the retinoids locally produced by radial glia in the LGE could be regulating striatal neuron differentiation, we made use of a serum-free striatal cell culture system in which only a minority of the LGE cells differentiate to express DARPP-32 (Nakao et al., 1996). Indeed, LGE cells that were grown with either 10 nM or 1 μM RA showed a significant increase in the proportion of neurons expressing DARPP-32 as compared to control, demonstrating that RA positively regulates striatal neuron differentiation. The few DARPP-32-expressing neurons that are present in control LGE cultures are likely due to the fact that striatal neurogenesis is just beginning and these cells may have already been specified by retinoids prior to dissection.

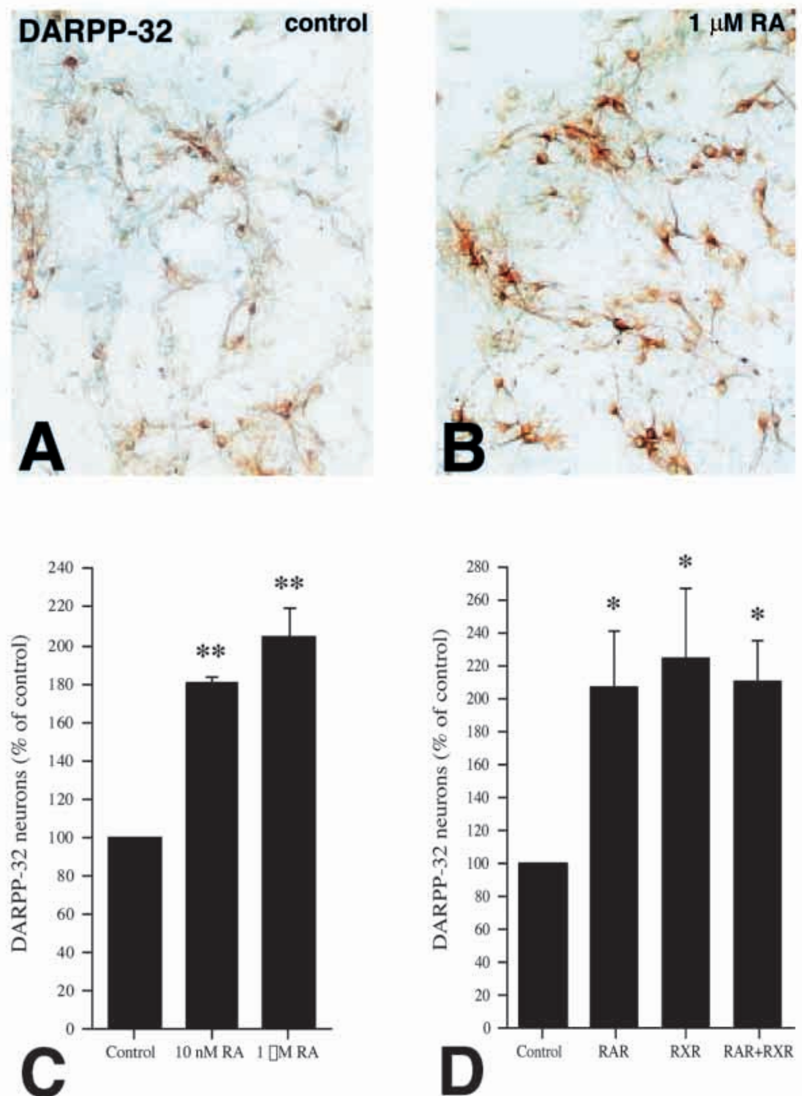

Fig. 8. RA enhances DARPP-32 expression in cultured LGE cells. (A) A fraction (approx. 6%) of primary cultured cells from the E13.5 LGE are observed to express DARPP-32 after 5 days in serum-free culture. (B) When these cells are grown in the presence of retinoids (in this case, 1 μM RA), an increase in both the proportion of DARPP-32-expressing cells and in the level of DARPP-32 expression per cell is observed. (C). Bar graph showing that the proportion of DARPP-32-expressing neurons grown in 10 nM or 1 μM RA is 180% and 205% of that seen in control LGE cultures, respectively. (D) Agonists specific for RARs (TTNPB, 100 nM) and RXRs (SR11237, 100 nM) enhance the proportion of DARPP-32 neurons to 207% and 224% of control, respectively. A combination of TTNPB and SR11237 does not enhance the proportion of DARPP-32-expressing neurons (210% of control) as compared to when they were added separately (D). ** $P<0.01$, * $P<0.05$, one-way ANOVA with Fisher PLSD test as a post-hoc.

Since retinoids can signal through both the RAR and RXR pathway, we were interested to determine their potential contribution to the RA effect. Both RAR and RXR activation (using agonists specific for each receptor type) was found to be equally capable of mediating the retinoid-induced increase in DARPP-32 neurons in our culture paradigm. This is interesting since both the explant and glial cultures only showed activation through the RAR pathway, suggesting that the retinoid produced in the LGE is all-*trans* RA (the ligand for RARs) and not 9-*cis* RA (which binds both RARs and RXRs). This does not exclude the possibility, however, that 9-*cis* RA could play a role in striatal neuron differentiation since high levels of all-*trans* RA can lead to partial isomerization into the 9-*cis* isoform (Levin et al., 1992). Our data, however, do not show a synergism between the two signalling pathways in regulating DARPP-32 expression in LGE cultures since combined RAR and RXR activation was only as efficient as either separately. This may be due to saturation of the RARs or RXRs at the concentration that the agonists were used (100 nM).

Retinoids have been shown to have essential functions in vertebrate development (Morriss-Kay and Sokolova, 1996) and effects of excess or deficiency have been described in many systems, including the developing CNS. In fact, recent studies have shown that in certain retinoid receptor double mutants (i.e. *RARα/RXRγ* and *RARβ/RXRγ*), the expression levels of genes characteristic of differentiated striatal projection neurons, such as the *dopamine D1* and *D2 receptors* as well as *proenkephalin* are altered (Samad et al., 1997; Krezel et al., 1998). Moreover, the promoter region of the *dopamine D2 receptor* gene, which is expressed in the LGE SVZ and developing striatum (Diaz et al., 1997), has recently been shown to contain a functional retinoic acid response element (RARE) (Samad et al., 1997). Although the authors of these retinoid receptor knock-out studies (Samad et al., 1997; Krezel et al., 1998) interpret their results as indicating a role for retinoids only in the adult striatum, no data are given from the embryonic or neonatal period. In light of the results presented here, the findings in *RAR/RXR* double mutants support our suggestion that retinoid signalling is required during development for the correct differentiation of striatal neurons.

The results of the present study demonstrate that the LGE is a novel site of local retinoid production within the developing brain and that retinoids are capable of regulating striatal neuron differentiation. Interestingly, the cellular source of retinoids within the LGE appears to be glial, likely radial glia. This raises the attractive possibility that, in addition to their well-documented role in neuronal migration, radial glia may also have an instructive role in regional neuronal differentiation.

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