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Research report

Molecular cloning of novel leucine-rich repeat proteins and their expression in the developing mouse nervous system

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Abstract

It is well established that leucine-rich repeat (LRR) proteins such as connectin, slit, chaoptin, and Toll have pivotal roles in neuronal development in *Drosophila* as cell adhesion molecules. However, to date, little information concerning mammalian LRR proteins has been reported. In the present study, we sought LRR proteins of the mouse brain, based on the assumption that fundamental mechanisms are conserved between different species. We screened a neonatal mouse brain cDNA library with a human partial cDNA encoding LRR protein as a probe. We obtained two independent cDNAs encoding LRR proteins, designated NLRR-1 and NLRR-2 (*N*euronal *L*eucine-*R*ich *R*epeat proteins). We analyzed the whole sequence of NLRR-1 and partial sequence of NLRR-2. Sequence analysis showed that these two clones are about 60% homologous to each other, and that NLRR-1 protein is a transmembrane protein. Northern blot analysis and in situ hybridization histochemistry showed that both NLRR-1 and NLRR-2 mRNAs were expressed primarily in the central nervous system (CNS); NLRR-1 mRNA was also detected in the non-neuronal tissues such as cartilage, while NLRR-2 mRNA expression was confined to the CNS at all developmental stages. These results suggest that there is at least one LRR protein family in the mouse and that these molecules may play significant but distinct roles in neural development and in the adult nervous system.

Keywords: Leucine-rich repeat protein; LRR; NLRR-1; NLRR-2

1. Introduction

In the morphogenesis of the nervous system, cell adhesion molecules play a pivotal role, for example in axon guidance and fasciculation [2,5,10,11,23,25,26], cell migration [19,20] and myelination [9,18,21]. These proteins have been shown to mediate cell-cell or cell-extracellular matrix interactions. Adhesion molecules are grouped into several families, e.g. the immunoglobulin superfamily [5], cadherin superfamily [30], integrin family [8], selectin family [17], and leucine-rich repeat superfamily [3,12,14,22,25].

It is well known that leucine-rich repeat (LRR) proteins play an essential role in the morphogenesis of the nervous system of *Drosophila* [6,24,25]. However, in mammalian species, the roles of LRR proteins in neural development have not been investigated, except for OMgp [21]. Based on the assumption that the fundamental mechanisms are conserved between *Drosophila* and mammalian species, we attempted to clone LRR proteins expressed in the developing mouse nervous system. We employed an EST clone as a probe for library screening. This was identified in the human brain cDNA sequencing project and encodes an LRR domain [1]. By screening a mouse brain cDNA library, we obtained two homologous but distinct clones designated NLRR-1 and -2 encoding proteins with LRR. In this report, we describe the characterization of the primary structures of these genes (whole sequence for NLRR-1 and partial sequence for NLRR-2) and show that these genes are expressed in the developing mouse nervous system. Possible functional implications of expression of these genes are discussed.

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Fig. 1. Nucleotide and predicted amino acid sequences of NLRR-1 (A) and NLRR-2 (B). The protein sequence is indicated below the DNA sequence. The cysteine-rich amino- and carboxy-flanking sequences are shown by underlines and the LRR sequences are shown by dashed underlines.

2. Materials and methods

2.1. Screening of mouse brain cDNA library

cDNA library screening was performed according to the methods described elsewhere [27]. The neonatal brain cDNA library of the ICR mouse (Stratagene) was screened with a human cDNA encoding LRR repeats (EST06184) [1] labeled by multiprimed labeling with [^{32}P]dCTP. Positive clones were excised with helper phage and recircularized to generate subclones in the pBluescriptII SK(−) phagemid vector (Stratagene).

2.2. Preparation of labeled cRNA probes

The *Bam*HI-*Hin*dIII fragment (0.8 kb) of NLRR-1 was subcloned into pBluescriptII SK(−). The plasmid was linearized with *Bam*HI for antisense RNA synthesis by T7 polymerase and with *Sal*I for sense RNA synthesis by T3 polymerase. The *Bst*XI-*Hin*cII fragment (1.0 kb) of NLRR-2 was subcloned into pBluescriptII SK(−). The plasmid was linearized with *Bam*HI for antisense RNA synthesis by T7 and with *Xho*I for sense RNA synthesis by T3 polymerase. In vitro synthesis was carried out using a Promega kit, and the probes were labeled with [^{35}S]UTP.

2.3. In situ hybridization histochemistry

The procedures were essentially the same as those described previously [31,32], except for the use of fresh frozen mouse brain sections in this study. Probes were hybridized to 18 μm thick sagittal cryostat sections of embryos at embryonic day (E) 13.5, E15.5 and horizontal sections of the brains of adult mice. After washing, the sections were dehydrated and laid in contact with X-ray film. After 2 days exposure, the films were developed and images were checked. The sections were then covered with photographic emulsion (Kodak NTB-2) and exposed for ten days. For semi-quantitative analyses of developmental changes in gene expression, we performed in situ hybridization experiments simultaneously with sections from mice at different ages to ensure that the hybridization and autoradiographic conditions were equivalent.

2.4. Northern blotting

Total RNA was prepared from various adult mouse tissues and whole embryos at various embryonic days as described previously [31]. RNA sample (adult tissues, 20 μg each; embryos, 30 μg each) were electrophoresed in formaldehyde-agarose gels, and transferred onto Immobilon-N membranes (Millipore). The cDNA inserts of NLRR-1 and NLRR-2 were labeled by multiprimed labeling with [^{32}P]dCTP. The membranes were hybridized with these radioactive probes and washed at a final stringency of 0.1 × SSC, 0.1% SDS, 65°C. And the expression of NLRR-2 mRNA was so weak in embryonic stages, we used the imaging plate-autoradiography system (BAS-2000, Fuji Photo Film Co., Ltd., Tokyo, Japan) to intensify the signals.

2.5. DNA sequencing

DNA was sequenced on both strands by the dideoxy chain termination method (Applied Biosystems Inc.) using AmpliTaq DNA polymerase. Nested deletions of selected subclones were made with exonuclease III.

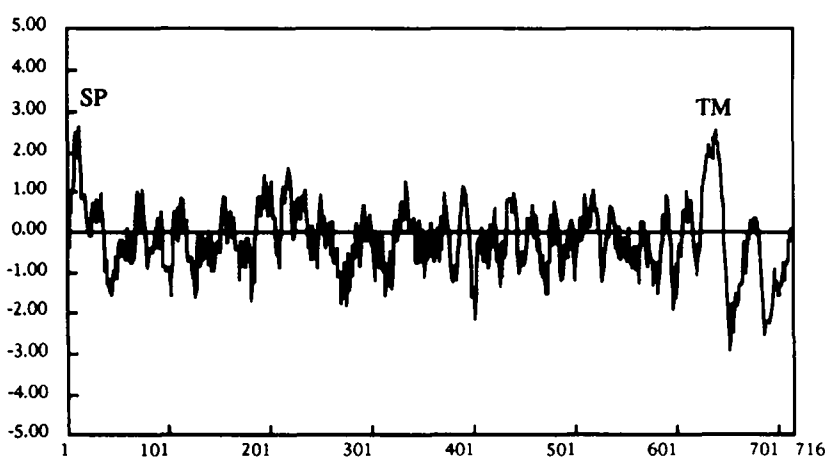

Fig. 2. Hydrophobicity plot of the predicted amino acid sequence of NLRR-1. The analysis was carried out by the method of Kyte and Doolittle [16] with a window of 10 residues. The putative hydrophobic signal peptide (SP) and transmembrane (TM) domain are indicated.

3. Results

3.1. Nucleotide sequence analysis of cDNA clones

Screening of the mouse brain cDNA library (1×10^6 plaques) yielded seven positive clones. Restriction enzyme mapping and partial nucleotide sequencing indicated that these seven clones could be grouped into two groups (NLRR-1 and NLRR-2) which were independent of each other. Partial nucleotide sequence analysis suggested that one of NLRR-1 group clones included the entire coding region. So, this clone was selected for complete nucleotide sequencing analysis. Fig. 1A shows the NLRR-1 nucleotide sequence and its deduced amino acid sequence. The cDNA contained a total of 3694 bases, including an open reading frame extending from nucleotide 717 to 2864. The continuous open reading frame encoded a polypeptide of 716 amino acids. In contrast, the clones in NLRR-2 group only spanned the part of the coding region. Fig. 1B shows the partial nucleotide sequence and deduced amino acid sequence of NLRR-2. The nucleotide sequences of NLRR-1 and NLRR-2 will appear in the GSDB, DDBJ, EMBL and NCBI nucleotide sequence database with following accession numbers D45913 and D49375, respectively.

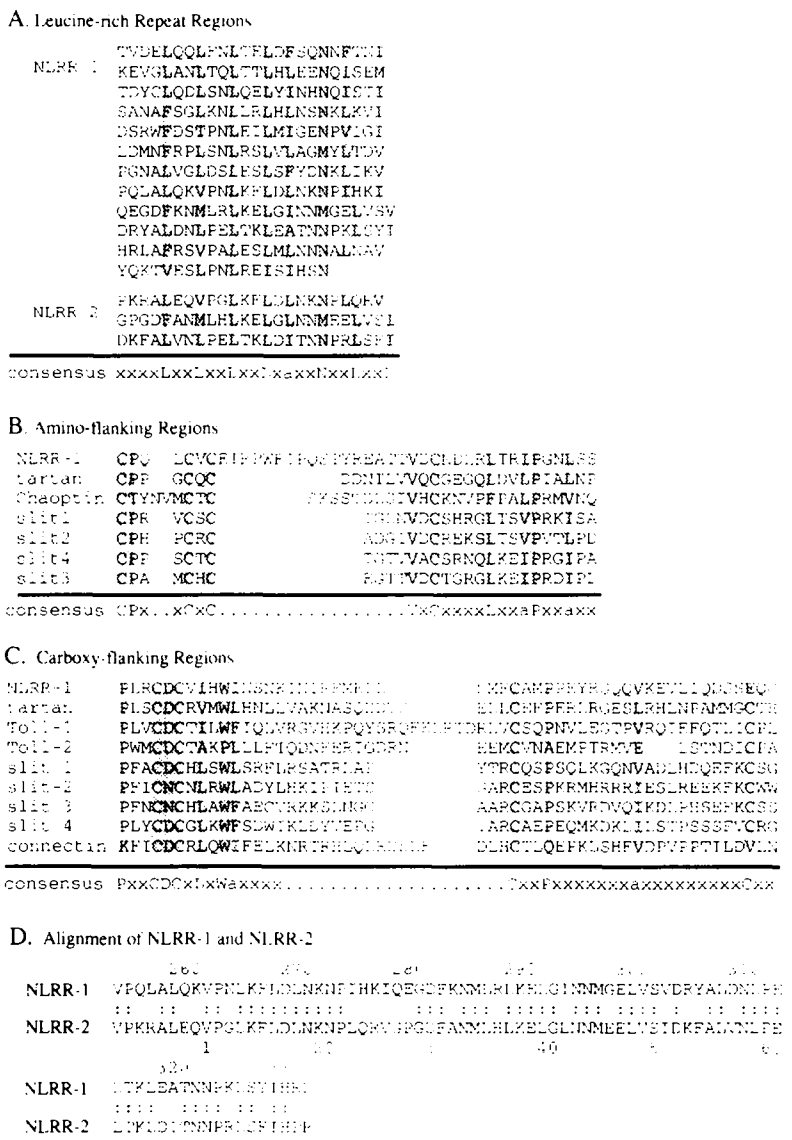

Fig. 3. A: LRR repeats in NLRR-1 and NLRR-2. B, C: Homology of the NLRR-1 amino- and carboxy-flanking region sequences to those of other LRR proteins. Consensus residues are shaded. The predominance of aliphatic residues (I, L, V) at the consensus positions is of note. a, Aliphatic residues. D: An alignment comparing the amino acid sequences of NLRR-1 and NLRR-2. The homology between NLRR-1 and NLRR-2 is about 70% at the amino acid sequence level. : indicates identical residue.

3.2. Structural features

3.2.1. NLRR-1

Hydrophobicity analysis [16] of the deduced amino acid sequence showed NLRR-1 has two hydrophobic regions (Fig. 2), one in the most N-terminal region suggesting that it is a signal peptide, while the other was situated relatively close to the C-terminus fulfilling conditions for a transmembrane domain.

The extracellular domain had an internally repeated sequence of 24–25 residues termed the leucine-rich repeat. This motif was present as 12 tandem repeats and was accompanied by typical amino-flanking and carboxy-flanking regions [3,14,22,25] (Fig. 3A,B,C).

3.2.2. NLRR-2

Partial amino acid sequence analysis showed that NLRR-2 had at least three LRR motifs (Fig. 3A). The homology with NLRR-1 was 62% at the nucleotide level and 70% at the amino acid sequence level (Fig. 3D).

3.3. Northern blotting

Fig. 4A,B shows the expression of NLRR-1 and NLRR-2 transcripts in various adult mouse tissues and Fig. 4C,D shows the expression in embryos at various embryonic days as revealed by Northern blot analysis. In adult tissues, both NLRR-1 (Fig. 4A) and NLRR-2 (Fig. 4B) mRNAs were detected as two bands only in the brain. Developmental analysis showed both NLRR-1 and NLRR-2 mRNA were detected in E11.5, E13.5 and E15.5 (Fig. 4C,D) as a single band.

3.4. Developmental expression examined by in situ hybridization

Throughout the present study, the specificities of each antisense probe were checked by using sections treated with sense probes. The sense probes gave no significant signals (data not shown). At the embryonic stage, intense labeling for NLRR-1 mRNA was detected in the CNS (the brain and the spinal cord), the dorsal root ganglia, and the cartilage. The expression in these regions became less intense as the embryo grew older (Fig. 5A,B).

In contrast to NLRR-1, the expression of NLRR-2 was confined exclusively to the central nervous system, and this pattern continued to adulthood. At E13.5 (the earliest stage examined in the present study), the expression of NLRR-2 was very weak but became more intense as development proceeded (Fig. 5C,D).

In adults, the expression of NLRR-1 mRNA was observed weakly all over the brain (Fig. 6A). Intense staining was detected in the granular cell layer of the dentate gyrus of the hippocampus, and in the granular and the Purkinje cell layers of the cerebellum. In the entorhinal cortex,

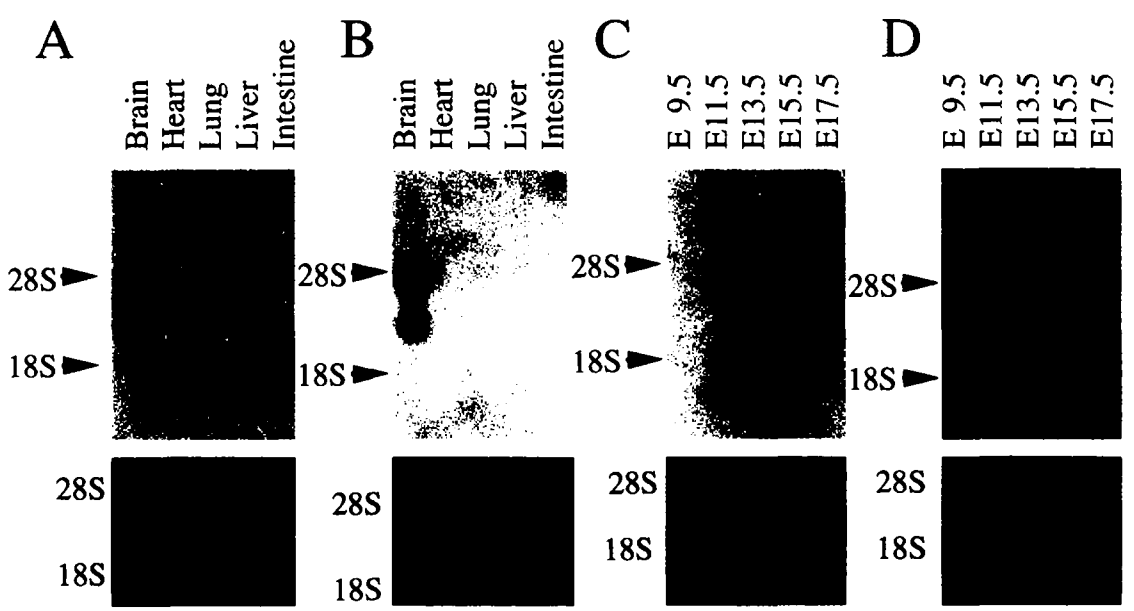

Fig. 4. Tissue distributions of NLRR-1 (A), NLRR-2 (B) mRNA and developmental expressions of NLRR-1 (C), NLRR-2 (D) mRNA. Northern blots were prepared as described in Materials and methods. The expression of NLRR-2 in embryonic stages was so weak, we used the imaging plate-autoradiography system (BAS-2000) to intensify the signals. Lower panels of ethidium bromide staining display sample loading. Markers at left indicate sizes of ribosomal RNAs. Arrows (D) show the bands of NLRR-2 mRNA.

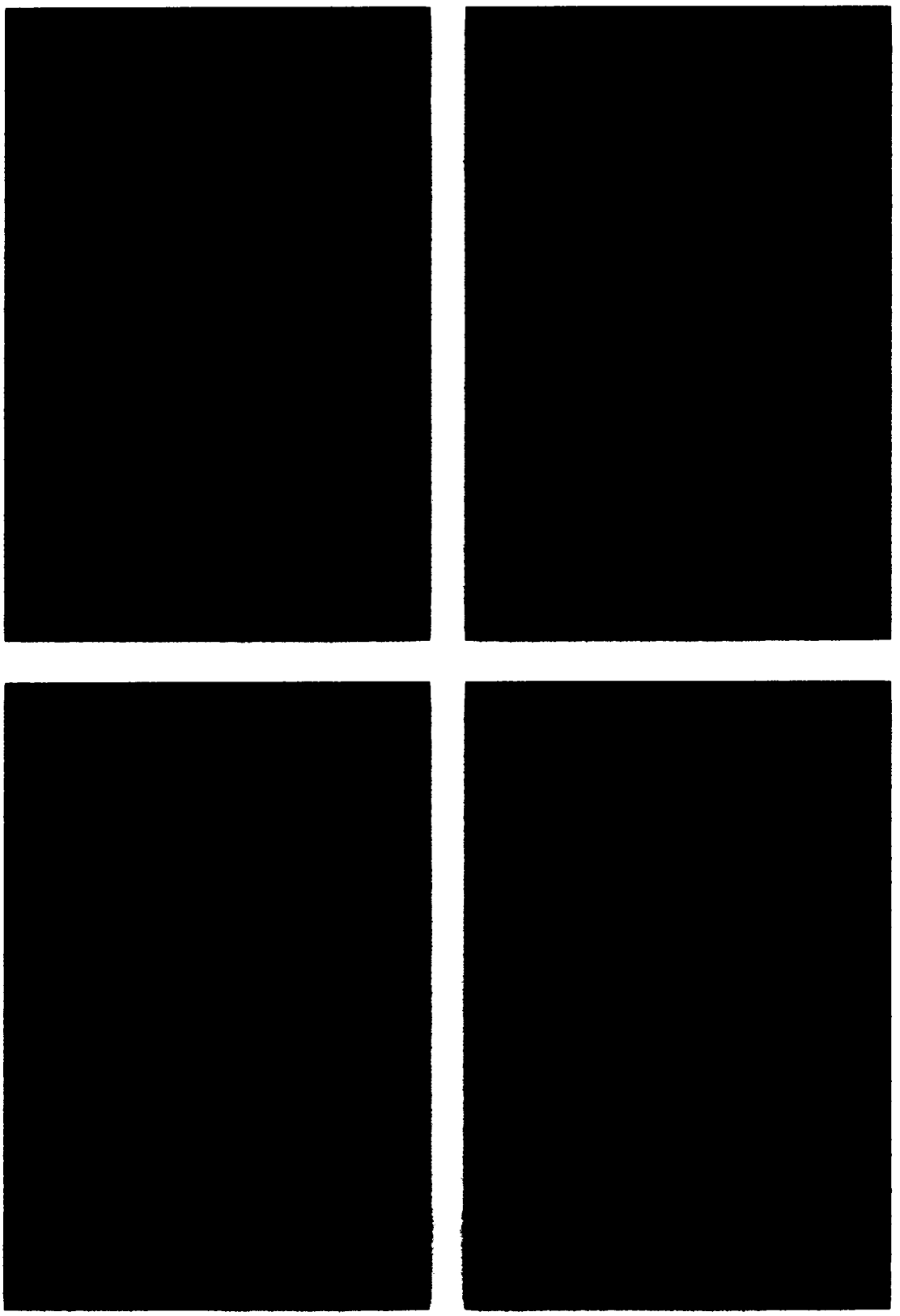

Fig. 5. Distribution of NLRR-1 mRNA (A, B) and NLRR-2 mRNA (C, D) in sagittal sections at embryonic day (E) 13.5 (A, C) and E15.5 (B, D). These pictures were obtained by film autoradiography. Because the expressions of NLRR-2 mRNA in E13.5 (C) and E15.5 (D) were so weak, we enhanced the signals of NLRR-2, accordingly it should be noted the autoradiographic conditions of NLRR-1 and NLRR-2 are not equal. Arrowheads show the spinal cord and small arrows show the brain. Note that the expression of NLRR-2 was confined exclusively to the central nervous system. The gaps in staining along the spinal cord in panel B and D are derived from parasagittal sectioning of embryos. B, brain; C, cartilage; D, dorsal root ganglia. Bars = 2 mm.

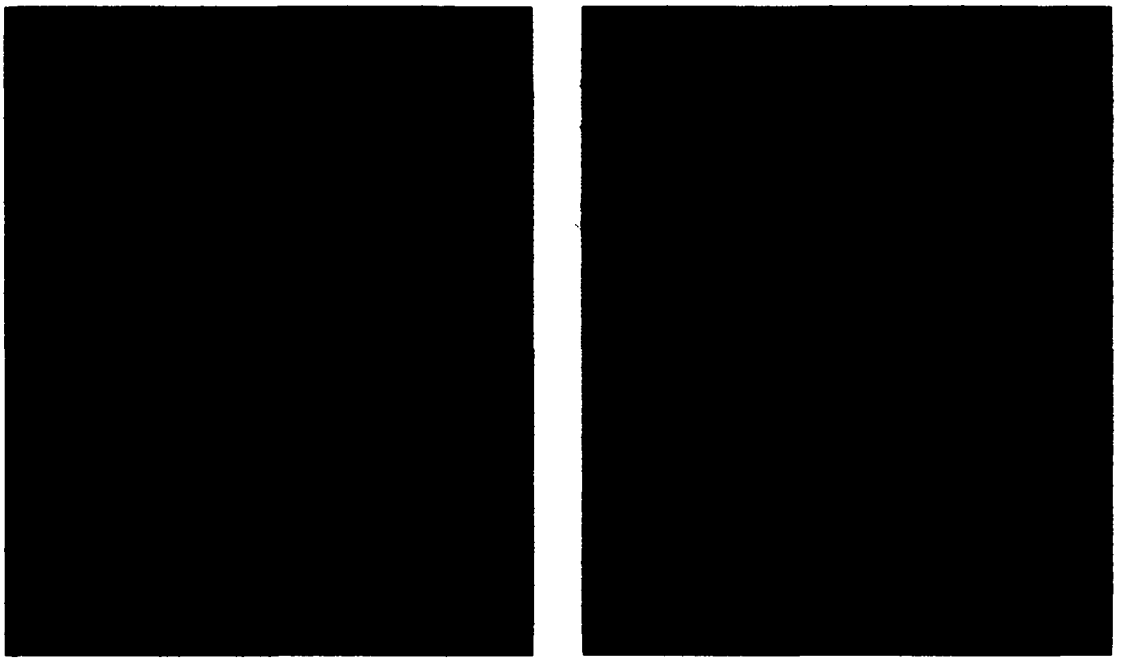

Fig. 6. Distribution of NLRR-1 mRNA (A) and NLRR-2 mRNA (B) detected by film autoradiography in horizontal sections of the adult brain. Hipp, hippocampus; Cer, cerebellum. Bar = 2 mm.

moderate staining was detected in the second layer (Fig. 7A,B).

At this stage, like that of NLRR-1, NLRR-2 mRNA was detected weakly all over the brain (Fig. 6B). However, in contrast to NLRR-1, intense staining was detected in the pyramidal cell layer of the hippocampus. In addition, in the cerebellum, intense staining was detected only in the Purkinje cell layer. In the entorhinal cortex, moderate staining was detected in all layers (Fig. 7C,D).

4. Discussion

We isolated two novel cDNAs from a neonatal mouse brain cDNA library encoding proteins containing tandem leucine-rich repeats. One of these, NLRR-1 is a transmembrane protein with amino-flanking region (AFR) and carboxy-flanking region (CFR). The LRR domain was first identified in human leucine-rich α2-glycoprotein [29] and now more than 40 proteins have been identified as members of the LRR superfamily [13]. This family contains diverse groups of molecules with different functions and cellular localizations in a variety of organisms. Many of the proteins of this family are located in the extracellular matrix or associated with the plasma membrane, while some members are cytoplasmic proteins (e.g., porcine RNase inhibitor [7]) or serum proteins (e.g., leucine-rich α2-GP [29]). Some members of this family are associated with and function in the nervous system of *Drosophila*; for example, connectin functions in neuromuscular junction formation [22], slit in axon development [25], chaoptin in photoreceptor-cell development [14], and tartan in neuronal and muscle development [3]. These molecules show various localization patterns in relation to the cell membrane; tartan is a transmembrane protein, connectin and chaoptin are membrane-anchored proteins, and slit is a secreted protein. When compared with these proteins, NLRR-1 is most close to tartan in terms of primary structure (Fig. 8). Moreover, NLRR-1, tartan, and slit have both AFR and CFR, while chaoptin has only AFR, and connectin has only CFR. Since all the LRR proteins with AFR or CFR (at least 18 proteins) are involved in cell adhesion or function as receptors [13], the NLRR-1 protein is likely to function as a cell adhesion molecule or signal transducing receptor. In mammalian species, it is noteworthy that there are several LRR proteins which are preferentially expressed in connective tissues, such as decorin [15,28] and biglycan [4]. However, NLRR-1 is distinct from these glycoproteins in that the homologies between NLRR-1 and these proteins at amino acid sequence level are less than 30%. In addition, NLRR-1 is a transmembrane protein with AFR and CFR, while decorin and biglycan are extra-

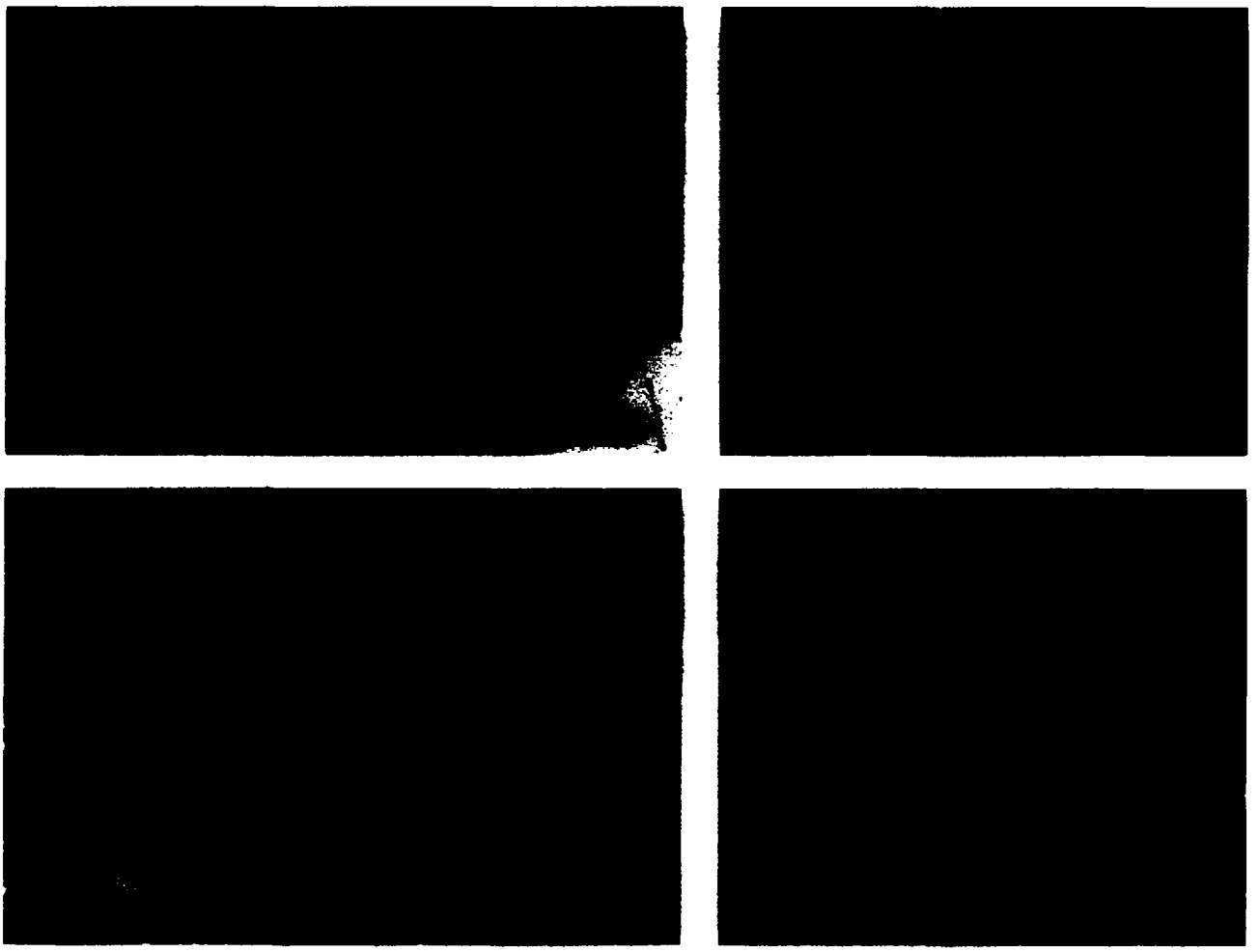

Fig. 7. Localization of NLRR-1 mRNA (A, B) and NLRR-2 mRNA (C, D) under dark-field illumination in the hippocampus (A, C) and the cerebellum (B, D) of the adult brain. Arrowheads (B, D) show the Purkinje cell layers of the cerebellum. CA1–CA4, CA1–CA fields of Ammon's horn; EC2, second layer of entorhinal cortex; GD, granular cell layer of dentate gyrus; GC, granular cell layer of the cerebellum. Bars = 0.5 mm.

cellular matrix proteins with only AFR. In adult mouse, Northern analysis shows NLRR-1 and NLRR-2 mRNA are detected only in brain while decorin mRNA is detected in various tissues: heart, lung, liver, kidney, spleen, bone and skin [28]. Taken together, NLRR-1 seems to belong to a LRR protein family which is distinct from known extracellular matrix protein family with LRR. NLRR-1 and NLRR-2 showed 62% homology at the nucleotide level and 70% at the amino acid sequence level, and these proteins have no significant homology to other LRR proteins. Taking account of these findings, NLRR-1 and NLRR-2 are likely to form a subfamily and to have similar functions. In this regard, it should be noted that multiple bands in adult brain and single band in embryonic stages detected by Northern blotting may indicate alternative splicing events or the existence of other family members with similar structures. Further studies are, therefore, required to test these speculations. Developmental analysis shows NLRR-1 and NLRR-2 proteins are first expressed in E11.5. These results suggest that both proteins have some functions from the mid-gestational period. Subsequent in situ hybridization analysis revealed that both NLRR-1 and NLRR-2 mRNAs are widely expressed in the developing mouse brain. This pattern is somewhat different from those of slit and connectin; the latter is expressed in certain subsets of neurons and muscles [22], while slit is primarily expressed in midline glial cells [25]. The differences between these expression patterns may indicate that NLRR-1 and -2 are involved in essential cell-cell interactions rather than in specific developmental events, such as formation of

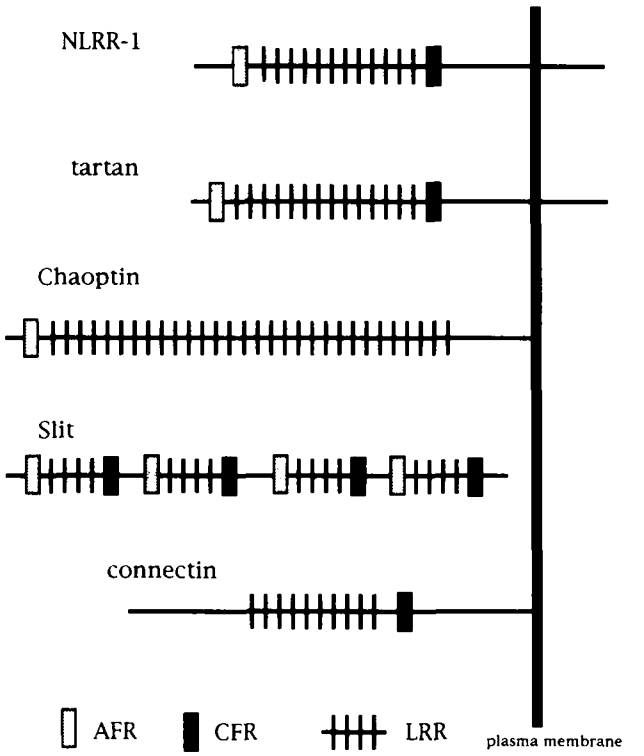

Fig. 8. Comparison of NLRR-1 protein structure to those of other LRR proteins known to function in the nervous system.

neuromuscular junctions [22] and of the commissural axon pathways [25]. In this context, tartan seems more similar to NLRR family than other *Drosophila* LRR proteins; tartan is broadly expressed in the nervous and skeletal systems [3]. If this is the case, null mutation of the NLRR-1 or -2 gene in mice will result in severe defects in the organization of the nervous system, similarly to null mutants of the tartan gene in *Drosophila* [3]. This kind of gene targeting experiment should give insight into the physiological functions of the NLRR gene family, and the present identification of these two cDNAs is the first step towards this end.

References

[1] Adams, M.D., Soares, M.B., Kerlavage, A.R., Fields, C. and Venter, J.C., Rapid cDNA sequencing (expressed sequence tags) from a directionally cloned human infant brain cDNA library, *Nature Genet.*, 4 (1993) 373–380.

[2] Bastiani, M.J., Harrelson, A.L., Snow, P.M. and Goodman, C.S., Expression of fasciclin I and II glycoproteins on subsets of axon pathways during neuronal development in the grasshopper, *Cell*, 48 (1987) 745–755.

[3] Chang, Z., Price, B.D., Bockheim, S., Boedigheimer, M.J., Smith, R. and Laughon, A., Molecular and genetic characterization of the Drosophila tartan gene, *Dev. Biol.*, 160 (1993) 315–332.

[4] Fisher, L.W., Termine, J.D. and Young, M.F., Deduced protein sequence of bone small proteoglycan I (biglycan) shows homology with proteoglycan II (decorin) and several nonconnective tissue proteins in a variety of species, *J. Biol. Chem.*, 264 (1989) 4571–4576.

[5] Harrelson, A.L. and Goodman, C.S., Growth cone guidance in insects: fasciclin II is a member of the immunoglobulin superfamily, *Science*, 242 (1988) 700–708.

[6] Hashimoto, C., Hudson, K.L. and Anderson, K.V., The Toll gene of Drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein, *Cell*, 52 (1988) 269–279.

[7] Hofsteenge, J., Kieffer, B., Matthies, R., Hemmings, B.A. and Stone, S.R., Amino acid sequence of the ribonuclease inhibitor from porcine liver reveals the presence of leucine-rich repeats, *Biochemistry*, 27 (1988) 8537–8544.

[8] Ignatius, M.J. and Reichardt, L.F., Identification of a neuronal laminin receptor: an Mr 200K/120K integrin heterodimer that binds laminin in a divalent cation-dependent manner, *Neuron*, 1 (1988) 713–725.

[9] Inuzuka, T., Fujita, N., Sato, S., Baba, H., Nakano, R., Ishiguro, H. and Miyatake, T., Expression of the large myelin-associated glycoprotein isoform during the development in the mouse peripheral nervous system, *Brain Res.*, 562 (1991) 173–175.

[10] Jessell, T.M., Adhesion molecules and the hierarchy of neural development, *Neuron*, 1 (1988) 3–13.

[11] Klambt, C. and Goodman, C.S., The diversity and pattern of glia during axon pathway formation in the Drosophila embryo, *Glia*, 4 (1991) 205–213.

[12] Kobe, B. and Deisenhofer, J., Crystal structure of porcine ribonuclease inhibitor, a protein with leucine-rich repeats, *Nature*, 366 (1993) 751–756.

[13] Kobe, B. and Deisenhofer, J., The leucine-rich repeat: a versatile binding motif, *Trends Neurosci.*, 19 (1994) 415–421.

[14] Krantz, D.E. and Zipursky, S.L., Drosophila chaoptin, a member of the leucine-rich repeat family, is a photoreceptor cell-specific adhesion molecule, *EMBO J.*, 9 (1990) 1969–1977.

[15] Krusius, T. and Ruoslahti, E., Primary structure of an extracellular matrix proteoglycan core protein deduced from cloned cDNA, *Proc. Natl. Acad. Sci. USA*, 83 (1986) 7683–7687.

[16] Kyte, J. and Doolittle, R.F., A simple method for displaying the hydropathic character of a protein, *J. Mol. Biol.*, 157 (1982) 105–132.

[17] Lasky, L.A., Selectins: interpreters of cell-specific carbohydrate information during inflammation, *Science*, 258 (1992) 964–969.

[18] Lemke, G., Unwrapping the genes of myelin, *Neuron*, 1 (1988) 535–543.

[19] Lindner, J., Rathjen, F.G. and Schachner, M., L1 mono- and polyclonal antibodies modify cell migration in early postnatal mouse cerebellum, *Nature*, 305 (1983) 427–430.

[20] McClay, D.R. and Ettensohn, C.A., Cell adhesion in morphogenesis, *Annu. Rev. Cell. Biol.*, 3 (1987) 319–345.

[21] Mikol, D.D., Gulcher, J.R. and Stefansson, K., The oligodendrocyte-myelin glycoprotein belongs to a distinct family of proteins and contains the HNK-1 carbohydrate, *J. Cell. Biol.*, 110 (1990) 471–479.

[22] Nose, A., Mahajan, V.B. and Goodman, C.S., Connectin: a homophilic cell adhesion molecule expressed on a subset of muscles and the motoneurons that innervate them in Drosophila, *Cell*, 70 (1992) 553–567.

[23] Patel, N.H., Snow, P.M. and Goodman, C.S., Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila, *Cell*, 48 (1987) 975–988.

[24] Reinke, R., Krantz, D.E., Yen, D. and Zipursky, S.L., Chaoptin, a cell surface glycoprotein required for Drosophila photoreceptor cell morphogenesis, contains a repeat motif found in yeast and human, *Cell*, 52 (1988) 291–301.

[25] Rothberg, J.M., Jacobs, J.R., Goodman, C.S. and Artavanis, T.S., slit: an extracellular protein necessary for development of midline glia and commissural axon pathways contains both EGF and LRR domains, *Genes Dev.*, 4 (1990) 2169–2187.

[26] Rutishauser, U. and Jessell, T.M., Cell adhesion molecules in vertebrate neural development, *Physiol. Rev.*, 68 (1988) 819–857.

[27] Sambrook, J., Fritsch, E.F. and Maniatis, T., *Molecular Cloning: A*

Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989, pp. 2.108–117.

[28] Scholzen, T., Solursh, M., Suzuki, S., Reiter, R., Morgan, J.L., Buchberg, A.M., Siracusa, L.D. and Iozzo, R.V., The murine decorin. Complete cDNA cloning, genomic organization, chromosomal assignment and expression during organogenesis and tissue differentiation, *J. Biol. Chem.*, 269 (1994) 28270–28281.

[29] Takahashi, N., Takahashi, Y. and Putnam, F.W., Periodicity of leucine and tandem repetition of a 24-amino acid segment in the primary structure of leucine-rich alpha 2-glycoprotein of human serum, *Proc. Natl. Acad. Sci. USA*, 82 (1985) 1906–1910.

[30] Takeichi, M., Cadherin cell adhesion receptors as a morphogenetic regulator, *Science*, 251 (1991) 1451–1455.

[31] Wanaka, A., Johnson, E.J. and Milbrandt, J., Localization of FGF receptor mRNA in the adult rat central nervous system by in situ hybridization, *Neuron*, 5 (1990) 267–281.

[32] Wanaka, A., Milbrandt, J. and Johnson, E.J., Expression of FGF receptor gene in rat development, *Development*, 111 (1991) 455–468.