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Research report

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Abstract

The low density lipoprotein receptor-related protein (LRP) is a multifunctional cell surface receptor that binds to the protease inhibitor α_2-macroglobulin (α_2M). LRP has also been identified as the apolipoprotein E (apoE) receptor that mediates lipid metabolism. Recently it has been reported that apoE4, one of three common isoforms of apoE, is a main risk factor of Alzheimer's disease (AD). Moreover, all three of these proteins are reported to accumulate in the senile plaques in the brains of Alzheimer's patients. To understand the roles of LRP in the normal development of the central nervous system (CNS) and in the pathogenesis of AD, we studied the developmental expression and localization of LRP mRNA in the CNS. We used Northern blot analysis to investigate the developmental profile of LRP mRNA in the rat brain. LRP mRNA was first detected as early as in 18-day-old embryonic rat brain and was continuously expressed thereafter. A particularly high level of expression of the mRNA was observed in the perinatal stage. We also determined the cellular distribution of LRP mRNA in the CNS of 20-day-old embryonic and 6-week-old adult rat brains by in situ hybridization using a digoxigenin-labeled antisense riboprobe to LRP mRNA. In the embryonic rat brain, LRP mRNA was highly expressed in most of the cells, mainly neurons and glial cells. In the adult rat, LRP mRNA was expressed mostly in neurons in both the brain and the spinal cord. These results suggest that LRP plays crucial roles in development of the brain.

Keywords: Low density lipoprotein receptor-related protein; α_2-Macroglobulin; Apolipoprotein E; Alzheimer's disease; In situ hybridization; Central nervous system

1. Introduction

Previously we reported that the protease inhibitor α_2-macroglobulin (α_2M) is an astrocyte-derived neurite-promoting factor in the rat central nervous system (CNS) [29,30,39]. α_2M inhibits the activity of the majority of proteases by means of protease trapping regardless of their catalytic mechanisms [2,12,48] and then exposes the receptor-binding site through a conformational change. The α_2M-protease complex binds to the cell surface α_2M receptor, and is internalized in the cell. α_2M receptor has been isolated from rat liver [27] and human placenta [1,20]. Partial protein sequence analysis of peptides generated by chemical and proteolytic cleavage of the purified receptor demonstrated that it is identical to the low density lipoprotein (LDL) receptor-related protein (LRP) [23,42] which has structural similarities to the LDL receptor [13]. α_2M receptor/LRP has been shown to bind to numerous other ligands, including plasminogen activator or its complex with plasminogen activator inhibitor [7,34,35], lactoferrin [17,18,46,49], lipoprotein lipase [5,8] and *Pseudomonas* exotoxin A [21]. Furthermore, LRP was also identified as a receptor for apolipoprotein E (apoE) [4,22,42]. In the CNS, apoE is synthesized in astrocytes [6,31,36] and oligodendrocytes [41], and it is a major apolipoprotein in the cerebrospinal fluid [36]. ApoE plays an important role in the transport and metabolism of lipids. Recently Nathan et al. reported that apoE3, one of the three common isoforms of apoE, increased neurite outgrowth in cultured dorsal root ganglia under the presence of β-migrating very low density lipoproteins, whereas apoE4 decreased outgrowth [33]. Moreover, apoE4 was shown to be a main risk factor of Alzheimer's disease (AD) [10,26,37,40,43]. It has been also demonstrated that α_2M, apoE and LRP are associated with senile plaques [3,32,38,45,47,50] in patients with AD. These observations suggest that all of these proteins are implicated in not only normal development of the CNS but

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also in the pathogenesis of AD. Knowledge on the location of LRP mRNA may be crucial for understanding the functions of α_2M and apoE in the CNS. In this study, we investigated the expression and distribution of LRP mRNA in the rat CNS by Northern blot analysis and in situ hybridization.

2. Materials and methods

2.1. Preparation of RNA probe

The template for LRP RNA synthesis was derived from a 604-base *Sma*I–*Bam*HI fragment corresponding to nucleotides 1,827–2,431 of a cDNA for human LRP (American Type Culture Collection). The cDNA fragment was subcloned into pGEM-1 (Promega) and linearized with *Eco*RI (antisense probe) or *Xba*I (sense-strand control). Digoxigenin (DIG)-labeled antisense and sense RNA probes were synthesized using SP6 and T7 polymerase, respectively, according to the supplier's instruction (Boehringer Mannheim) from corresponding linearized plasmid templates.

2.2. RNA purification and Northern blot analysis

The brains were removed from fetal (stages 14, 16, 18, 21), neonatal (postnatal day 1, 3, 5, 11) and adult (maternal) Wistar rats and rapidly frozen in liquid nitrogen. For developmental analysis, total RNA was isolated from the brains according to the method of Chirgwin et al. [9]. Total RNA (20 μg per lane) was electrophoresed on a 1.2% agarose-2.2 M formaldehyde gel, blotted to a nylon membrane (Hybond-N$^+$; Amersham) and hybridized with a UTP-DIG-labeled LRP antisense RNA probe (50 ng/ml) in 5 × saline-sodium citrate (SSC) at 65°C, 10 × Denhardt's solution, 10 mM Na_2PO_4 (pH 6.5), 0.1 mg of salmon sperm DNA per ml, 0.5% sodium dodecyl sulfate (SDS) and 50% formamide. After hybridization, the membrane was washed at high stringency with 2 × SSC and 0.1% SDS for 10 min at room temperature and then twice with 0.2 × SSC and 0.1% SDS at 65°C for 20 min. Hybridization signals were detected with a DIG nucleic acid detection kit (Boehringer Mannheim). The membrane was washed briefly with Buffer 1 (100 mM Tris-HCl pH 7.5, 150 mM NaCl), incubated for 1 h with Buffer 1 containing 1% blocking reagent (provided in the kit) (Buffer 2) and for 30 min with an alkaline phosphatase-coupled DIG antibody (1:10,000) in Buffer 1 that contained 0.2% Tween 20. After unbound antibody conjugate was removed by washing twice with Buffer 1 containing 0.2% Tween 20 for 15 min, the membrane was equilibrated for 3 min with Buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$), and then for 5 min with 100 mM diethanolamine and 2 mM $MgCl_2$. Detection of DIG-labeled nucleotide was accomplished with a chemiluminescent reagent, Lumiphos-530 AP detection reagent (Amersham). The membrane was incubated with this reagent at 37°C for 15 min. For detection of the chemiluminescent signal, the membrane was exposed to X-ray film, X-Omat (Kodak). The same filter was rehybridized by a β-actin probe as described previously [44].

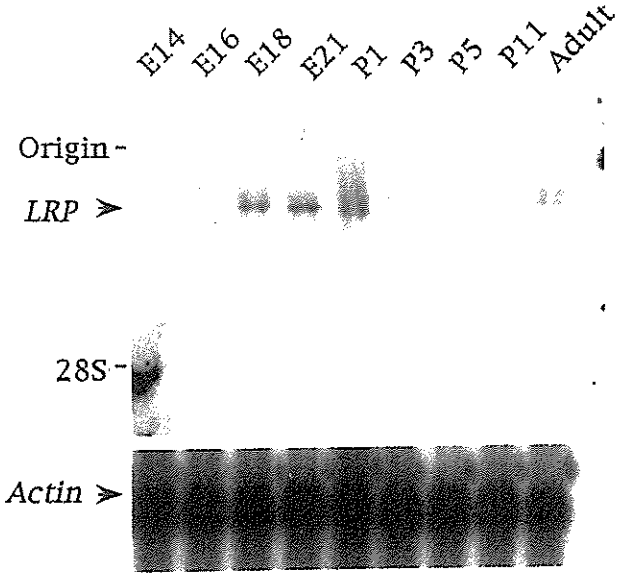
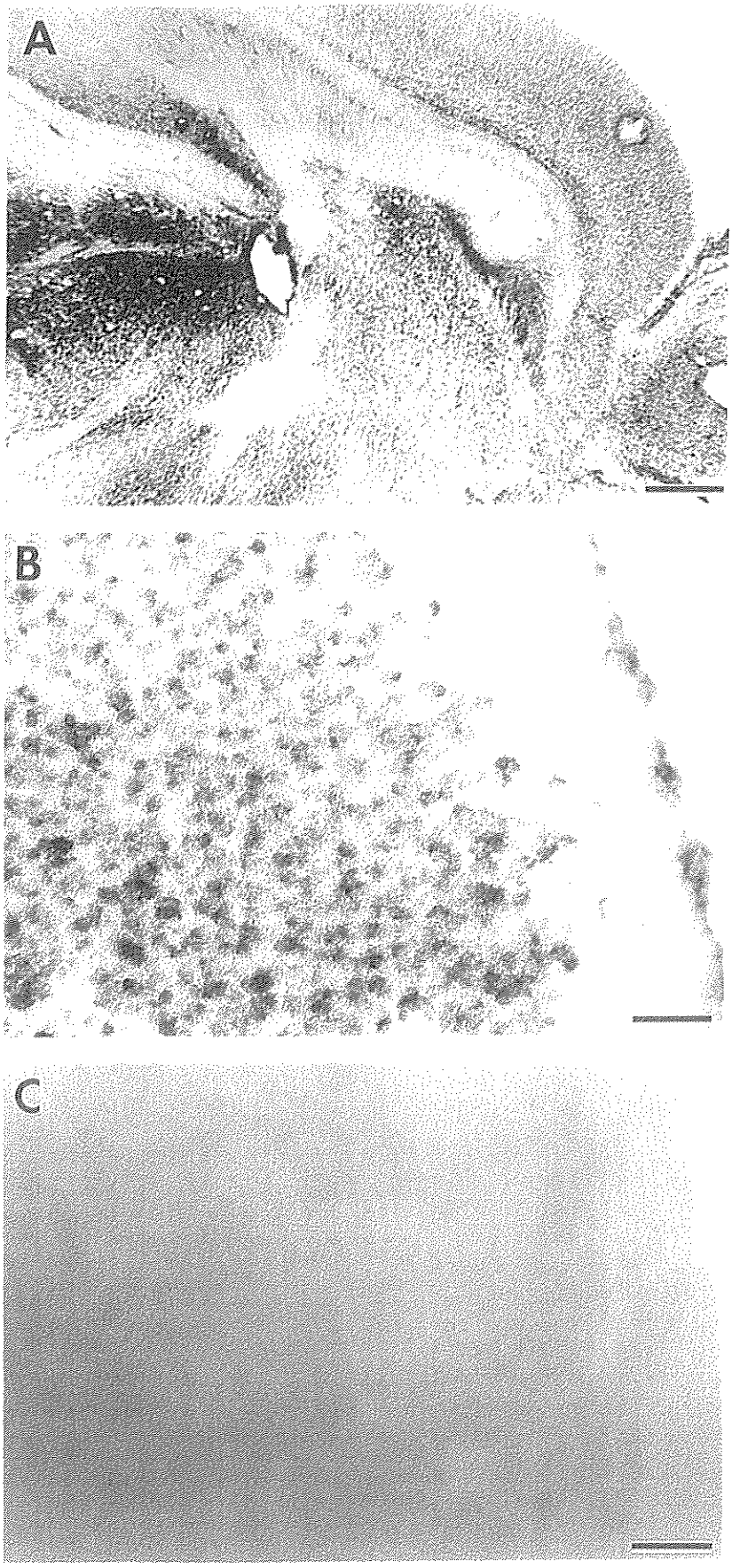


Fig. 1. Northern blot analysis of LRP mRNA in the brain at different developmental stages. Embryonic (E) and postnatal (P) days are indicated. Approximately 20 μg of total RNA was loaded in each lane. The same blot was rehybridized with a β-actin probe.

2.3. In situ hybridization

The brain from a 20-day embryonic Wistar rat was fixed in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) and then equilibrated in 30% sucrose/PBS at 4°C. Six-week-old Wistar rats (male) were anesthetized and then perfused with PBS through the heart to remove blood before fixation with 4% PFA/PBS. Cryostat sections (10–16 μm thick) were processed by the following steps: refixing in 4% PFA/PBS; proteinase K treatment (0.01 mg/ml at 37°C for 10 min); 4% PFA/PBS; washing with PBS; 200 mM HCl; washing with PBS; 100 mM triethanolamine-HCl pH 8.0 (TEA) for 1 min; TEA/0.25% acetic anhydride treatment for 10 min; washing with PBS;

Fig. 2. In situ hybridization of LRP mRNA in the embryonic rat brain. A: sagittal sections of a 20-day embryonic rat were hybridized with a DIG-labeled antisense RNA probe to LRP mRNA. B: high magnification photograph of (A) in the cortex. No hybridization was observed with the sense probe (C). Bars = 500 μm (A) and 50 μm (B and C).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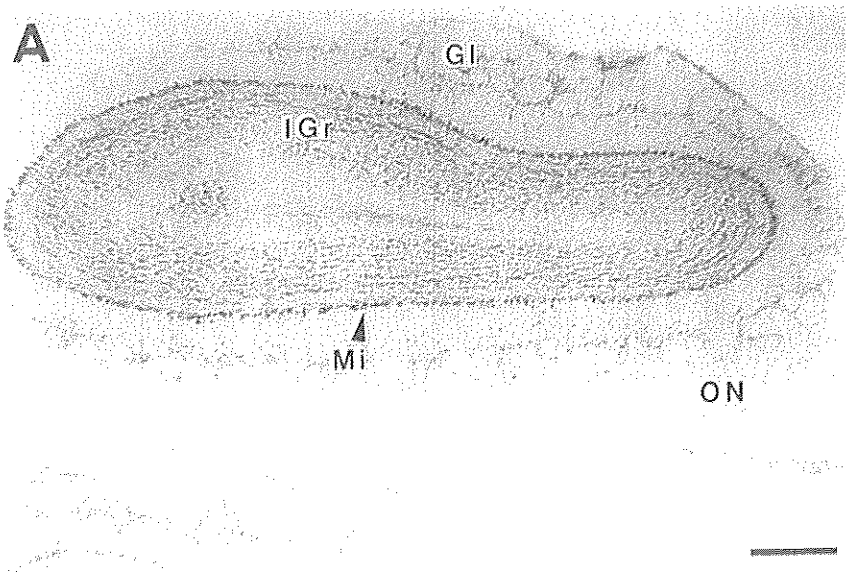
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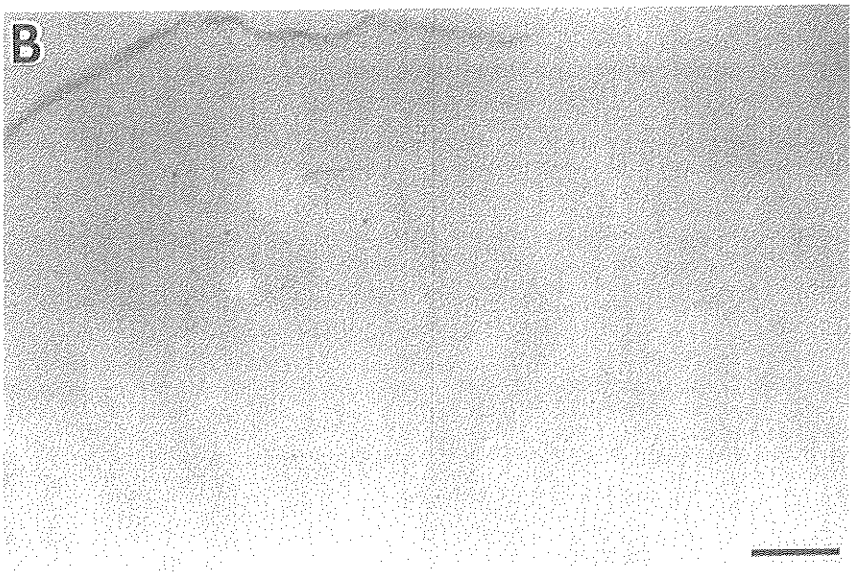
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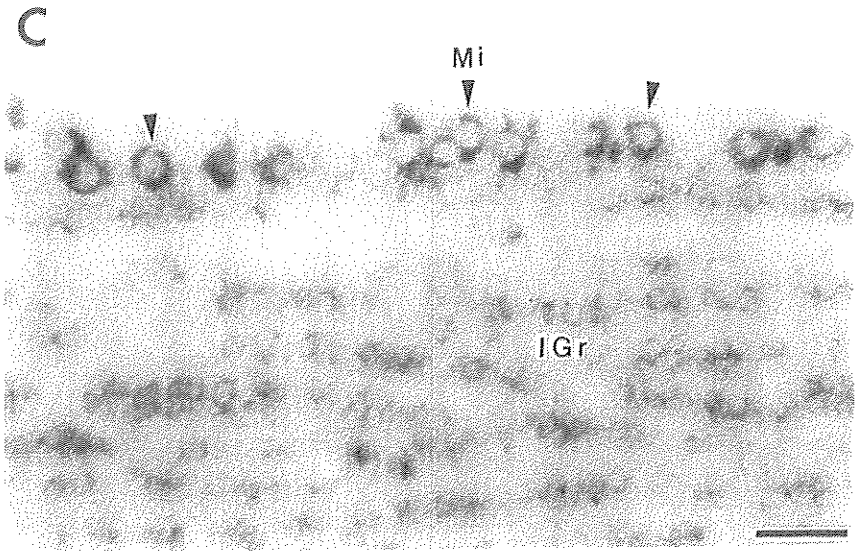
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dehydration. The freshly denatured DIG-labeled RNA probes (5 min/90°C) were added to the hybridization solution (5 × SSC, 2% blocking reagent, 50% formamide) at a final concentration of 0.75 μg/ml. The hybridization mixture was applied directly onto the sections, which were then covered with a parafilm. As a negative control, the adjacent sections were hybridized with the DIG-labeled sense RNA probe or the hybridization solution alone. The RNA–RNA hybridization was allowed to proceed overnight in a humid chamber at 50°C. Then the following washing procedures were used: 5 × SSC at 50°C; 2 × SSC/50% formamide at 50°C for 30 min; 10 mM Tris-HCl pH 7.6, 500 mM NaCl, 1 mM EDTA (TNE) at 37°C for 10 min; 0.02 mg of RNase A per ml in TNE at 37°C for 30 min; TNE at 37°C for 10 min; 2 × SSC at 50°C for 20 min; twice 0.2 × SSC at 50°C for 20 min. For detection of LRP mRNA, the following protocol was used. The sections were washed briefly (5 min) with Buffer 1, blocked with Buffer 2 (1.5% blocking reagent in Buffer 1) at room temperature for 60 min, incubated with an alkaline phosphatase-coupled DIG antibody (diluted 1:500 in Buffer 1) at room temperature for 30 min, washed twice with Buffer 1 at room temperature for 15 min, equilibrated with Buffer 3 for 3 min and developed with Buffer 3 containing nitro-blue-tetrazoliumchloride (450 μg/ml) and 5-bromo-4-chloro-3-indolylphophate (175 μg/ml). The sections were incubated in the dark in a humid chamber at room temperature for 40 h. They were then washed with Buffer 4 (10 mM Tris-HCl pH 7.6, 1 mM EDTA), fixed with 4% PFA/PBS for 10 min, dehydrated and covered with a coverslip.

3. Results

3.1. LRP mRNA expression in the developing rat brain

To determine the pattern of LRP mRNA expression during development of the brain, Northern blot analysis was performed (Fig. 1). Analysis of total RNA from the whole brain revealed that the transcripts were clearly present as early as embryonic day 18 (E18). Two bands corresponding to LRP mRNA in length were detected. The larger transcript at the position of 15 kb is similar in molecular size to that reported by other groups. The larger transcript was expressed continuously in the brain throughout the developmental stages (E18 to P5) and adult. Intense expression of the smaller transcript was observed through the perinatal (E21 to P1) and adult stages.

3.2. Localization of LRP mRNA in the rat CNS

To determine the cellular distribution of LRP in the perinatal and adult rat CNS, in situ hybridization assays were performed on the cryostat sections with a DIG-labeled antisense riboprobe to LRP mRNA. The RNA probe was synthesized with the same template and transcription system as used in the Northern blot analysis. In the embryonic rat brain, most of the cells, mainly neurons and glial cells, exhibited strong labeling of LRP (Fig. 2A,B).

No significant signal was detected when the DIG-labeled sense probe to LRP mRNA was hybridized to the adjacent sections (Fig. 2C). In the adult rat, the expression of LRP mRNA was detected in various regions of the brain. In the olfactory bulb (Fig. 3A), LRP mRNA was observed in the glomerular, internal granular and mitral cell layers of the olfactory bulb and olfactory nerve layers. A high level of LRP mRNA was observed in the mitral cells (arrowheads in Fig. 3C). These signals were not observed in the adjacent sections hybridized with the sense probe (Fig. 3B). In the hippocampus, both the pyramidal cells and the dentate gyrus were hybridized with the LRP antisense probe. Fig. 4A shows abundant hybridization in the pyramidal cells of the hippocampus CA3 region. LRP mRNA signals were also detected in smaller cells around the pyramidal layer, presumably glial cells. In the cerebral cortex (Fig. 4B), LRP mRNA was observed in the cortical layers (II–VI) but little in the molecular layer. In the cerebellum, LRP mRNA was detected in both the granular and molecular layers (Fig. 4C). The Purkinje cells were strongly labeled with the LRP probe (Fig. 4D). LRP mRNA was also observed in the septal nuclei, caudate putamen and hypothalamus (data not shown). LRP mRNA was detected not only in the brain but also in the spinal cord (Fig. 5A). Motor neurons in the anterior gray horn were strongly labeled with the LRP probe (Fig. 5B). The cells in the white matter, possibly glial cells, also showed the signals (Fig. 5C).

4. Discussion

Previous studies from our laboratory demonstrated that α_2M, which is produced in astrocytes [11,39], promotes neurite outgrowth in cultured embryonic rat neocortical neurons [29] and raised the possibility that specific receptors against α_2M are involved in the neurotrophism of α_2M [30]. This hypothesis was recently confirmed by other research groups showing that the α_2M receptor is

Fig. 3. In situ hybridization of LRP mRNA in the olfactory bulb. A: coronal sections of the olfactory bulb were hybridized with a DIG-labeled antisense RNA probe to LRP mRNA. B: control study with the sense probe. C: high magnification photograph of (A). Gl, IGr and Mi designate glomerular, internal granular and mitral cell layers of the olfactory bulb, respectively (mitral cells, arrowheads in A and C). ON designates olfactory nerve layer. Bars = 500 μm (A and B) and 50 μm (C).

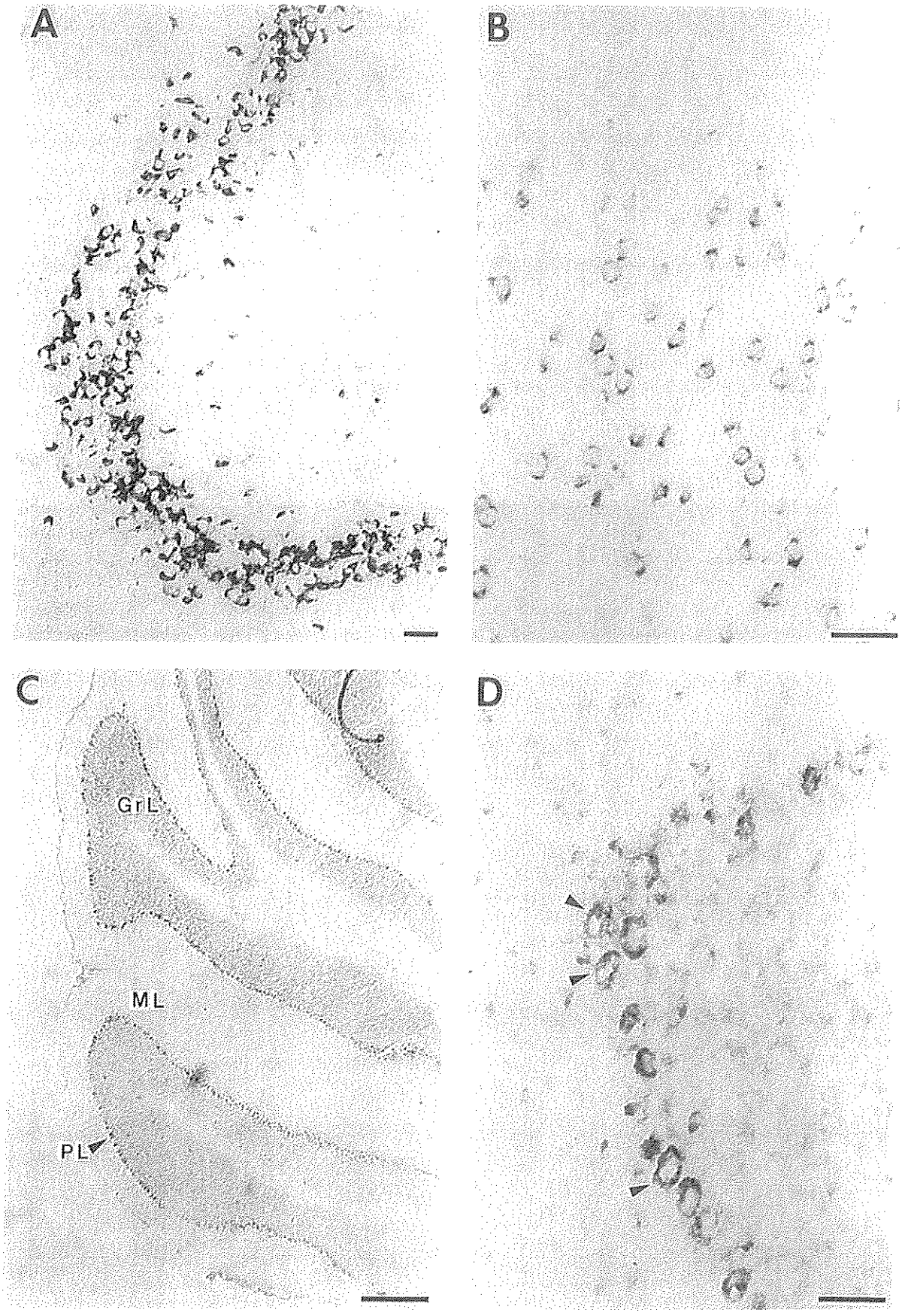

Fig. 4.

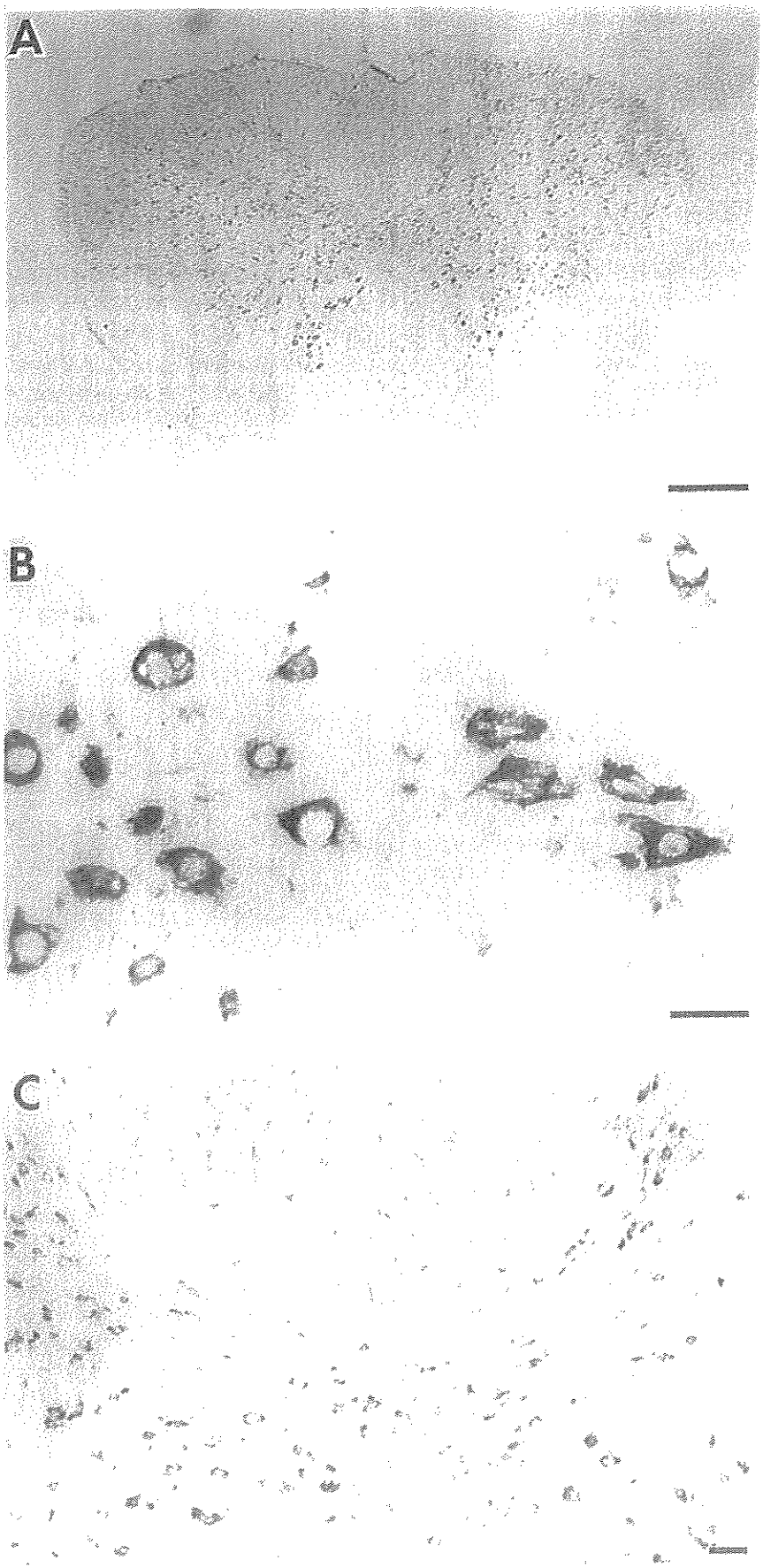

Fig. 5.

LRP [23,42]. Moreover, LRP was shown to be distributed in neurons in the human brain by immunohistochemical staining [28,51]. These findings gave rise to the idea that astrocyte-derived α_2M plays certain roles in neurite extension during perinatal development.

In the present studies, strong expression of LRP mRNA was found in the perinatal stage of rat CNS, and the two transcripts were detected at around 15 kb. The existence of LRP mRNA with different sizes suggests the possibility of an alternative splicing of the mRNA at this stage. A dramatic increase of LRP mRNA in the perinatal stage of rat brain and its quick decrease after birth may indicate the physiological importance of LRP during embryogenesis and the perinatal development of the CNS. In fact, Herz et al. [14,15] reported that homozygous mice lacking LRP showed delayed embryonal development and died prenatally around day 13.5 of the development.

By in situ hybridization of 20-day embryonic rat, an intensive expression of LRP mRNA was found in almost all the cells in the brain. Our findings on the distribution of LRP mRNA in the embryonic rat brain agree with those found by the immunohistochemical study of Moestrup et al. [28]. They revealed that the sections of human fetal brain were more intensely stained with monoclonal antibodies against the LRP α-chain than those of the adult brain. We also demonstrated the distribution of LRP mRNA in the adult rat CNS by in situ hybridization. LRP mRNA was constitutively expressed in adult rat neurons in both the brain and spinal cord. The expression was also detected in the glial cells from various regions, but to a minor extent (data not shown). These results are also consistent with the observation by the immunohistochemical analysis of Moestrup et al. [28]. However, some inconsistent results were observed in the cerebellum and spinal cord in our study. We detected a strong labeling of Purkinje cells with the RNA probe to LRP mRNA, whereas, they observed only minimal staining of LRP in the cells by the immunohistochemical analysis. In the spinal cord, a pronounced labeling of LRP mRNA was found in the cells of the posterior funiculus, possibly oligodendrocytes. This is a new finding because no immunoreactivity of LRP has so far been detected in the oligodendrocytes by other studies. Our data indicate the ubiquitous expression of LRP mRNA in the CNS cells, although the immunohistochemical analysis demonstrated only the restricted distribution of LRP in other organs [28,51].

In a recent study, we found induction of α_2M in the rat brain after treatment with kainic acid (KA) [16]. This observation suggests an important involvement of α_2M during repair processes following brain injury. By in situ hybridization, we detected an intensive signal of LRP in glial cells of the corpus callosum and in other glial fibrillary acidic protein-immunoreactive regions (data not shown) after brain injury by KA indicating that the reactive astrocytes express and produce LRP. It was reported that, unlike quiescent astrocytes in normal brain, activated astrocytes in the gray and white matter were consistently immunoreactive for LRP [24]. It is postulated that, since α_2M is synthesized in astrocytes [11,39], autocrine interaction of the ligand with LRP may take place on the reactive astrocytes in addition to the paracrine interaction on neurons. Furthermore, the secretion of apoE by astrocytes [6,31,36] is reported to be up-regulated in response to neural damage [19]. These findings lead to the hypothesis that α_2M and apoE are involved in the nerve regeneration following lesioning [16,19,25].

The studies presented here demonstrated a significantly high expression of LRP in most of the cells in the rat CNS. It is likely that LRP plays a significant role in mediating the biological effects of α_2M and apoE in the CNS, although the signal transduction mechanism mediated by LRP remains to be solved. The roles of α_2M and apoE in the CNS will be important areas for future investigations.

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Fig. 4. In situ hybridization of LRP mRNA in the rat brain. A: the hippocampus CA3 region. B: cerebral cortex. C and D: cerebellum. No hybridization was observed with the sense probe (data not shown). D: high magnification photograph of the cerebellum. GrL, ML and PL designate granular, molecular and Purkinje cell layers of the cerebellar cortex, respectively. Purkinje cells, arrowheads in D. Bars = 50 μm (A, B and D) and 500 μm (C).

Fig. 5. In situ hybridization of the rat spinal cord (A). High magnification photographs of the anterior gray horn (B) and posterior funiculus (C). Bars = 500 μm (A) and 50 μm (B and C).

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