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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, chaptin, 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 (Neuronal Leucine-Rich Repeat 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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A

1 CACGAGCAGGAGGCGCGGGAGCAAGCCGTAAGCCAGGCGGGGGGCGTCGCGCCGGGTTTCAGCCGT
 72 TGTGGGCGGGAGCCGGGCGCGCGGCGCCTCCGAGACAGTGGATCGTCCGACCGGAGCTGGAGGGCC
 143 CTCGGGGCGTTCCTTTGAGACCGATCCCTGGGCGCGCCCGCCTTCCAGCCCAATTCTCCAGCCCTTTT
 214 CTAATCCGGCCTGTTCACAGACCCCAAGAACGGATTTAGCCGAGCCCGGGAGGAGCTTGGCT
 285 CGCGGGGCTTCTGGCGTTGATCTGGTGTCTGCTGGGGTCCGCCAAGGAGCCGATGGCCGCTCGGG
 356 CTGGGTGAGGGGCTCTGTGTCCGGCTCCATCCCGGGGCTAGTGGTCTGCTCCACCAACCCCGGTC
 427 CGTGGCACCCCAACACCCCTGTGAGCCGAGCCCTAGGACAGCTTCCAGACTTTGATTTGGCTGAGTAA
 498 CTCCCAAGAAGCCTGTGACATGCTCCGGATTTGATGATAGCGTTCAGGACTTCCAGATTGCTTCCTCCGGC
 569 TCTTGGGAAGTTCAGAAAACTTCAGACTCCGGAAATTTCTTTCAGTGAGACTTCGTGGTAAATTACAC
 640 TATCTGCTTGTGTCTCTCCCGCATGAAATCGTGTTCAGATCCGGCTCAGGAACTGGGTGGTGGCAA
 711 GCCACC

717 ATG GCC AGA TTG AGC ACT GGG AAA GCA GCT TGG CAG GTG GTC TGT GGC TTG GTG
 1 M A R I : S T A A C Q V Y D G I D

771 ATA ACT TCA TTA ACT GAA TGT TCC ATA CTG ACT AGT GAG GGT TGG GAG TTG TGL
 19 I T S L T E S I L T S E P F Q L C

825 GTT TGT GAA ATT CGT TGG TTT ACC CCA CAG TCC AGC TAT AGA GAA GCC AGC
 37 V C E T R P W E T P Q C A G R E A T

879 ACC GTG GAT TGT AAT GAC CTC CGC CTA ACC AGG ATC CCT GGC AAC CTC TGT AGT
 55 T V D C N D L R L G T H I P G N L S R

933 GAC ACA CAA GTG CTT CTC TTA GAC AAG AAT AAC ATC GTC AAC ACC GTG SAC GAG
 73 D T Q V L L T Q S N N I A K I V D E

987 CTG CAG CAG CTC TTC AAC TTG ACC GAG CTG GAT TTC TGG CAG AAC AAC TTT ACA
 91 L Q Q L F N L T D F S Q N N F T

1041 AAC ATC AAA GAG CTA GGG CTG GCA AAC TTG ACC CAG CTC AGA ACC CTG CAT CTA
 109 N : K E V G L A N I T Q L D T D R E

1095 GAG GAA AAT CAG ATT TCA GAG ATT ACC GAT TAG TGT GTG CAA GAC CTC AGC AAC
 127 E E N Q L F E M T D Y C Q D L S N

1149 CTT CAA GAA CTC TAC ATC AAC CAC AAC CAG ATC AGC ACA ATG TGT GGT AAC GGC
 145 : Q E L Y : N H N Q : S T : S A N A

1203 TTT TCT GGC TTG AAA AAT CTC TTG AGG CTG CAC CTG AAT TGC AAT AAG TTG AAA
 163 F S G L R N L L R L H I N S N K I K

1257 GTG ATC GAT AGC GGC TGG TTT GAT TCC ACT GGC AAC CTG GAA ATT CTG ATG ATT
 181 V L D S H W F D S T P N I E L N L

1311 GGG GAA AAC CCT GTG ATT GFA ATC CTG GAC ATG AAC TTG AGA CCT CTC TCC AAC
 199 G E N P V : G : L D M N F R P L S N

1365 CTG AGA AGC CTA GTG TTA GCA GAA ATG TAG CTC ACT GAT GTP CCC GGA AAC GGC
 217 L R S L V L A L A G M Y L T D V P G N A

1419 TTG GTA GGC TTG GAT AOC CTC GAG AGC CTG TCT TTC TAT GAC AAC AAA GTG ATC
 235 L V G L D S L E S L S F Y D N K L I

1473 AAA GTC CCT CAA CTT GCC CTG CAA AAA GTC CCA AAT TTG AAA TTT TTA GAC CTC
 253 K V P Q L A L Q K V P N L K E I D D

1527 AAT AAA AAT CCT ATT CAC AAA ATC CAA GAA GGA GAC CTT AAA AAT ATG GTT GGG
 271 N K N P : R K : Q E G D F K N M D R

1581 TTA AAA GAA CTG GGA ATC AAC AAC ATG GGA GAG CTG GTT TCC GTC GAC GGC TAT
 289 : K E L G : N N M G E L V S Y D R Y

1635 GGC CTG GAT AAC TTG CCA GAA TTG ACA AAG CTC GAA GCA ACC AAT AAC GGC AAA
 307 A : D H L P E L T K L E A R N N P K

1689 CTG TCA TAG ATC CAG CCT TTG GCT TTC GGT AGT GTG CCG TGT CTA GAA AGT TTA
 325 E S Y I H R L A F R S V P A D F S

1743 ATG TTG AAC AAC AAT GCC TTG AAT GGC GTG TAG CAA AAA ACA GTG GAA TCG TTT
 343 M L N N N A L N A V Y Q K T V E S I

1797 CCC AAT CTG CGT GAG ATC AGC ATC CAC AGC AAC GCG CCG AGG TGT GAC TGT TGT
 361 P N L R E : S I H S N P L R C D C V

1851 ATC CAC TGG ATC AAC TCC AAC AAA ACC AAC ATT CGC TTC ATG GAG CCC CTC TGC
 379 I H W I N S N K T N I R F M E P L S

1905 ATG TTC TGT GCC ATG CCA CCG GAA TAC AGG GGC CAG CAG GTG AAG GAG GTC CTA
 397 M F C A M P P E Y R G Q Q V K E V L

1959 ATC CAG GAC TCA AGT GAG CAG TCC CTC CCG ATG ATA TCG CAC GAT ACA TTC CTT
 415 I Q D S S E Q C L P M I S H D T F P

2013 AAT CAT CTA AAC ATG GAC ATT GGT AGA AGA CTT TTC CTA GAC TGT GGC GCC ATG
 433 N H L N M D I G T T I F I D G R A M

2067 GCT GAG CCA GAA CCA GAG ATT TAC TGG GTC ACT CCC ATT GGA AAT AAG ATA ACC
 451 A E P E P E I Y W V T P : G N K : T

2121 GTG GAA ACT CTT TCA GAC AAA TAC AAG CTG ACT AGT GAG GGC ACC TTG GAG ATA
 469 V E T L S D K Y K L S S E G T D H :

2175 GCA AAC ATA CAG ATT GAA GAC TCA GGA AGG TAC ACC TGG GTT GCC CAG AAT GTP
 487 A N I Q : E D S G R Y T C V A Q N V

2229 CAA GGA GCA GAC ACT CGA GTG GCA ACT ATC AAG GTT AAT GGA ACC CTT CTA GAT
 505 Q G A D T R V A T : K V N G T : L D

2283 GGT GCA CAG GTG CTG AAA ATA TAT GTC AAA CAG ACA GAA TCT CAG TGG ATT TTA
 523 G A Q V L K : Y V K Q T E S H S I D

2337 GTA TCT TGG AAA GTT AAT TCC AAT GTC ATG ACA TGG AAC TTG AAA TGG TCA TCT
 541 V S W K V N S N V M I S N : K W S S

2391 GCC ACT ATG AAG ATC CAG AAC CCC CAC ATA ACA TAC ACA GCC AGC GTG CCA GCA
 559 A T M K : D N P H I T Y T A R V P V

2445 GAT GTT CAT GAA TAT AAC CTA ACA CAT CTG CAG CCT TCT ACC GAT TAT GAA GTG
 577 D V H E Y N L T H L Q P S T D Y F V

2499 TGT CTG ACG GTG TCC AAC ATC CAC CAG CAG ACT CAG AAG TCA TGT GTC AAT GTC
 595 C L T V S N : H Q Q T Q K S C V R V

2531 ACA ACC AAA ACC GGT GCG TTT GCG CTG CAG ATC TCT GAC CAT GAA ACC AGC ACC
 611 T T K T A A F A L D I S D H E S C

2607 GCC CTC GGT GCG GTC ATG GGG TCC ATG TTT GGC CTC ATC AGT CTA GCA TCC ATC
 637 A : A A V M G S M F A V : S L A S :

2661 GGT ATT TAC ATC GCG AAA AGG GTT AAG ASG AAA AAT TAC CAC CAT TGG CTG AAA
 685 T T K T A A F A L D I S D H E S C

2715 AAG TAT ATG GAA AAA ACC TCG TCC ATC CCA CTG AAC GAG CTG TAC CCC CCA CTC
 667 K Y M Q K T S S : P I N E L Y P P L

2769 ATT AAC CTG TCG GAA GCT GAC AGT GAC AAG GAC AAA GAT GGC TGG GCA GAC ACC
 885 T T K T A A F A L D I S D H E S C

2825 AAG CCA ACC CAG GTT GAC ACA TCC AGA ACC TAT TAC ATG TGG TAA
 703 K P T Q V D I S R S Y Y M W 9:00

2868 CTCACGTGGTGTTTTGGCTTCGTGGTAGTAAGGACACAAAGAGATTTCGTTTTATTCTGCAAAAAGTAAGAA
 2939 GGTGAAGAGACTTTTGTACTTTTGACTTTGTCTAGTTGGCAGAGCCGAGAGATGGGTGGATAATTCAA
 3010 AGTTTTTTAGTATAGCGTATCGCAAGGGGGTGGACACAGCTGGTGGTGACTTCAGCTTCAGTTTGTGT
 3081 TGCTTTTATCGCTATCAATTATTATATATATTATTTATTTTATTATCATATTATTTTATTTTATGTTGA
 3152 TTCCTAACCTCAGTAAATGCTGTCTTAAC TACGATGCTCAATGATTAATGACAGGTTGGGGGTTCTCCTGTC
 3223 CTTCAGCCCTTCTTCTGAAAGCCATAACAGCAACCGGACCCGCGCTCAACAGCTAACATAG
 3294 GGTTTGAAGCAGGATTAGATCCTTTGACCACTGGTCTACAGACTTAACTCAAGCTAAGCTAAACT
 3365 GCTACTTTGTGAATGATTAGTTAGTTGACTGTACTGTAATGTGTGTAACAATGAACTGAAATGTTCCTTTC
 3436 AGCAACAATCTTTTCCTCTCTTTCCTTTTCCTTTTAAATAGTAAAGAGGCTTAGAACAAGCTAACAGCGAA
 3507 TAGAAATGTATATCAGACTTTTCTAAATGAACTCACTGTAATGTATTACTATTTCTTTTATCTT
 3578 TAGTAGACACTTCTAAAGAAGAAAAGATAATTTTGTTGTGTTTTAACTCAACCAACTTGGTATTAAGAGA
 3649 CAGAAGTAAATAAATTAGTTCTTCTGATTTCTTAAAAAAA

B

1 GTG GCC AAG CGG GGA TTG GAG CAG GTG CCT GGG CTC AAG TTC CTA GAC CTG AAC
 3 V P K R A L E Q V P G G I K F L D I N

55 AAA AAC CCA CTG CAG CCG GTC GGA CCG GGA GAC TTT GCC AAC ATG CTG CAC CTC
 59 K N P L Q R V G P G D F A N M L H L

109 AAG GAA CTA GGA CTA AAC AAC ATG GAG GAA CTG CTC TCC ATC GAC AAG TTT GCT
 37 K E L G L N N M E E L V S : D K F A

163 CTG GTA AAT CTC CCG GAG CTG ACC AAG TTG GAC ATC ACC AAC AAC CCA CCG CTC
 55 L V N L P E L T K L D I T N N P R L

217 TCT TTC ATC CAT CCT GCG
 24 S F I H P R

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 [³²P]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-*Hind*III 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-*Hinc*II 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 [³⁵S]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 [³²P]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.

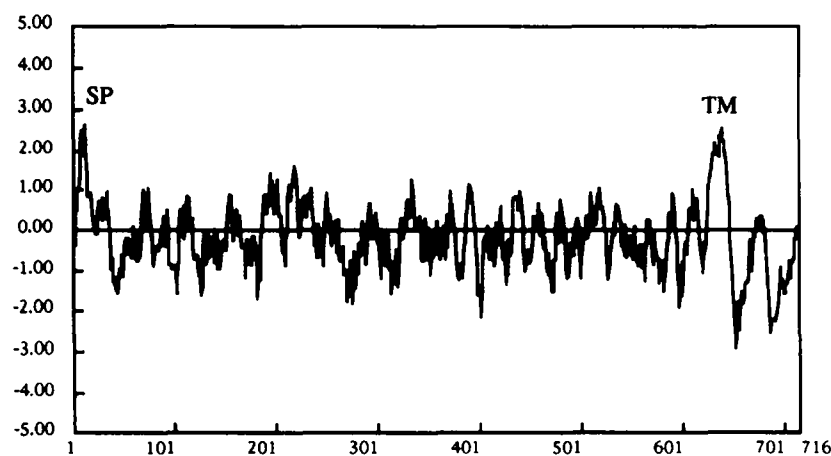


Fig. 2. Hydrophobicity plot of the predicted amino acid sequence of NLRR-1. The analysis was carried out by the method of Kyte and Doolittle [16] with a window of 10 residues. The putative hydrophobic signal peptide (SP) and transmembrane (TM) domain are indicated.

3.2. 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,

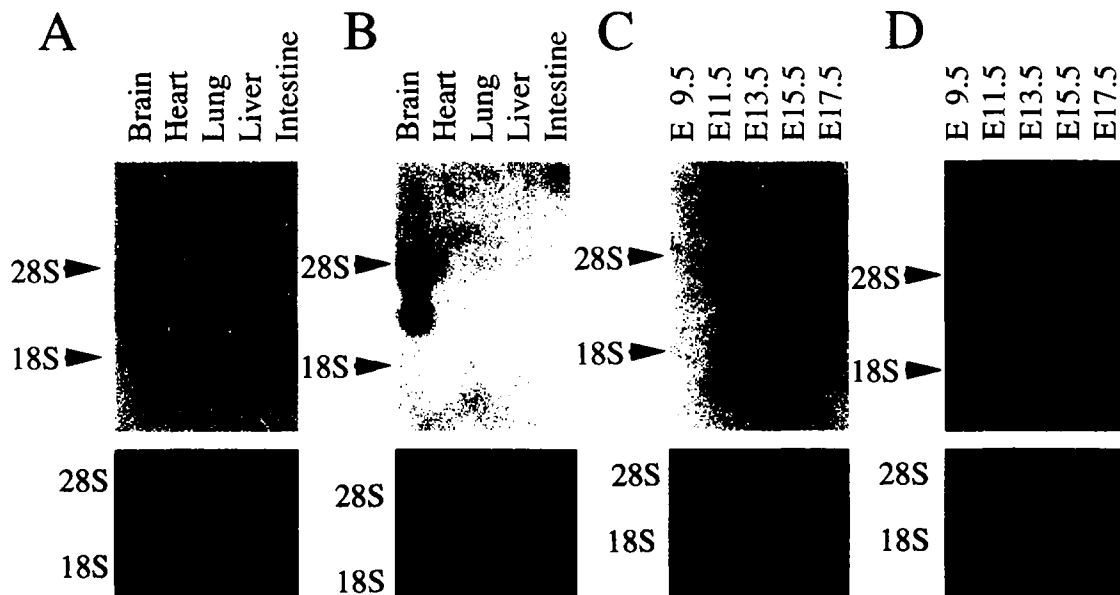


Fig. 4. Tissue distributions of NLRR-1 (A), NLRR-2 (B) mRNA and developmental expressions of NLRR-1 (C), NLRR-2 (D) mRNA. Northern blots were prepared as described in Materials and methods. The expression of NLRR-2 in embryonic stages was so weak, we used the imaging plate-autoradiography system (BAS-2000) 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.

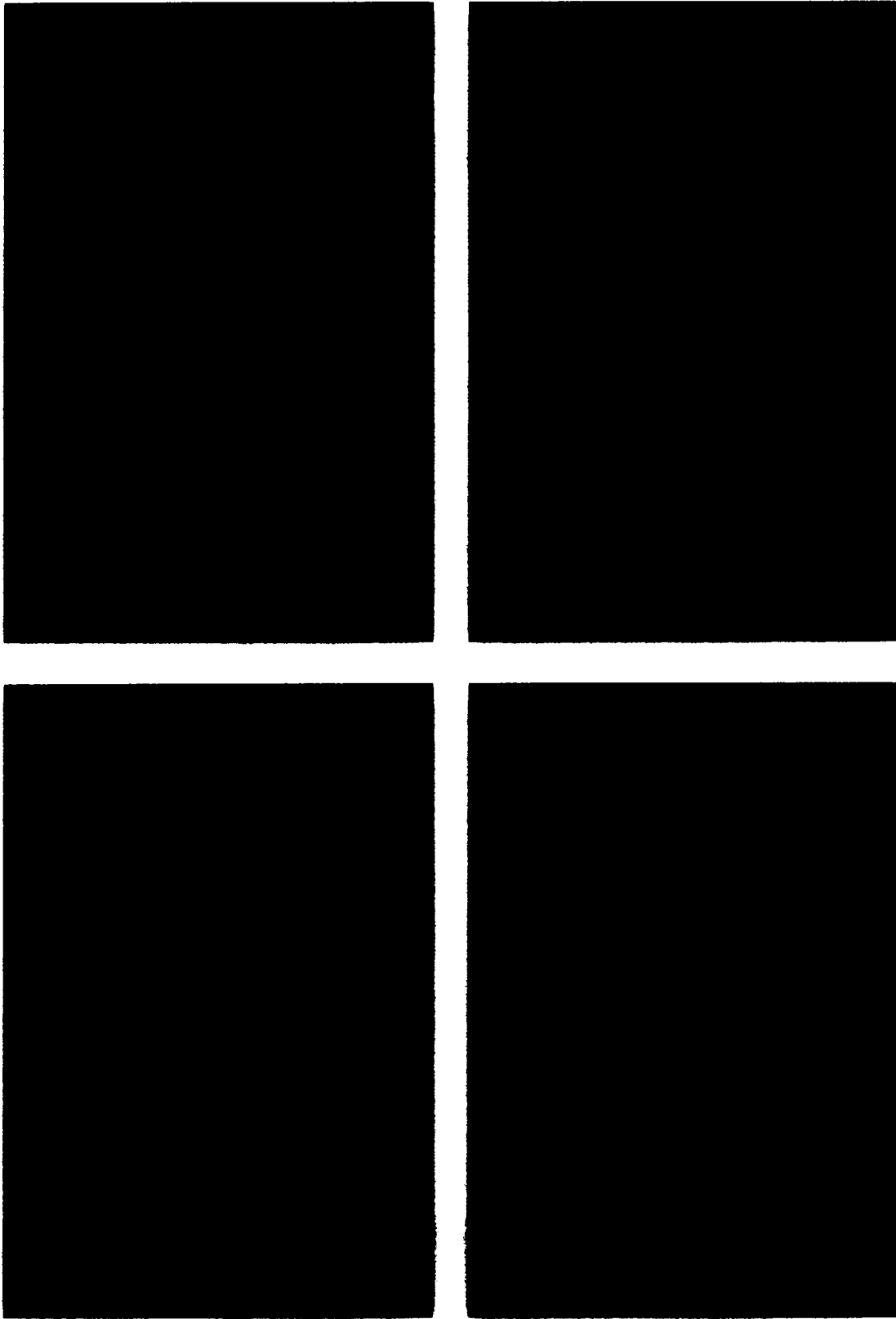


Fig. 5. Distribution of NLRR-1 mRNA (A, B) and NLRR-2 mRNA (C, D) in sagittal sections at embryonic day (E) 13.5 (A, C) and E15.5 (B, D). These pictures were obtained by film autoradiography. Because the expressions of NLRR-2 mRNA in E13.5 (C) and E15.5 (D) were so weak, we enhanced the signals of NLRR-2, 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.

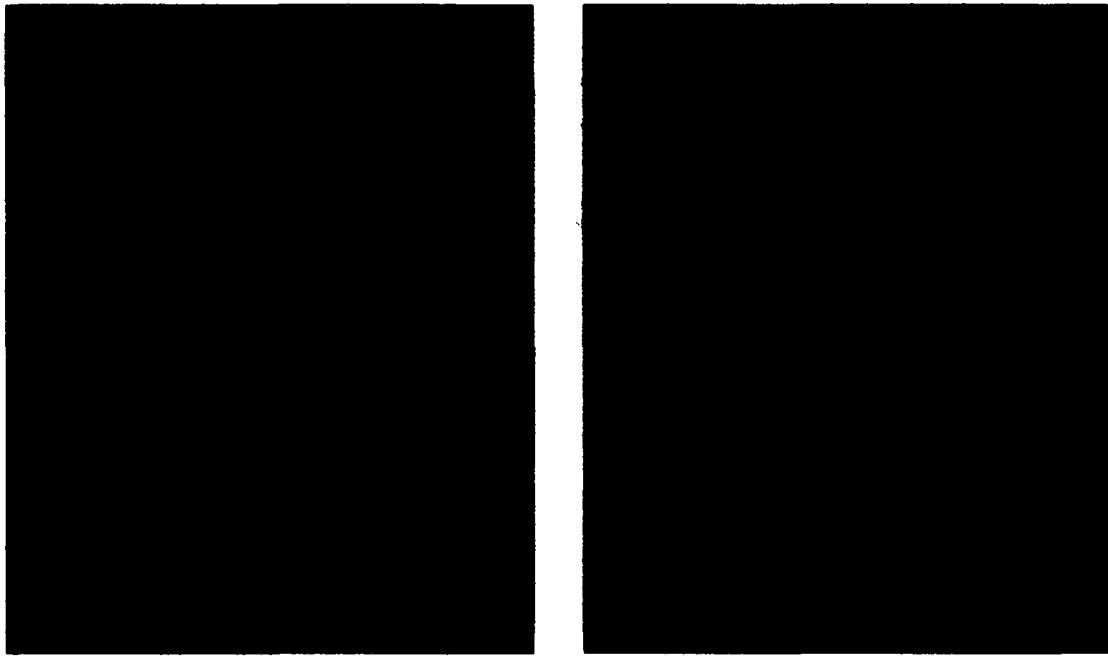


Fig. 6. Distribution of NLRR-1 mRNA (A) and NLRR-2 mRNA (B) detected by film autoradiography in horizontal sections of the adult brain. Hipp, hippocampus; Cer, cerebellum. Bar = 2 mm.

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 α 2-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 α 2-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-

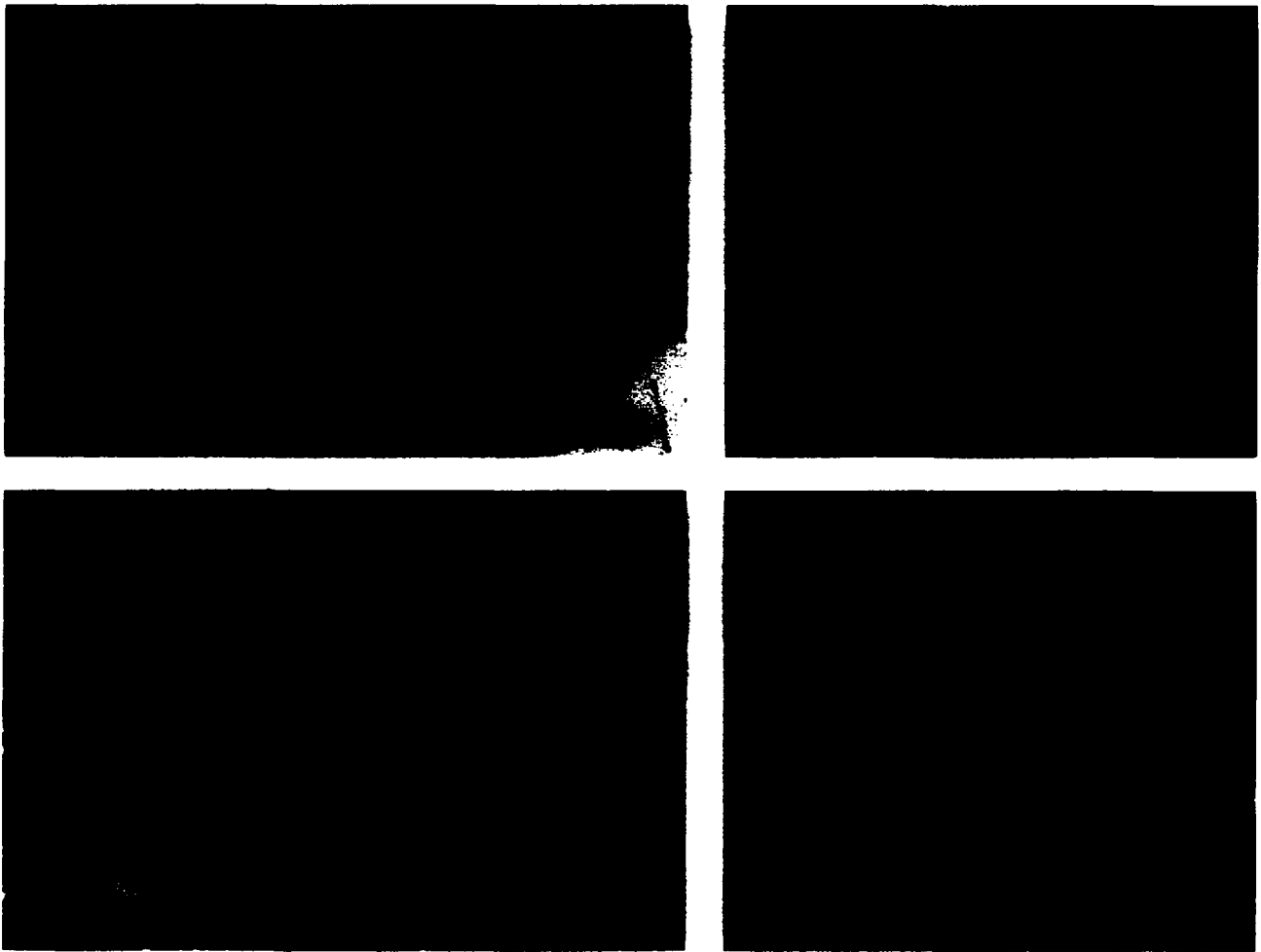


Fig. 7. Localization of NLRR-1 mRNA (A, B) and NLRR-2 mRNA (C, D) under dark-field illumination in the hippocampus (A, C) and the cerebellum (B, D) of the adult brain. Arrowheads (B, D) show the Purkinje cell layers of the cerebellum. CA1–CA4, CA1–CA fields of Ammon's horn; EC2, second layer of entorhinal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

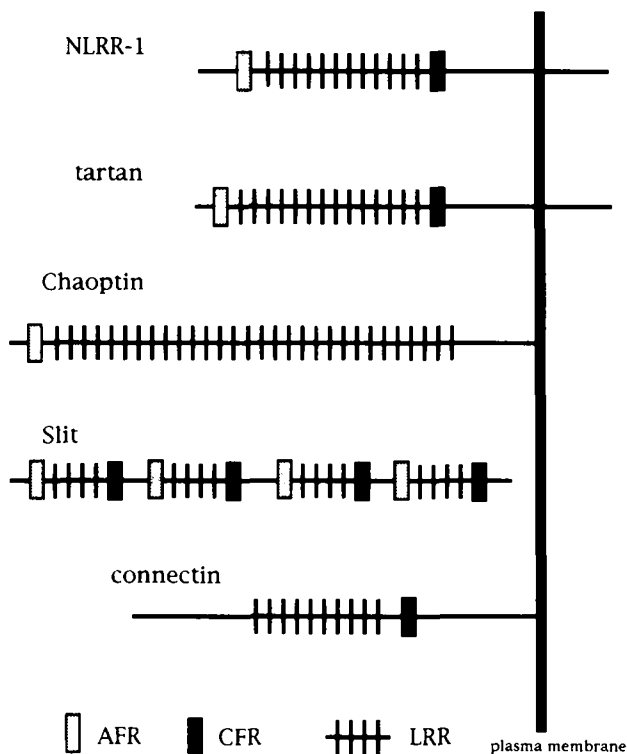


Fig. 8. Comparison of NLRR-1 protein structure to those of other LRR proteins known to function in the nervous system.

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.

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