Molecular Characterization and In Situ mRNA Localization of the Neural Recognition Molecule J1-160/180: a Modular Structure Similar to Tenascin

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Abstract. The oligodendrocyte-derived extracellular matrix glycoprotein J1-160/180 is a recognition molecule expressed exclusively in the central nervous system. J1-160/180 has been shown to be adhesive for astrocytes and repellent towards neurons and growth cones. We report here the complete nucleotide sequence of J1-160/180 in the rat. The predicted amino acid sequence showed a structural architecture very similar to tenascin: a cysteine-rich amino terminal region is followed by 4.5 epidermal growth factor-like repeats, 9 fibronectin type III homologous repeats and a domain homologous to fibrinogen. Sequence comparison analysis revealed highest homology of rat J1-160/180 to mouse tenascin and chicken restrictin with a similarity of 66% and 85%, respectively. The J1-160/180-coding mRNA is derived from a single copy gene. Using the polymerase chain reaction we could show that two J1-160/180 isoforms are generated by alternative splicing of the sixth fibronectin type III homologous repeat. Localization of J1-160/180 mRNA by in situ hybridization in the cerebellum, hippocampus and olfactory bulb confirmed the expression of J1-160/180 by oligodendrocytes with a peak of transcription at 7–14 d after birth, indicating a functional role during myelination. In addition, J1-160/180-specific RNA was found in a small subset of neurons in all three structures of the CNS analyzed. These neurons continue to express J1-160/180 in the adult.

Cell–cell and cell–extracellular matrix interactions have been shown to play crucial roles in the development and maintenance of a multicellular organism. The search for the molecular basis of these interactions led to the characterization of an increasing number of adhesion molecules (for reviews see Edelman, 1986; Jessell, 1988; Schachner, 1990, 1991). Recently, it has become clear that not only adhesive, but also inhibitory or repellent processes represent important features of active cell–cell and cell–extracellular matrix interactions (for review see Keynes and Cook, 1992). Several molecules have been identified which are involved in the inhibition of neural interactions, demonstrable as local paralysis of growth cone motility followed by growth cone collapse (Cox et al., 1990; Davies et al., 1990; Schwab, 1990). Both adhesive and repellent properties have been demonstrated for the extracellular matrix proteins tenascin and J1-160/180 (Pesheva et al., 1989; Spring et al., 1989; Faissner and Kruse, 1990; Morganti et al., 1990).

Tenascin, also known as hexabrachion, cytotactin and J1-200/220 (for review see Erickson and Bourdon, 1989), and J1-160/180 constitute members of the J1 family of extracellular matrix glycoproteins. The members of the J1 family were first described as neural adhesion molecules involved in the short-term interaction between neurons and glial cells (Kruse et al., 1985). In electron micrographs of rotary shadowed molecules, tenascin appears as hexamers (Erickson and Iglesias, 1984), whereas J1-160/180 consists of trimers (J1-180) and dimers or monomers (J1-160) (Pesheva et al., 1989). During development, there is a shift from the higher (tenascin) to the lower J1-160/180 molecular weight forms, resulting in hardly detectable levels of tenascin in the adult brain. Tenascin is expressed widely by fibroblasts, astrocytes, and Schwann cells (Gatchalian et al., 1989; Faissner et al., 1988; Seilheimer and Schachner, 1988; Bartsch et al., 1992), whereas J1-160/180 has been reported to be an oligodendrocyte-derived molecule (Pesheva et al., 1989). Beside these differences structural similarities between J1-160/180 and tenascin have been shown to exist by immunochemical methods (Faissner et al., 1988; Pesheva et al., 1989) and similar functional properties have been described in vitro (Faissner and Kruse, 1990; Pesheva et al., 1989; Taylor, J., P. Pesheva, M. Schachner, submitted for publication).

The similar functional and structural properties of tenascin and J1-160/180 led to the question, whether these similarities are due to common structural motifs. Therefore, we isolated a J1-160/180-specific cDNA clone from an expression library of rat spinal cord oligodendrocytes (Fuss et al., 1991). Here we report the complete coding sequence of rat J1-160/180 as determined from this cDNA clone and from a

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fragment obtained by polymerase chain reaction (PCR)[1] and encoding the 5′ end of the molecule. The predicted amino acid sequence revealed highest homology to the sequences of mouse tenascin (Weller et al., 1991) and chicken restrictin (Nörenberg et al., 1992), with a structural architecture common to all three molecules. The existence of at least two J1-160/180 isoforms, identified by PCR, suggests that, in analogy to the isoforms of tenascin, the difference between J1-160 and J1-180 may be due to alternative splicing. The localization of J1-160/180 mRNA in the cerebellum, hippocampus, and olfactory bulb by in situ hybridization confirmed the expression of J1-160/180 by oligodendrocytes. In addition, some neurons were shown to express J1-160/180–coding mRNA in all three brain regions that were analyzed. Some of these results have been reported previously in abstract form (Fuss and Schachner, 1991).

Materials and Methods

DNA Sequence Analysis

DNA was sequenced by the dideoxynucleotide chain-termination method described by Sanger (1977) with double-stranded Bluescript clones as templates using T7 DNA polymerase (Pharmacia LKB, Uppsala, Sweden). The sequence of the cDNA clone was determined by constructing nested unidirectional deletions using exonuclease III (Pharmacia LKB). Subsequently, parts of the sequence which were still missing were determined by using specific oligonucleotides as primers. For sequence analysis the UWGCG Sequence Analysis Software Package (Devereux et al., 1984) was used.

Polymerase Chain Reaction

Amplification of the 5′ end of the J1-160/180–coding sequence, which was missing in the cDNA clone originally described (Fuss et al., 1991) and amplification of the alternatively spliced FNIII repeat was achieved by the PCR (Saiki et al., 1988) on reverse transcribed RNA, synthesized with Moloney murine leukemia virus RNase H^- reverse transcriptase according to the manufacturer's instructions (Gibco/BRL, Basel, Switzerland).

For amplification of the 5′ end the following oligonucleotides were used: 5′-GGACATAGTCCAGTTGTCCTGTGGC-3′ (nucleotide [nt] 675-651 in Fig. 1) as primer for the single stranded cDNA synthesis; 5′-AACTTC-TGTCTGTATGTGTTCTAGC-3′, synthesized according to the sequence of the genomic mouse J1-160/180 clone (kindly provided by Dr. Dirk Montag, in this department) as 5′ primer and 5′-GCATCTCGATTCGGCTCAAC-3′ (nucleotides 589-570 in Fig. 1) as 3′-primer. The amplifying reaction was carried out in the presence of 1.5 mM $MgCl_2$ by repeating the cycle (93°C for 1 min, 55°C for 1 min, and 72°C for 3 min) 25 times.

For amplification of the alternatively spliced fibronectin type III (FNIII) repeat, the oligonucleotide 5′-GTGGAGAAGTTGGTGGCGAT-3′ (nt 3268-3249 in Fig. 1) was used as primer for single stranded cDNA synthesis, 5′-CTGACAGATCTAGAGCCTGGAGCAG-3′ (nt 2376-2400 in Fig. 1) as 5′-primer and 5′-CTTGGGTGGTCGATAGGATACTCGA-3′ (nt 2860-2839 in Fig. 1) as 3′-primer. The cycle (93°C for 1 min, 68°C for 1 min, and 72°C for 5 min) was repeated 35 times in the presence of 0.5 mM $MgCl_2$. Analysis of PCR products was performed after reannealing to remove artificial heteroduplices according to the method described by Wenger et al. (1991).

Southern Blot Analysis

Isolation of genomic DNA was performed in combination with the purification of RNA according to the method of Chirgwin et al. (1979; see also Sambrook et al., 1989) by omitting steps that could lead to sheared DNA. 15 μg DNA were used for digestion with various restriction enzymes each. The DNA fragments were separated on a 0.8% agarose gel and subsequently transferred to Biodyne A nylon membranes (Pall, Dreieich, Germany). For hybridization, a 122-bp fragment, representing nt 558-680 of the rat J1-160/180 sequence (see Fig. 1), was labeled with digoxigenin-11-dUTP according to the manufacturer's instructions (Boehringer Mannheim GmbH, Mannheim, Germany). Hybridization was carried out in 50% formamide at 42°C. The washing conditions were the following: two washings, each at room temperature, in 2× SSC (0.3 M NaCl/0.03 M Na-citrate, pH 7.5) containing 0.1% SDS; and two washings at 65°C in 0.5× SSC containing 0.1% SDS. For detection of bound DNA, antidigoxigenin antibodies coupled with alkaline phosphatase and the chemiluminescent substrate AMPPD (3-[2′-spiroadamantane]-4-methoxy-4-(3″-phosphoryloxy)-phenyl-1,2-dioxetane) were used as described (Boehringer Mannheim GmbH).

In Situ Hybridization

For in situ hybridization, digoxigenin-11-UTP-labeled cRNA probes (antisense and sense) were generated according to the manufacturer's instructions (Boehringer Mannheim GmbH) and hydrolyzed under alkaline conditions to obtain fragments of ~250 nt in length. The J1-160/180–specific cRNA probe represents the entire length of the cDNA clone originally isolated (Fuss et al., 1991). The cRNA probe specific for myelin-associated glycoprotein (MAG) represents the whole coding region of the 72-kD protein (Lai et al., 1987) and was kindly provided by Ulrich Dörries (this department). The cRNA probe specific for L1 (Moos et al., 1988) represents the amino-terminal part of the protein including the first 2.5-immunoglobulin (Ig)-like domains and was kindly provided by Hasan Mohajeri (this department). Negative controls, hybridized with the respective sense cRNA probes, showed no significant signals.

Fixation and hybridization of fresh-frozen cryostat sections was carried out as described previously (Bartsch et al., 1992). Briefly, sections were thaw mounted onto 3-aminopropyltriethoxy-silane–coated coverslips and fixed in 4% paraformaldehyde in PBS, pH 7.3. After treatment with 0.1 M HCl and subsequently with 0.1 M triethanolamine, pH 8.0, containing 0.25% acetic anhydride, sections were dehydrated in an ascending alcohol series. Prehybridization and hybridization were carried out in 50% formamide at 55°C. Washing conditions were as follows: two washings in 0.2× SSC and three washings in 0.1× SSC containing 50% formamide at 55°C. Detection of bound cRNA was performed by incubation with alkaline phosphatase–coupled antibodies to digoxigenin and subsequent development with NBT (4-nitroblue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3-indolylphosphate) in the presence of levamisol.

Cell Suspensions

Cerebella, hippocampi, and olfactory bulbs were carefully prepared from 2-wk-old rats, avoiding contamination with tissues adherent to these brain regions. Tissues were cut into pieces, incubated in Ca^{2+}- and Mg^{2+}-free (CMF) HBSS containing 0.1% DNase, 1% trypsin for 15 min at room temperature and resuspended in CMF-HBSS containing 0.05% DNAse. After pipetting up and down with a fire-polished glass pipette, cell suspensions were washed with CMF-HBSS and plated on glass coverslips coated with Cell-Tak (Collaborative Research Incorporated, Bedford, MA). After 2 h cells were fixed in PBS, pH 7.3, containing 4% paraformaldehyde and used for in situ hybridization and indirect immunofluorescence as described (Wintergerst et al., 1993). For indirect immunofluorescence, the mAb D3 (Schlosshauer, 1989) and a mixture of mAbs SMI-31 and SM-32 (Sternberger Monoclonals Inc., Baltimore, MD) were used, recognizing N-CAM-180, the neuron-specific isoform of N-CAM (Noble et al., 1985) and the phosphorylated and non-phosphorylated forms of neurofilament, respectively. Primary antibodies were visualized by FITC-conjugated goat anti-mouse (Dynatech Laboratories, Inc., Alexandria, VA) IgG antibodies.

Results

Sequence Analysis of J1-160/180

Isolation and identification of the rat cDNA clone encoding almost the whole coding region of J1-160/180 has been described previously (Fuss et al., 1991). The clone was identified as J1-160/180 specific by the following criteria: First, the fusion protein encoded by this cDNA clone is recognized by three different J1-160/180-specific mAbs; second, the cDNA clone contains the nucleotide sequence coding for an

1. *Abbreviations used in this paper*: CNS, central nervous system; FNIII, fibronectin type III; GABA, γ-amino butyric acid; J, J1-160/180; MAG, myelin-associated glycoprotein; nt, nucleotides; PCR, polymerase chain reaction.

amino acid sequence which is identical to the amino-terminal amino acid sequence derived from a tryptic peptide of the mouse J1-160 glycoprotein; third, Northern blot analysis revealed that the tissue distribution and developmental occurrence of the mRNA recognized by the cDNA clone is in agreement with previous immunocytochemical and immunochemical observations. The sequence of this cDNA clone is represented by nucleotides 509-6455 in Fig. 1. The missing amino-terminal sequence was determined by using PCR with a 5′ primer derived from the genomic mouse J1-160/180 sequence and reverse transcribed RNA from the brains of 15-d-old rats. The resulting PCR fragment of 603 bp was cloned into a Bluescript vector to determine the rat-specific amino-terminal sequence of J1-160/180. The predicted ATG start codon at nucleotide positions 159-161 (Fig. 1) is flanked by the most conserved nucleotides of the consensus sequence for translation initiation sites in eukaryotes (Kozak, 1984). This location is further confirmed by the existence of stop codons in all three reading frames further upstream. The location of the putative signal sequence and the first amino acid of the mature protein at residue 32 was predicted according to the method of von Heijne (1988) and from the hydrophobicity profile (Kyte and Doolittle, 1982; and data not shown) of the translated sequence which indicates one hydrophobic region at the amino terminus (residues 9-25; Fig. 1). A potential polyadenylation signal followed by a poly(A) tail could not be found within the 3′-untranslated sequence. The deduced amino acid sequence revealed a protein of 1,356 amino acid residues (Fig. 1) with a predicted molecular mass of 149 kD.

The J1-160/180 sequence is composed of different modules: a cysteine-rich amino-terminal region, followed by 4.5 EGF-like domains, 9 FNIII repeats, and a domain homologous to fibrinogen. This modular structure is very similar to the one described for tenascin in different species (Spring et al., 1989; Jones et al., 1989, Weller et al., 1991). Mouse tenascin shows an overall identity of 47% and a similarity of 66% to rat J1-160/180, whereby calculation of the similarity takes into account mismatches based on the evolutionary distance between the amino acid residues (Gribskov and Burgess, 1986). Comparison of the rat J1-160/180 sequence with the recently published sequence of the 170-kD extracellular matrix molecule chicken restrictin (Nörenberg et al., 1992) revealed an overall similarity of 85% and an identical modular structure.

It has been shown for tenascin, but not for restrictin that alternatively spliced FNIII repeats are inserted between the fifth and third last FNIII repeat (see Fig. 9) (Prieto et al., 1990; Gulcher et al., 1991; Siri et al., 1991; Weller et al., 1991). The alignment of the FNIII repeats of rat J1-160/180 showed that the first five repeats correspond to the first five and the last three correspond to the last three FNIII repeats of mouse tenascin, whereby each repeat in rat J1-160/180 is more homologous to the corresponding repeat in mouse tenascin than to one of its own repeats (Fig. 2). Similar results were obtained for chicken restrictin, where each FNIII repeat is more similar to a repeat in chicken tenascin than to one of its own repeats (Nörenberg et al., 1992). The only exception to this principle is represented by the sixth repeat of rat J1-160/180 which has no clear counterpart in tenascin. It showed highest homology to repeat three of mouse tenascin with a similarity of 49% and to the third last repeat of human tenascin with a similarity of 55%. Highest homology of the sixth repeat in rat J1-160/180 with any other FNIII repeat of J1-160/180 was found for repeat two with a similarity of 52%.

Characterization of Two Isoforms of Rat J1-160/180

The position of corresponding FNIII repeats in J1-160/180 and tenascin suggested the existence of alternatively spliced FNIII repeats in J1-160/180 also. To characterize putative J1-160/180 isoforms generated by alternative splicing of FNIII repeats, PCR was performed using oligonucleotides priming in the fifth and seventh repeat of J1-160/180 and reverse transcribed RNA from brains of 17-d-old embryonic, 15-d-old postnatal, and adult rats. After reannealing three PCR products were visible at all stages tested (Fig. 3 *A*). The largest product (482 bp) corresponded in size to the product derived from the sequence that is represented in the isolated cDNA clone (Fig. 3 *A*, lanes *100 fg*, *A*, *P15*). Sequence analysis of the 215-bp product revealed an isoform missing the sixth FNIII repeat of J1-160/180 (Fig. 3 *B*). The additional PCR product of 258 bp showed no significant homology to the J1-160/180 sequence, but turned out to be identical to the 5′ sequence of rat dynamin-1, a GTP-binding protein (Obar et al., 1990).

These results demonstrate that rat brain contains two J1-160/180 isoforms derived from a single copy gene (Fig. 4) and generated by alternative splicing of a FNIII repeat at all ages studied.

Localization of J1-160/180 mRNA in the Cerebellum, Hippocampus, and Olfactory Bulb by In Situ Hybridization

Previous immunocytological and immunohistological investigations showed that J1-160/180 is associated with oligodendrocytes in vitro and is predominantly detectable in myelinated regions of the CNS in vivo (Pesheva et al., 1989; Morganti et al., 1990). To elucidate whether this immunocytochemical distribution of the secreted J1-160/180 glycoprotein reflects the previously concluded synthesis of J1-160/180 mRNA by oligodendrocytes, in situ hybridization experiments were performed using the immunohistochemically well-characterized cerebellum (Pesheva et al., 1989). Only few J1-160/180–positive cells were detectable in the cerebellum of neonatal rats, predominantly situated at the base of the cerebellum and in the proximal part of prospective white matter tracts extending into the folia (Fig. 5 *A*). A similar distribution of positive cells was observed after in situ hybridization with the MAG-specific probe (Fig. 5 *B*). More J1-160/180- than MAG-positive cells were detectable (compare Fig. 5, *A* and *B*). At 7 and 14 days after birth J1-160/180 mRNA containing cells were detectable along the entire length of the developing white matter tracts and in the internal granular layer (Fig. 5, *D* and *G*) in a distribution comparable to that of MAG (Fig. 5, *E* and *H*). For both J1-160/180 and MAG mRNA, there is an increase in the number of positive cells between postnatal days 7 and 14. In the adult, however, fewer cells were J1-160/180 than MAG mRNA positive in white matter tracts and in the internal granular layer, indicating that J1-160/180 expression in mature oligodendrocytes is down regulated. The cellular localization of J1-160/180 mRNA in developing and adult white matter supports the formerly suggested oligodendrocyte-

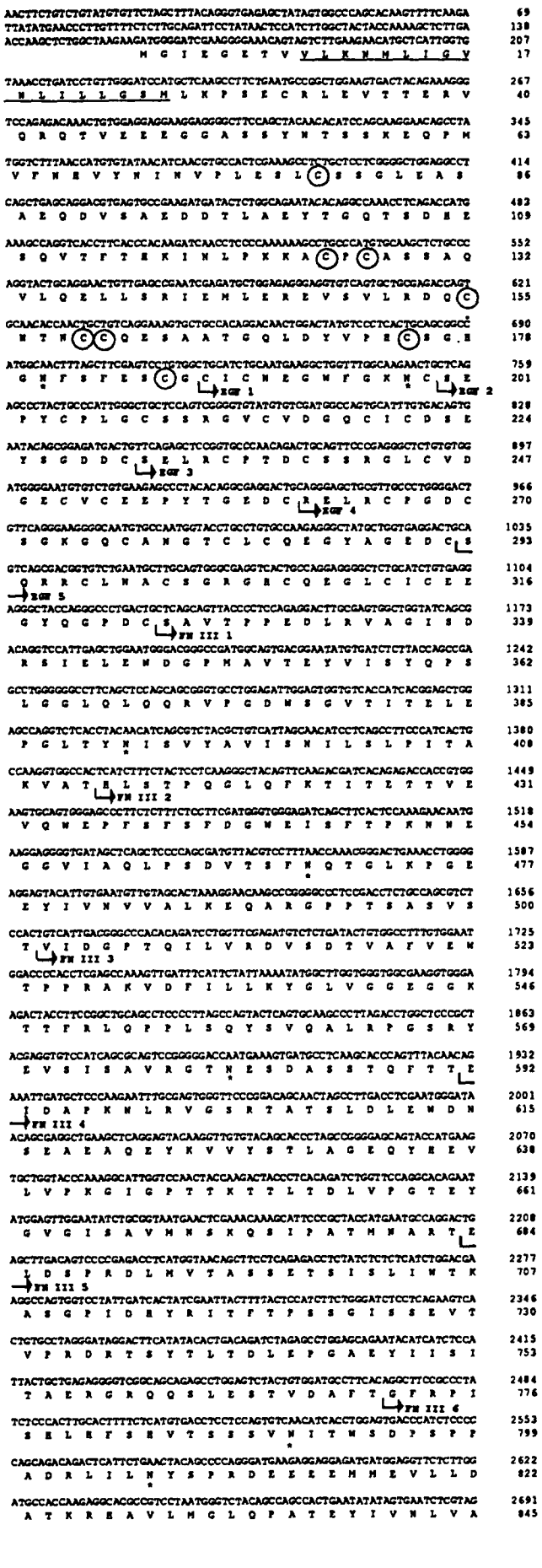


Figure 2. Alignment of the constitutive FNIII repeats between rat J1-160/180 (*rJ*) and mouse (*mTN*) and human (*hTN*) tenascin. The RGD in the third FNIII repeat of the human sequence is marked by a surrounding box. Conserved amino acid residues are given in the last row and marked by a star. The alignment was performed with the program "pileup" of the UWGCG DNA analysis software package.

specific expression of J1-160/180. No expression by Golgi epithelial cells could be detected at any stage. These cells represent a subclass of cerebellar astrocytes which can be unequivocally identified by the position of their cell bodies, intercalated among the unlabeled cell bodies of Purkinje cells.

Synthesis of J1-160/180 mRNA was, however, not restricted to oligodendrocytes. J1-160/180 mRNA-positive cells were also seen in the molecular layer and first detectable at 7 d after birth in a row of cells just above the Purkinje cell layer (Fig. 5 *D*). At 14 d after birth, the hybridization signal spread more upwards in the molecular layer towards the meninges (Fig. 5 *G*). In the adult, J1-160/180-positive cells covered almost the whole molecular layer, fading slightly towards the meninges (Fig. 5, *K* and *L*). These J1-160/180-positive cells in the molecular layer showed a similar distribution as L1-positive cells in adjacent sections (compare Fig. 5, *G* and *I*). Since L1 is specifically expressed by postmitotic neurons (Rathjen and Schachner, 1984; Persohn and Schachner, 1987) and since no other cell types have their cell bodies in the molecular layer, the position, size, and density of the labeled cells unequivocally identifies them as the basket and stellate cells, the small inhibitory interneurons of the cerebellar cortex (Palay and Chan-Palay, 1974; Altman, 1982). In contrast to oligodendrocytes, expression of J1-160/180 mRNA in these neuronal cells was not downregulated in the adult (Fig. 5 *K*, *L*). Purkinje and granule cell neurons were J1-160/180 mRNA negative at all developmental stages studied.

To further investigate the cell type-specific expression of J1-160/180, we performed in situ hybridization experiments with two other, anatomically well characterized structures of the CNS, the hippocampus, and olfactory bulb. Studies were carried out at different postnatal stages, including the adult. In the following, only the results observed in 14-d-old animals will be documented.

J1-160/180-positive cells in the fimbria and fornix, which are composed of myelinated fibers, were already detectable in 2- and 6-d-old animals (not shown) in a distribution similar to the one of MAG-positive cells in 14-d-old animals (Fig. 6, *E* and *F*). No MAG expressing cells were visible at the younger developmental stages. In 14-d-old animals there were more J1-160/180-positive than MAG-positive cells in the white matter of fimbria and fornix (compare Fig. 6, *A* and *B* with *E* and *F*). In the adult, a reduction in number of J1-160/180-positive cells were seen (not shown).

Figure 1. DNA and deduced amino acid sequence of J1-160/180. The hydrophobic region corresponding to the putative signal sequence is underlined. The beginning of the homologous repeats, EGF-like domains (*EGF 1-5*), FNIII repeats (*FNIII 1-9*) and the domain homologous to fibrinogen (*FG*), are indicated by arrows. The cysteine residues in the amino terminal region are encircled. The terminal stop codon (*TGA*) is marked by a surrounding box. Potential *N*-glycosylation sites are marked by a star. These sequence data at the nucleotide level are available from EMBL/GenBank/DDBJ under accession number Z18630.

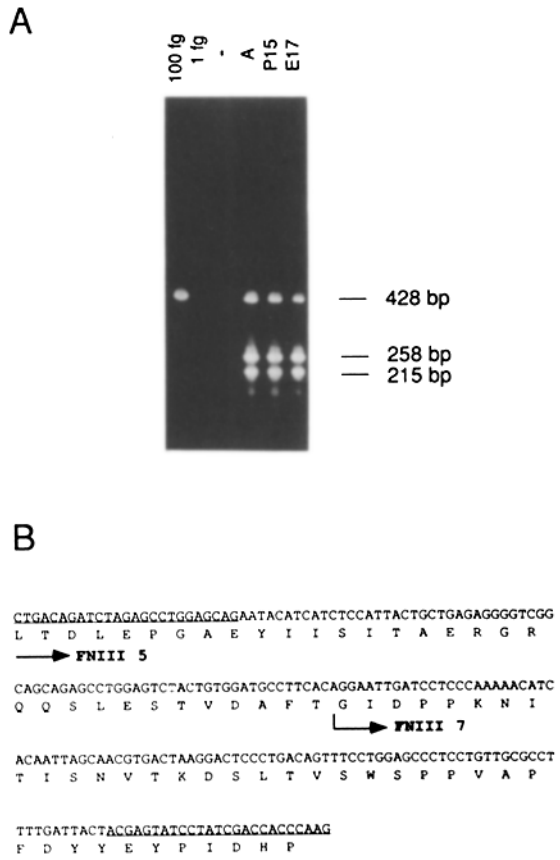


Figure 3. Alternative splicing of a FNIII repeat in Jl-160/180. The existence of an alternatively spliced FNIII repeat could be demonstrated by PCR using the oligonucleotides represented by the underlined sequences in *B* and total RNA from 17-d-old embryonic (*A*, *E17*), 15-d-old (*A*, *P15*) and adult rats (*A*, *A*) as indicated above each lane. Control amplifications were performed without template (*A*,−) and with 1 fg (*A*, *1 fg*), and 100 fg (*A*, *100 fg*) of the isolated cDNA clone as template. The amplification products were analyzed after reannealing. In *A*, the 1.5% agarose gel of these products, stained with ethidium bromide is shown. The molecular weights of the fragments obtained are marked at the right margin in base pairs (*bp*). The nucleotide and derived amino acid sequences of the 215-bp PCR product is shown in *B*. The arrows mark the FNIII repeats represented by the sequence according to the numerical order given in Fig. 1.

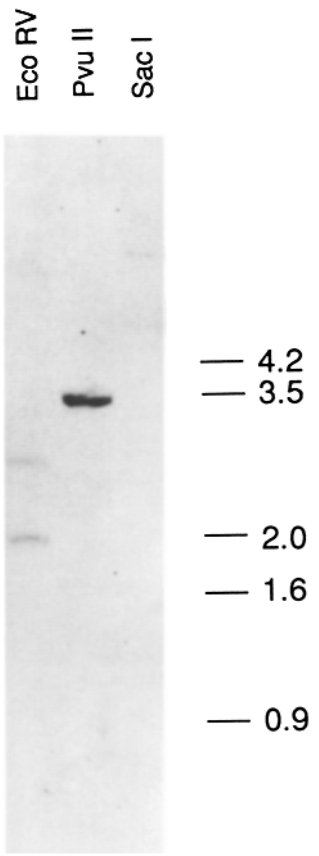
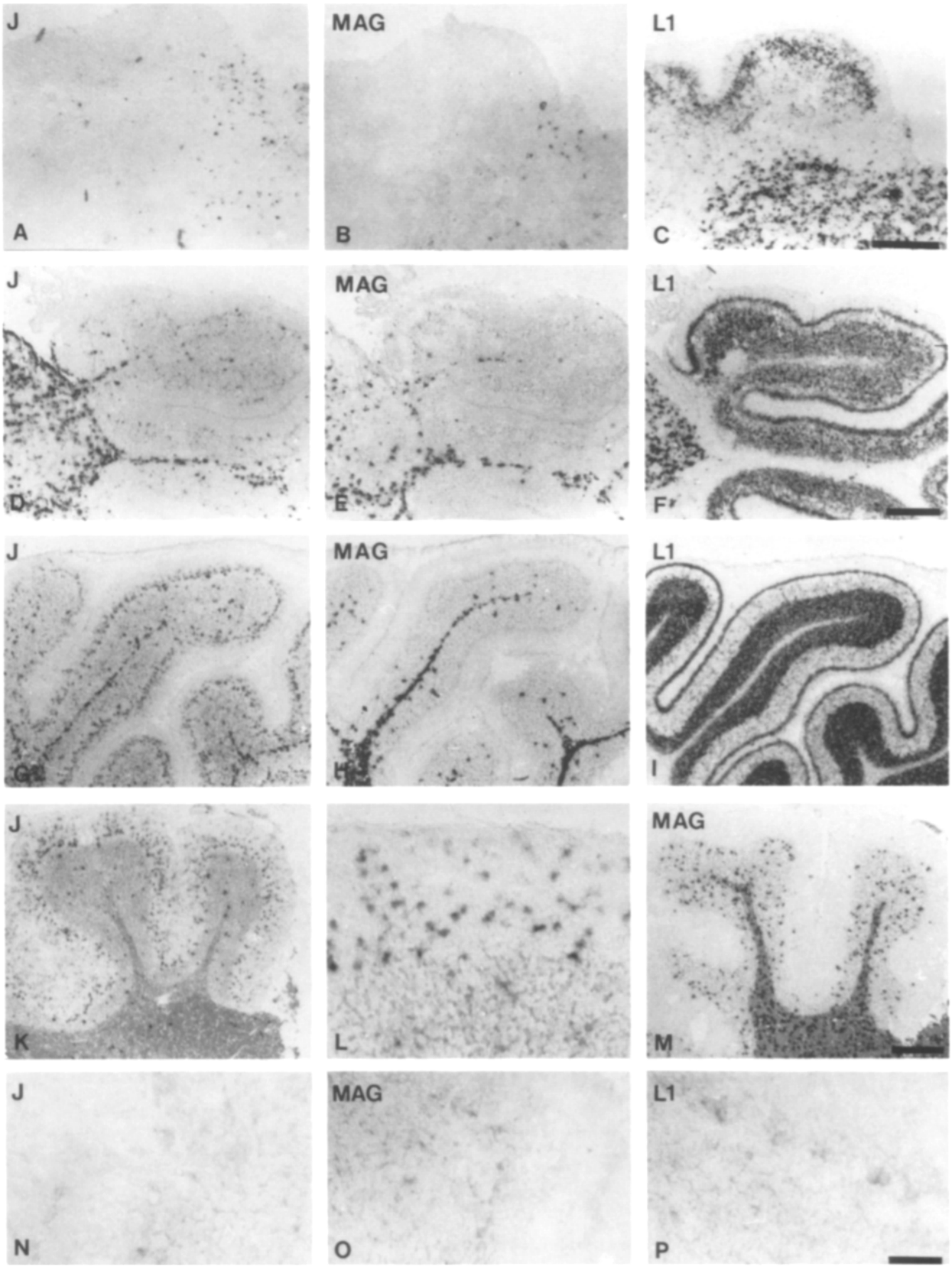


Figure 4. The Jl-160/180–specific cDNA is derived from a single copy gene. Total genomic rat DNA was digested with the Eco RV, Pvu II, and Sac I as indicated above each lane. Hybridization was performed with a digoxigenin-11-dUTP–labeled 122-bp fragment representing nucleotides 558-680 of the rat Jl-160/180 sequence shown in Fig. 1. Specifically bound, labeled DNA was detected by chemiluminescence. The molecular weight markers are indicated at the right margin in kb.

Pyramidal cells of the hippocampal fields CA1, CA2, CA3, and granule cells of the dentate gyrus were Jl-160/180 mRNA-negative at all developmental stages tested. However, some cells in the hilus of the dentate gyrus became Jl-160/180 mRNA positive in 6-d-old animals, a developmental stage where the dentate gyrus becomes a distinct anatomical structure (Altman and Bayer, 1975). These cells are also Jl-160/180 mRNA positive in 14-d-old (Fig. 6, *A*, *B*, and *H*) and adult animals (not shown) in a distribution similar to the one of Ll-positive neurons (compare Fig. 6, *H* and *G*). Since the Jl-160/180–positive hilar region of the dentate gyrus is popu-

Figure 5. Localization of Jl-160/180 (J)- (*A*, *D*, *G*, *K*, and *L*), MAG- (*B*, *E*, *H*, and *M*), or Ll- (*C*, *F*, and *I*) specific mRNA in the cerebellum of neonatal (*A–C*), 7-d-old (*D–F*), 14-d-old (*G–I*), and adult (*K–M*) rats. In neonatal rats, cells expressing Jl-160/180 are restricted to the base of the cerebellum (*A*) and have penetrated the developing cerebellum more deeply than cells containing MAG transcripts (*B*). In 1-wk-old rats, numerous Jl-160/180- (*D*), and MAG- (*E*) positive cells are present at the base of the cerebellum and along the entire length of developing white matter tracts of the folia. In difference to MAG positive cells, Jl-160/180 positive cells are also detectable in the internal granular layer. In the cerebellar cortex of 2-wk-old animals, a Jl-160/180 signal becomes visible in the developing molecular layer (*G*). In contrast to the Ll (*I*) signal present in basket and stellate cells, the Jl-160/180 signal appears to be more restricted to cells located just above the Purkinje cell layer. Cells expressing MAG (*H*) are present in the internal granular layer, although not as numerous as cells expressing Jl-160/180. In adults, the number of cells containing Jl-160/180 (*K*) transcripts and their labeling intensity decreased in white matter tracts and in the internal granular layer. In contrast, the number of labeled cells located in the molecular layer (*K*) increased when compared with 2-wk-old animals (*G*). They were detectable over the entire width of the molecular layer (*L*) with a signal intensity similar to that observed in 2-wk-old animals. Sections incubated with Jl-160/180- (*N*), MAG- (*O*), or Ll- (*P*) sense cRNA probes showed no labeling. Bars: (*A–C*, and *D–F*) 300 μm; (*G–I*, *K*, and *M*) 400 μm; and (*L*, and *N–P*) 100 μm.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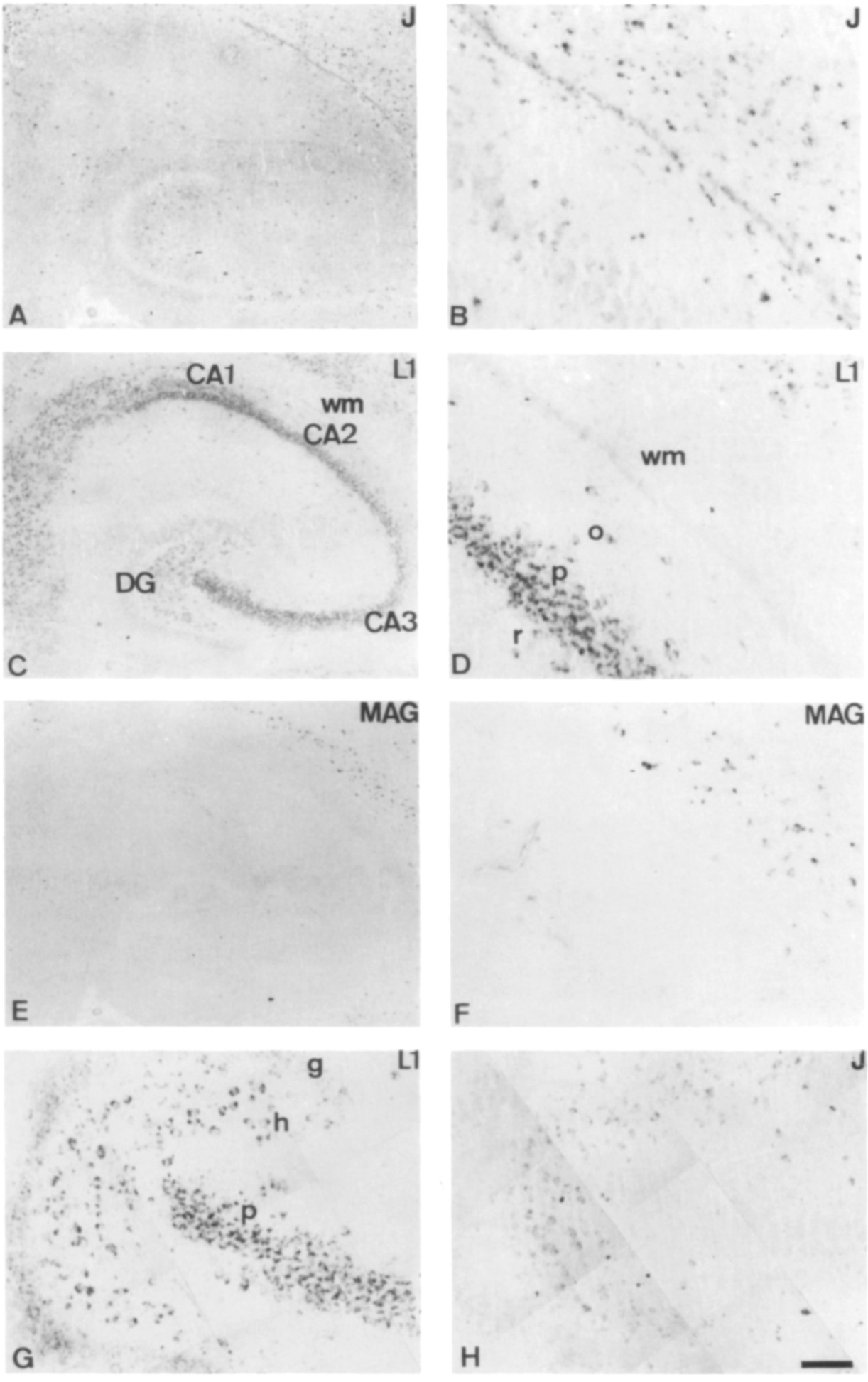
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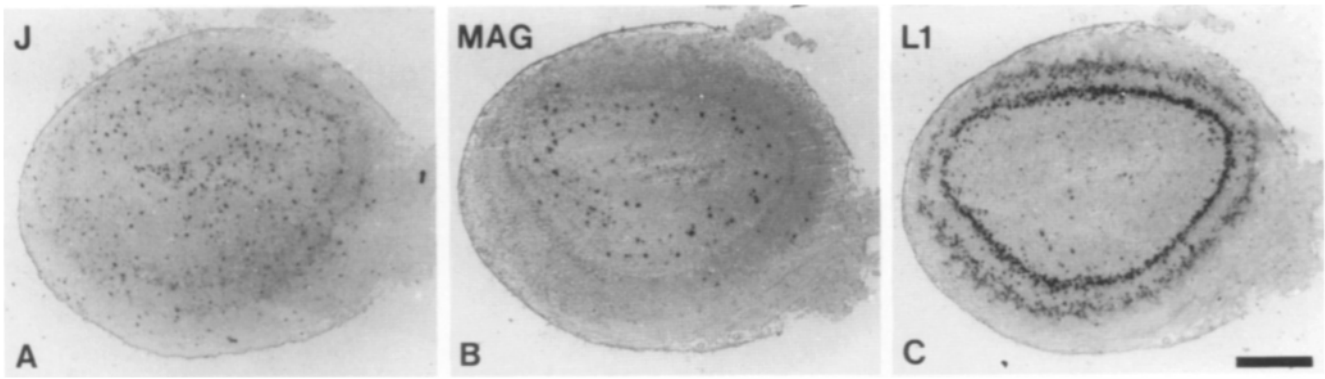

Figure 7. Localization of J1-160/180 (J)- (*A*), MAG- (*B*), and L1- (*C*) specific mRNA in consecutive sections in the olfactory bulb of 14-d-old rats by in situ hybridization. Cells that are strongly labeled with the J1-160/180 cRNA probe are mainly located in the internal granular layer whereas only few labeled cells are visible above the mitral cells layer (*A*) with a distribution very similar to that of MAG expressing cells (*C*). In addition, cells that are weakly labeled with the J1-160/180 probe are located in the mitral cell layer, external plexiform layer and in a periglomerular position (*A*). The distribution of these cells is similar to that of cells expressing L1-specific mRNA (*C*). Bar, 0.5 mm.

lated by several neuronal cell types (Amaral and Witter, 1989), a more precise characterization by solely anatomical criteria was not possible. A few J1-160/180–positive signals were also detectable in hippocampal layers below and superficial to the pyramidal cell layer, the stratum oriens, stratum radiatum, and stratum lacunosum-moleculare (Fig. 6, *A* and *B*). The distribution of these cells was comparable to that of L1-positive cells in adjacent sections (compare Fig. 6, *A*, *B*, and *H* with *C*, *D*, and *G*, respectively). Thus, we conclude that not only in the cerebellum, but also in the hippocampus J1-160/180 is not exclusively expressed by oligodendrocytes, but also by certain subtypes of neurons. As in the cerebellum, the neuron-derived expression of J1-160/180 mRNA was not down-regulated in the adult (not shown).

In the olfactory bulb of neonatal rats, cells expressing J1-160/180 were only weakly labeled and located mainly in the mitral cell layer with a distribution of the hybridization signal similar to that of L1 (not shown). Few cells were labeled in the internal and external granular layers (not shown). No cells were labeled with the MAG anti-sense cRNA probe in the olfactory bulb of neonatal rats (not shown). In the olfactory bulb of 14-d-old rats, cells strongly expressing J1-160/180 mRNA became visible in the internal granular layer (Fig. 7 *A*). Few strongly labeled cells were also detectable above the mitral cell layer, in the external plexiform layer and in the glomerular layer. In general, cells that were strongly labeled with the J1-160/180 anti-sense cRNA probe were distributed in a pattern similar to that of cells containing MAG transcripts (Fig. 7 *C*). As in the white matter tracts of the cerebellum and the fimbria and fornix of the hippocampus there were more J1-160/180- than MAG-positive cells detectable at this stage.

Cells that were weakly labeled with the J1-160/180 probe were located in the mitral cell layer, the external plexiform layer and in a periglomerular position (Fig. 7 *A*) with a distribution similar to that of cells containing L1 transcripts (Fig. 7 *B*). This indicates that the weakly labeled cells correspond to neurons. Since there is no neuronal cell type known that shows this characteristic distribution, it is not possible to determine the identity of the J1-160/180 mRNA containing neuronal cell types. The distribution of cells containing the transcripts of J1-160/180, L1 or MAG in adult animals was similar to that of 14-d-old animals, except that the number of labeled cells was reduced mainly in the granular layer (not shown), indicating that the oligodendrocyte-derived, but not the neuron-derived expression of J1-160/180 is down regulated in the adult.

Characterization of J1-160/180 Expressing Cell Types In Vitro

To further investigate the cell type–specific expression of J1-160/180 in the three regions of the CNS by in situ hybridization, we hybridized acutely dissociated single cell suspensions from the cerebellum, hippocampus, and olfactory bulb of 14-d-old rats with the J1-160/180–specific cRNA probe and identified the neurons by indirect immunofluorescence with neuron-specific antibodies. We chose antibodies specific for N-CAM 180, the neuron-specific isoform of the neural cell adhesion molecule, and neurofilament, since in contrast to L1, the astrocyte-specific marker GFAP and the

Figure 6. Localization of J1-160/180 (J)- (*A*, *B*, and *H*), MAG- (*E* and *F*), and L1- (*C*, *D*, and *G*) specific mRNA in consecutive sections of the 14-d-old rat hippocampus by in situ hybridization. In *B*, *D*, and *F* the hippocampal field CA2 and in *G* and *H* the denate gyrus (*C*, *DG*) are presented in higher magnification. Labeled cells are detectable in the white matter (*C*, *wm*) with the J1-160/180- and MAG-specific probe, whereby more cells are J1-160/180- than MAG-positive (compare *A* and *B* with *E* and *F*). In addition, there are J1-160/180–positive signals detectable in a distribution similar to the one of L1-positive cells in the hilar region (*G*, *h* compared to *H*) of the dentate gyrus, the stratum oriens (*D*, *o* compared to *B*) and the stratum radiatum of the hippocampus (*D*, *r* compared to *B*). Pyramidal cells of the pyramidal layer (*D* and *G*, *p* compared to *B* and *H*), and granule cells of the granular layer (*G*, *g* compared to *H*) of the dentate gyrus are J1-160/180-negative. Bar: (*A*, *C*, and *E*) 360 μm; (*B*, *D*, and *F*) 90 μm; and (*G* and *H*) 15 μm.

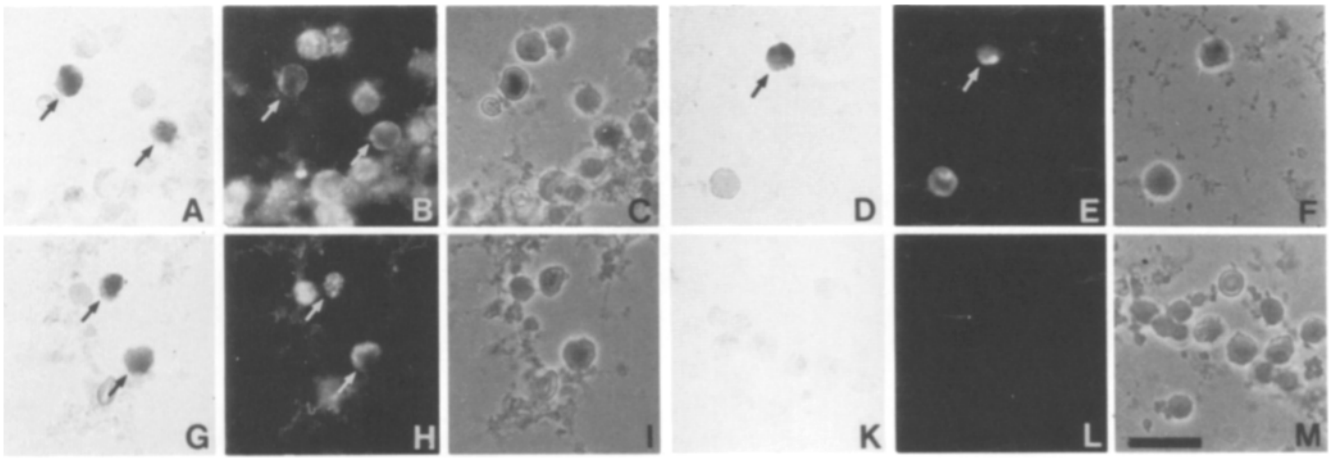

Figure 8. Identification of J1-160/180 mRNA expressing cell types by in situ hybridization in acutely dissociated single cell suspensions of cerebellum (*A–C*), hippocampus (*D–F*), and olfactory bulb (*G–I*) of 14-d-old rats using antibodies to neurofilament by indirect immunofluorescence (*A–I*). Cells from the three brain regions contained cells expressing J1-160/180 mRNA (*A*, *D*, and *G*, *arrows*) and some but not all J1-160/180 expressing cells were also neurofilament positive (*B*, *E*, and *H*, *arrows*). The less uniform labeling pattern of neurofilament immunofluorescence of J1-160/180 mRNA positive cells is the result of quenching by the alkaline phosphatase reaction product. This is evident from the complementarity of the two labels. Cell suspensions incubated with J1-160/180 sense cRNA (*K*) or cell suspensions in which primary antibodies were omitted (*L*) showed no labeling. *C*, *F*, *I*, and *M* represent the phase contrast microphotographs to corresponding in situ hybridization and immunofluorescence images shown in *A* and *B*, *D* and *E*, *G* and *H*, and *K* and *L*, respectively. Bar, 25 μm.

oligodendrocyte-specific marker MAG the epitopes recognized by these antibodies were not destroyed or did not disappear during the treatment used for preparation of the single cell suspensions.

In the single cell suspensions of all three CNS regions, there were always some cells detectable that contained J1-160/180 mRNA (Fig. 8, *A*, *D*, and *G*). Many of these labeled cells were at the same time N-CAM 180- (not shown) or neurofilament immunoreactive (Fig. 8, *B*, *E*, and *H*), indicating that these cells represent nerve cells. These results unequivocally confirm that J1-160/180 is not only synthesized by oligodendrocytes, but also by neurons.

Discussion

Primary Structure of J1-160/180

As expected from previous immunochemical studies (Kruse et al., 1985; Faissner et al., 1988), J1-160/180 and tenascin are structurally very similar, with 47% identity and 66% similarity between rat J1-160/180 and mouse tenascin. The overall molecular architecture of both molecules is identical in that the putative signal peptide is followed by a cysteine-rich amino-terminal region, EGF-like domains, FNIII repeats, and a calcium binding domain homologous to fibrinogen (Fig. 9). The lower molecular weight of J1-160/180 can be explained by the smaller numbers of EGF-like domains which amount to 4.5 in rat J1-160/180 and to 14.5 in mouse tenascin (Weller et al., 1991). The recently published sequence of chicken restrictin (Nörenberg et al., 1992) showed an overall similarity of 85% to rat J1-160/180 and an identical modular structure. As shown for the FNIII repeats in tenascin of different species, each FNIII repeat in rat J1-160/180 is more homologous to the corresponding one in chicken restrictin than to one of its own repeats, suggesting that rat J1-160/180 and chicken restrictin represent species homologs. In agreement with the extracellular localization of J1-160/180, sequence analysis predicts a protein without a transmembrane domain. For tenascin, it was suggested by protein structure predictions that hydrophobic heptad peptides in the amino terminal part of the protein are directly involved in the association of the monomeric polypeptide chains with each other to form a hexameric complex (Spring et al., 1989). For both tenascin and J1-160/180, cysteine residues were suggested to participate in the stabilization of the multimeric complex by forming disulfide bridges (Spring et al., 1989; Pesheva et al., 1989). The cysteine residues and three hydrophobic heptad peptides (residues 134-155) are located in the amino-terminal sequence of J1-160/180 at the same positions as in the tenascin sequence (Fig. 9). It is therefore likely that these structures are also involved in the formation of the trimers and dimers of J1-160/180.

J1-160/180 Isoforms Generated by Alternative Splicing

Two J1-160/180 isoforms are generated from one single copy

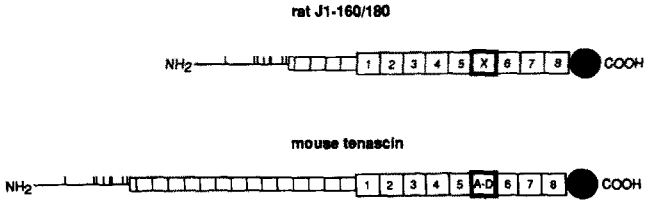

Figure 9. Schematic representation of the modular structure of rat J1-160/180 in comparison to mouse tenascin. The cysteine residues in the amino-terminal region are indicated by vertical lines. EGF-like domains are symbolized by small boxes, FNIII repeats by large boxes, and the domain homologous to fibrinogen by a filled circle. The bold-bordered box within the FNIII repeats represents alternatively spliced FNIII repeats.

gene by alternative splicing of a FNIII repeat located between the fifth and seventh repeat. The position of the alternatively spliced FNIII repeat and the fact that each of the constitutive FNIII repeats of J1-160/180 is most homologous to the corresponding one in tenascin may suggest that J1-160/180 and tenascin are derived from a common ancestor during evolution. Unexpectedly, the alternatively spliced sixth repeat in J1-160/180 did not reveal highest homology with one of the alternatively spliced FNIII repeats of tenascin from mouse (Weller et al., 1991) and human (Gulcher et al., 1991). Because of the structural similarities between J1-160/180 and tenascin in alternative splicing of FNIII repeats, we would like to introduce a nomenclature of the FNIII repeats in J1-160/180 as recently described by Vrucinic-Filippi and Chiquet-Ehrismann (1992) for tenascin, where the constitutively expressed repeats are designated by numerals and the alternatively spliced ones by capital letters. Since there is no counterpart for the sixth J1-160/180 FNIII repeat in tenascin, we termed it FNIII repeat X. This repeat was not found to be significantly more similar to any other J1-160/180 repeat than to those in tenascin and it seems, therefore, unlikely that it was generated through a recent reduplication of an FNIII unit as suggested for four of the alternatively spliced FNIII repeats in tenascin (Gulcher et al., 1990). The result of this comparison could indicate that the alternatively spliced repeats are either not highly conserved or that in tenascin additional, yet undiscovered alternatively spliced repeats exist. In case of the first alternative, it is possible that the alternatively spliced FNIII repeat in J1-160/180 may code for functional properties that distinguish J1-160/180 from tenascin.

It is at present difficult to determine the relationship between the isoforms described at the protein level with an apparent molecular weight of 180 and 160 kD and the two isoforms found at the DNA level, with calculated molecular masses of 149 and 139 kD. Removal of N-linked carbohydrates by glycopeptidase F treatment yielded protein bands at ~160 and 140 kD by SDS-PAGE (B. Fuss and P. Pesheva, unpublished results). Since *O*-glycosidically bound oligosaccharides have been suggested for J1-160/180 (Wing et al., 1992), it is possible that these are responsible for the difference between the calculated molecular masses and the apparent molecular weights of the glycopeptidase F-treated proteins. We therefore propose that the 160-kD glycoprotein isoform corresponds to the smaller DNA-derived isoform and that the larger glycoprotein isoform of 180 kD may then correspond to the larger DNA-derived isoform.

The existence of additional J1-160/180 isoforms generated by alternative splicing cannot be excluded and it is still unclear whether the glycoprotein component of ~220 kD, known to copurify with the 160 and 180 kD components of J1-160/180 (Pesheva et al., 1989), represents such an isoform. Additionally, alternative splicing may occur in the amino-terminal portion of the protein, since it is likely that the difference in the association of the monomeric arms of J1-160/180, resulting in the structural appearance of trimers, dimers and monomers, could be generated by different amino terminal structures. Attempts to determine the amino-terminal sequences of the two major J1-160/180 glycoprotein isoforms (160 and 180 kD) by protein sequencing have so far failed, most probably due to blocked amino termini. Thus, it remains to be conclusively shown which alternatively spliced regions are represented in the different glycoprotein isoforms and whether alternative splicing alone is responsible for generating the different J1-160/180 isoforms.

For restrictin, alternative splicing in the cysteine-rich amino-terminal part was described, suggesting that the generation of these isoforms plays an important role in the modification of multimerization, i.e., formation of trimers and dimers, observed for J1-160/180 and restrictin by electron microscopic analysis of rotary shadowed molecules (Pesheva et al., 1989; Nörenberg et al., 1992). Because of the genomic organization of J1-160/180 (D. Montag, unpublished observations) it appears unlikely that isoforms differing in the amount of amino-terminal cysteine residues are generated by alternative splicing in J1-160/180 at the same position as in restrictin. Alternative splicing of FNIII repeats has not been described for restrictin. It remains to be seen whether both types of isoforms are generated in both species and whether additional isoforms exist.

Cellular Synthesis of J1-160/180

For a secreted extracellular matrix protein, such as J1-160/180, it is not only important to know which cells carry detectable levels of the protein, but also which cell types synthesize the molecule. The J1-160/180 glycoprotein has so far been detected immunocytochemically in association with oligodendrocytes and white matter tracts in the central nervous system (Pesheva et al., 1989; Morganti et al., 1990). In agreement with these observations, the present study has localized J1-160/180–specific mRNA in cells with a similar distribution as the MAG-expressing oligodendrocytes (Sternberger et al., 1979). The spatio-temporal appearance of J1-160/180–expressing cells in the prospective white matter and in the white matter tract of the developing and adult cerebellum closely resembles the immigration of oligodendrocytes from the subependymal layer of the 4th ventricle via the superior medullary velum and the cerebellar peduncles (Reynolds and Wilkin, 1988), further confirming the oligodendrocyte-derived expression of J1-160/180. Interestingly, there is the tendency at earlier developmental stages for more cells showing detectable levels of J1-160/180 coding mRNA than for cells showing MAG coding mRNA. Thus, in the three brain regions analyzed expression of J1-160/180 in oligodendrocytes precedes the expression of MAG. The oligodendrocyte-derived expression of J1-160/180 is highest between the second and third postnatal week and low in the adult. These findings are in agreement with in vitro studies demonstrating that oligodendrocyte precursor cells and immature oligodendrocytes express J1-160/180 before they express MAG, whereas J1-160/180 expression decreases with increasing maturity of the oligodendrocytes (Jung et al., submitted for publication). The observations are also consistent with the results obtained by in situ hybridization in the optic nerve and spinal cord (Wintergerst et al., 1993), by immunocytochemistry and immunochemistry in the retina and optic nerve (Bartsch, U., and P. Pesheva, manuscript submitted for publication) and by Northern blot analysis of CNS RNA (Fuss et al., 1991). The synthesis of J1-160/180–specific mRNA coinciding with the time of active myelination is suggestive of an important role of J1-160/180 during myelination (for further discussion see Bartsch et al., submitted for publication).

Astrocytes, with the exception of type-2 astrocytes which

are present in optic nerve cell cultures and of which the existence in vivo is still a matter of discussion (Noble, 1991), have so far never been seen to synthesize J1-160/180, neither by immunocytochemical nor by in situ hybridization studies (Pesheva et al., 1989; Wintergerst et al., 1993; Jung, M., P. Pesheva, M. Schachner, and J. Trotter, manuscript submitted for publication).

J1-160/180 is not only expressed by oligodendroglial cells, but also by subpopulations of neurons. The neuronal expression of J1-160/180 could be demonstrated in tissue sections of brain regions with unequivocally identifiable localization of neuronal cell bodies, such as the cerebellum, hippocampus, and olfactory bulb, and by comparison of the pattern of distribution of J1-160/180–positive cells with that of cells expressing the neuronal marker L1 and the oligodendroglial marker MAG. Furthermore, acutely dissociated single cell suspensions of these brain regions were double labeled with the J1-160/180 anti-sense RNA by in situ hybridization and with the neuronal markers N-CAM180 and neurofilament by indirect immunofluorescence. These experiments clearly demonstrated that J1-160/180 is expressed by some subtypes of neurons in the three brain regions analyzed. The presence of J1-160/180–specific mRNA in the cerebellar stellate and basket cells was first detectable during the first postnatal week at a time where these cells are born and differentiate (Altman, 1982). In the hippocampus and olfactory bulb, neuronal cell types expressing J1-160/180 could be identified by their distribution being similar to that of L1 expressing nerve cells. However, a more precise identification of the neuronal subtypes expressing J1-160/180 by anatomical criteria was not as unequivocal as in the cerebellum, since in the hippocampus J1-160/180–positive neurons are located in a region of high diversity in neuronal cell types (Amaral and Witter, 1989) and in the olfactory bulb the distribution of J1-160/180-positive neurons is not in conformity with the main neuronal cell types. Nevertheless, our observations indicate, that only subpopulations of neurons express J1-160/180. It is presently not clearly apparent why certain subpopulations of neurons synthesize J1-160/180 in different regions of the CNS. Whether particular lineage relationships between oligodendrocytes and subpopulations of neurons, as suggested during neurogenesis in the cerebral cortex (Williams et al., 1991), are instrumental in the expression of J1-160/180 by such diverse cell types, or whether the J1-160/180–positive neuronal subpopulations display particular functional features, remains to be elucidated.

J1-160/180 was originally characterized as an oligodendrocyte-derived extracellular matrix protein (Pesheva et al., 1989), whereas restrictin was described as an extracellular matrix protein associated with axonal surfaces (Rathjen et al., 1991). Immunohistological localization of restrictin was performed using sections of embryonic chick tissues, localizing it in the prospective white matter of the cerebellum, the inner and outer plexiform layer of the retina, and around motor neurons in the spinal cord (Rathjen et al., 1991). This distribution is similar to the one described for J1-160/180 in the rat and in the mouse by immunohistological methods and by in situ hybridization (Pesheva et al., 1989; Wintergerst et al., 1992). It remains to be seen whether in situ hybridization using a restrictin-specific probe yields similar results as those for J1-160/180. Thus, rat J1-160/180 and chicken restrictin have a similar molecular structure and appear to be expressed in similar patterns in different structures of the CNS. However, not only similar but also different functional properties have been described for the two molecules. Both molecules interact or copurify with the neuronal cell adhesion molecule F3/11 (Rathjen et al., 1991; Pesheva et al., 1992). No repellent properties for neurons have been reported for restrictin, neither for cell bodies nor for neurites (Rathjen et al., 1991), but have been observed for J1-160/180 (Pesheva et al., 1989, 1992). However, since the two molecules were tested under different experimental conditions, further investigations are necessary to show whether the two molecules are not only structurally, but also functionally highly related.

Since the expression of J1-160/180 and tenascin is differentially regulated during development, it is tempting to speculate that an important determinant of the molecules' functions, namely whether they are repellent or adhesive, could reside in the temporal and spatial context of their expression. With the cDNA clones now available it is possible to study these contrary phenomena at the cellular and molecular levels. In addition, the common structural motifs of the known members of the J1 family will assist the search for other members of the family.

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Note Added in Proof. Recently we have renamed J1-160/180 glycoprotein Janusin (Fawcett, J. W., N. Fersht, L. Housden, M. Schachner, P. Pesheva. 1992. *J. Cell Sci.* 103:571–579; Wintergerst, E.-S., B. Fuss, U. Bartsch. 1993. *Eur. J. Neurosci.* In press; Bartsch, U., P. Pesheva, M. Raff, M. Schachner. 1993. *Glia.* In press). This name will be used in the future to designate the protein.

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