

Localization of Janusin mRNA in the Central Nervous System of the Developing and Adult Mouse

Eva Sabine Wintergerst, Babette Fuss and Udo Bartsch

Department of Neurobiology, Swiss Federal Institute of Technology, Hönggerberg, 8093 Zürich, Switzerland

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Abstract

Janusin (formerly termed J1-160/180) is an oligodendrocyte-derived extracellular matrix molecule which is restricted to the central nervous system and which is expressed late during development (Pesheva *et al.*, *J. Cell Biol.*, 1765–1778, 1989). To gain insights into the molecule's morphogenetic functions and to identify its cellular source *in vivo*, we have studied the localization of janusin messenger RNA in the optic nerve, retina and spinal cord and the expression of janusin protein in the spinal cord of developing and adult mice. Moreover, we have analysed optic nerve cell cultures and retinal cell suspensions in double-labelling experiments using a janusin-specific anti-sense complementary RNA probe and cell type-specific antibodies to identify the cell types containing janusin transcripts. In developing animals, oligodendrocytes were strongly labelled with the janusin anti-sense cRNA probe during the period of myelination. The number of labelled cells and intensity of the hybridization signal decreased significantly with increasing age. Interestingly, expression of janusin was not confined to oligodendrocytes. Some neuronal cell types and type-2 astrocytes present in optic nerve cell cultures also contained janusin transcripts. In contrast to oligodendrocytes, the number and labelling intensity of neurons containing janusin transcripts remained constant during postnatal development and into adulthood. Expression of janusin protein in the spinal cord was developmentally regulated, with a peak of expression in 2- or 3-week-old animals. The molecule was visible in the white and grey matter. In myelinated regions, it was associated with myelinated fibres and accumulated at nodes of Ranvier. These observations suggest that janusin may be of functional relevance for myelination.

Introduction

Janusin (formerly termed J1-160/180) represents the lower molecular weight group of the J1 complex of extracellular matrix glycoproteins described in the mouse central nervous system (CNS). It is composed of two major molecular weight forms of 160 and 180 kD (Kruse *et al.*, 1985; Faissner *et al.*, 1988; Pesheva *et al.*, 1989). Electron microscopic investigations using the rotary shadowing technique revealed a trimeric structure for the 180-kD component, whereas the 160-kD component is organized as monomers and dimers (Pesheva *et al.*, 1989). Functional studies revealed repellent substrate properties towards neuronal cell bodies for both janusin components, in a mixture with laminin (Pesheva *et al.*, 1989) and towards growth cones when offered as a sharp border (Taylor *et al.*, 1993). When offered as a homogeneous substrate in a mixture with laminin, the molecule accelerates growth cone advance of dorsal root ganglion cells but inhibits axonal elongation of retinal ganglion cells (Taylor *et al.*, 1993). In contrast to these repellent properties towards nerve cells, janusin has been shown to be adhesive for astrocytes (Morganti *et al.*, 1990). Such a dual behaviour has also been demonstrated for tenascin (formerly designated J1–200/220; Kruse *et al.*, 1985; Faissner and Kruse, 1990; Lochter *et al.*, 1991; S. Bartsch *et al.*, 1992), which represents the other member of the J1 glycoproteins and which is also known as hexabrachion (Erickson and Iglesias, 1984)

and cytотactин (Grumet *et al.*, 1985). Besides common adhesive/repellent properties, janusin and tenascin possess a similar structural architecture, as indicated by complementary DNA (cDNA) sequence data (Spring *et al.*, 1989; Jones *et al.*, 1989; Fuss and Schachner, 1991; Weller *et al.*, 1991; Fuss *et al.*, 1993). Rat janusin is composed of a cysteine-rich amino-terminal region which is followed by 4.5 epidermal growth factor-like repeats, 9 fibronectin type III homologous repeats and a carboxy-terminal region homologous to fibrinogen (Fuss and Schachner, 1991; Fuss *et al.*, 1993). Restrictin, a recently described neural extracellular matrix molecule in the chicken, reveals the same structural architecture and an amino acid sequence which shows 85% similarity to rat janusin. Restrictin can therefore be considered as the species homologue of janusin (Nörenberg *et al.*, 1992; Fuss *et al.*, 1993).

In contrast to tenascin, which is expressed in the CNS (Grumet *et al.*, 1985; Kruse *et al.*, 1985; Prieto *et al.*, 1990; S. Bartsch *et al.*, 1992; U. Bartsch *et al.*, 1992), in the peripheral nervous system (Daniloff *et al.*, 1989; Martini *et al.*, 1990) and in various non-neuronal structures (see e.g. Chiquet and Fambrough, 1984; Erickson and Iglesias, 1984; Bourdon *et al.*, 1985; Chiquet-Ehrismann *et al.*, 1986; Aufderheide *et al.*, 1987), expression of janusin is restricted to the CNS (Pesheva *et al.*, 1989). Janusin becomes detectable late during development and is

Correspondence to: Eva Sabine Wintergerst, as above

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predominantly associated with developing white matter tracts (Pesheva *et al.*, 1989). In cell cultures prepared from early postnatal mouse cerebellum or spinal cord, oligodendrocytes but not fibroblasts, astrocytes or neurons, were identified as janusin-immunoreactive cell types (Pesheva *et al.*, 1989). Using an anti-J1 antiserum, increased immunoreactivity associated with nodes of Ranvier in adult rat optic nerves has been described, and immunoreactive oligodendrocytes and type-2 astrocytes have been found in optic nerve cell cultures (ffrench-Constant *et al.*, 1986; ffrench-Constant and Raff, 1986a). In immunoblots of adult rat optic nerves this same antiserum recognized a broad band between 160 and 180 kD. Janusin immunoreactivity at nodes of Ranvier and of oligodendrocytes and type-2 astrocytes in optic nerve cell cultures has recently been confirmed using monoclonal anti-janusin antibodies (Bartsch *et al.*, 1993). The possibility that janusin expression is not restricted to oligodendrocytes is strengthened by the observation that the oligodendrocyte-free retina of mice and rats is janusin-immunoreactive (Bartsch *et al.*, 1993). However, since janusin is a secreted molecule these findings may also indicate that type-2 astrocytes and retinal cells express janusin receptor(s) which bind oligodendrocyte-derived janusin. To decide between these possibilities and to gain insight into possible morphogenetic functions of janusin, we have studied the molecule's cellular source in the developing and adult mouse optic nerve, retina and spinal cord and in optic nerve cell cultures and retinal cell suspensions by employing a non-radioactive *in situ* hybridization technique using a janusin-specific digoxigenin-labelled complementary RNA (cRNA) probe.

Material and methods

Animals

For *in situ* hybridization neonatal, 7-, 14-, 21- and 28-day-old and adult (i.e. older than 60 days) ICR ZUR mice were used. For optic nerve cell cultures and retinal cell suspensions, we used 6–8-day-old and 1-month-old mice, respectively. All animals were bred at the departmental animal facilities.

Antibodies

As primary antibodies for indirect immunofluorescence microscopy, we used monoclonal mouse antibodies 619, recognizing the 160- and 180-kD component of janusin (Morganti *et al.*, 1990), O1, recognizing an oligodendrocyte-specific cell surface antigen (Schachner *et al.*, 1981; Sommer and Schachner, 1981; Trotter and Schachner, 1989), D3, recognizing the 180-kD isoform of the neural cell adhesion molecule (N-CAM) (Schlosshauer, 1989) and polyclonal rabbit antibodies to glial fibrillary acidic protein (GFAP; a kind gift of Dr L. Eng, Stanford University, USA).

Primary antibodies were visualized by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse (Dynatech) or tetramethylrhodamine isothiocyanate (TRITC)-conjugated pig anti-rabbit (Dakopatts) IgG antibodies. Digoxigenin-labelled cRNA probes for *in situ* hybridization were visualized using alkaline phosphatase-conjugated Fab fragments to digoxigenin (Boehringer Mannheim).

Nucleic acid probes for *in situ* hybridization

For synthesis of the janusin-specific cRNA probe the 5946-base pair janusin cDNA clone was used which was ligated into the Bluescript KS vector (Stratagene, Zürich; Fuss *et al.*, 1991; Fuss *et al.*, 1993). This cDNA clone encodes nearly the whole coding region. A L1-specific DNA clone comprising the first 952 base pairs of the cDNA (Moos *et al.*, 1988) was subcloned into the pBluescript KS vector, giving rise to the

pBlue L1-Acc plasmid. Digoxigenin-labelled anti-sense and sense cRNA probes were generated according to the manufacturer's recommendations using the RNA Labelling Kit (Boehringer Mannheim). After transcribing, probes were digested under alkaline conditions to obtain an average fragment length of ~300 nucleotides.

Optic nerve cell cultures

Preparation of optic nerve cell cultures was performed as described (Raff *et al.*, 1983). In brief, optic nerves were removed from 6–8-day-old mice, chopped and incubated in 1 ml Hank's buffered salt solution (HBSS⁻), 1 ml trypsin (0.25% in Ca²⁺- and Mg²⁺-free Dulbecco's modified Eagle's medium; DMEM) and 50 µl collagenase (2% in distilled water) for 30 min at 37°C, followed by 1 ml Ca²⁺- and Mg²⁺-free DMEM containing 0.02% ethylenediamine tetraacetic acid (EDTA) and 50 µl collagenase for 20 min at 37°C. Then, 1 ml fluid was removed and an equal volume of Ca²⁺- and Mg²⁺-free DMEM containing 0.04 mg/ml DNase, 0.5 mg/ml soybean trypsin inhibitor, 3 mg/ml bovine serum albumin (BSA) was added. The tissue was dissociated by gentle trituration through a fire-polished Pasteur pipette and a 25 G needle. Cells were washed, plated on poly-L-lysine-coated glass coverslips at a density of ~500 000 cells/ml, and cultured in a humidified atmosphere of 5% CO₂ and 95% air for 3 days at 37°C, in either DMEM containing 10% fetal calf serum (FCS) or in modified Sato medium [DMEM containing 0.2% (w/v) sodium bicarbonate, 0.01 mg/ml insulin, 0.1 mg/ml transferrin, 2 mM glutamine, 200 nM progesterone, 100 µM putrescine, 220 nM sodium selenite, 400 nM triiodothyronine, 500 nM thyroxine and 0.025 mg/ml gentamycin sulphate]. Cultures were fixed in phosphate-buffered saline (PBS; pH 7.4) containing 4% paraformaldehyde for at least 30 min at room temperature, before they were subjected to *in situ* hybridization.

Retinal cell suspensions

Four-week-old mice were enucleated and the retinae were isolated and incubated in HBSS⁻ containing papain (14 U/ml) for 30 min at 37°C. After trituration with a fire-polished glass pipette, cell suspensions were washed with HBSS⁻ and plated on glass coverslips coated with Cell-Tak (WAK-Chemie Medical GmbH). Cells were allowed to settle for 2 h and were then fixed in PBS (pH 7.4) containing 4% paraformaldehyde.

In situ hybridization

In situ hybridization was performed as described elsewhere (S. Bartsch *et al.*, 1992). Cryostat sections, 14 µm in thickness, from fresh-frozen mouse retinae, optic nerves and spinal cords were thaw-mounted on coverslips coated with 3-aminopropyltriethoxy-silane (Sigma) and fixed in PBS containing 4% paraformaldehyde for 30 min at room temperature. After fixation, tissue sections, retinal cell suspensions or optic nerve cell cultures were washed three times in PBS, once in 70% ethanol and twice in distilled water. After incubation with 0.1 M HCl for 5 min, they were washed with PBS, immersed in 0.1 M triethanolamine (pH 8.0) containing 0.25% acetic anhydride for 20 min, washed again in PBS and dehydrated in an ascending series of ethanol. Prehybridization was carried out by incubating the sections, cell suspensions or cell cultures in prehybridization buffer containing 50% deionized formamide, 25 mM EDTA, 50 mM Tris-HCl (pH 7.6), 2.5× Denhardt's solution, 0.25 mg/ml transfer RNA (Boehringer Mannheim) and 20 mM NaCl, overnight at 37°C. Hybridization was carried out overnight in hybridization buffer containing 50% formamide, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1×Denhardt's solution, 0.5 mg/ml transfer RNA, 0.1 mg/ml poly(A) RNA, 0.1 M dithiothreitol, 10% dextran sulphate and the sense or anti-sense cRNA probes at concentrations of ~400

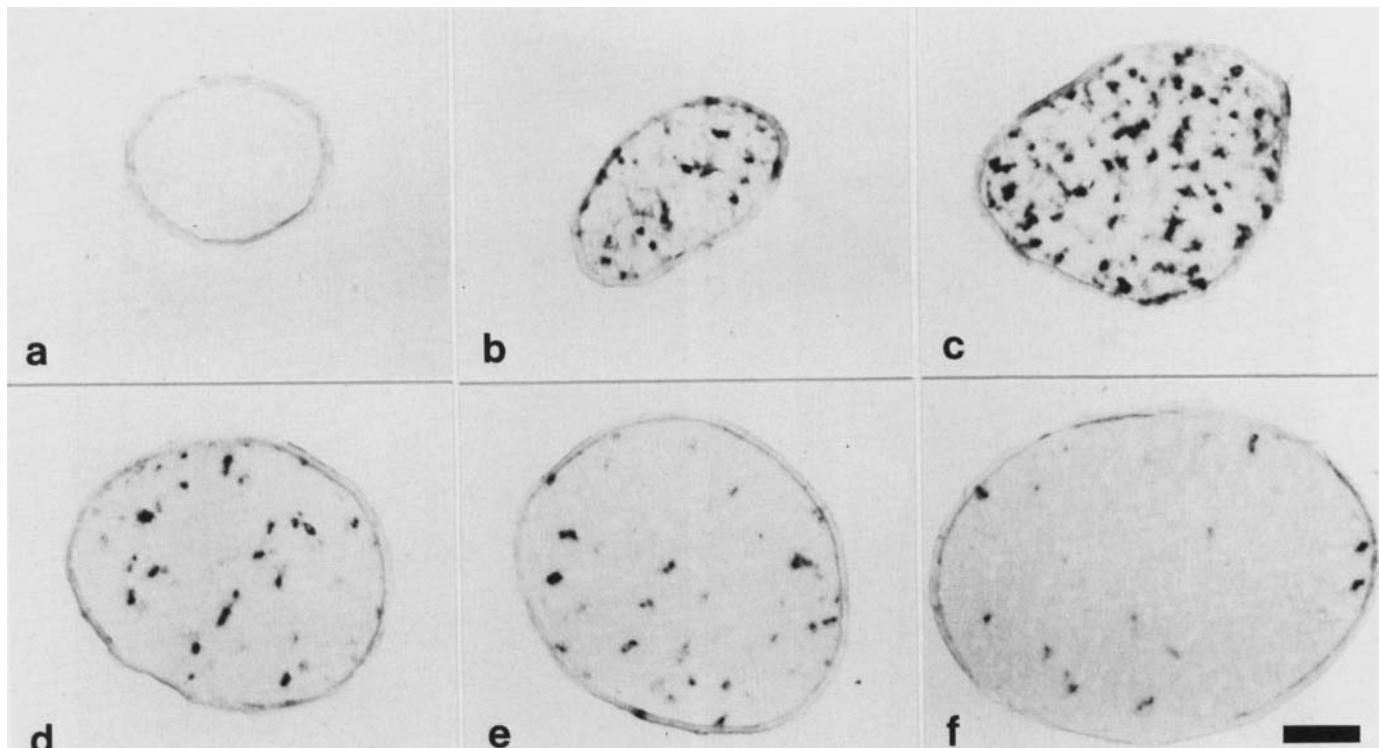


FIG. 1. Localization of janusin mRNA in the developing and adult mouse optic nerve. Janusin-expressing cells are not detectable in cross-sections of optic nerves from neonatal animals (a). Labelled cells are present in optic nerves from 1-week-old mice (b) and increase in density up to the second postnatal week (c). In 3-week-old (d) and 4-week-old (e) optic nerves, the density of labelled cells decreases. In adults (f), only a few cells contain janusin-specific transcripts and most cells are weakly labelled. Bar: 70 μ m.

pg/ μ l hybridization buffer. After hybridization, coverslips were washed twice in 30 mM NaCl and 3 mM Na-citrate (pH 7.0; 0.2 \times SSC) at 55°C for 30 min each and three times for 1.5 h each in 0.2 \times SSC containing 50% formamide at 55°C. Sections or cells were then washed with 0.2 \times SSC, equilibrated with buffer 1 (100 mM Tris-HCl, 150 mM NaCl; pH 7.5) for 10 min and blocked for 30 min with blocking buffer (1% Boehringer blocking reagent and 0.5% BSA in buffer 1), all at room temperature. Subsequent incubation with alkaline phosphatase-conjugated Fab fragments to digoxigenin (diluted 1:350 in blocking buffer) was carried out overnight at 4°C. Sections were washed twice for 15 min in buffer 1 and equilibrated for 2 min in buffer 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM MgCl₂; pH 9.5). The colour reaction was initiated by adding buffer 3 supplemented with 0.34 mg/ml 4-nitroblue tetrazolium chloride, 0.175 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.25 mg/ml levamisole. Colour development was performed overnight in the dark. Cell suspensions or cell cultures were washed twice with buffer 4 (10 mM Tris-HCl, 1 mM EDTA) for 10 min each and processed further for immunocytochemical analysis (see next paragraph). Cryostat sections were washed in distilled water, air-dried and analysed with an Axiophot microscope (Zeiss). As a control, hybridization of sections, cell cultures and cell suspensions was carried out in the presence of the janusin and L1 sense cRNA probes and no labelled cells were found.

Immunocytochemistry

Following *in situ* hybridization, cell suspensions and cell cultures were analysed immunocytochemically with cell type-specific antibodies to identify the cell types expressing janusin. After washing in buffer 4,

coverslips were incubated in PBS containing 1% BSA for 30 min at room temperature. Coverslips were then incubated with primary antibodies for 2 h, washed in PBS, incubated with FITC- or TRITC-conjugated secondary antibodies for 1 h, washed again, and mounted onto slides, all at room temperature. Immunohistochemical analysis of janusin expression in cryostat sections of the cervical level of the spinal cord was carried out as described elsewhere (Bartsch *et al.*, 1989).

Results

Localization of janusin messenger RNA in the developing and adult mouse optic nerve

To analyse the developmental regulation of janusin expression in the mouse optic nerve at the messenger RNA (mRNA) level and to identify the cellular source of janusin *in vivo*, we investigated a developmental series of optic nerve cross-sections using *in situ* hybridization. Signal intensity and density of labelled cells in tissue sections from different developmental ages are directly comparable, since sections were mounted on the same coverslip.

Cells containing janusin transcripts were not detectable in optic nerves from neonatal animals (Fig. 1a). Labelled cells were visible in 1-week-old optic nerves (Fig. 1b) and their density increased significantly up to the second postnatal week (Fig. 1c). In older animals, the density of labelled cells decreased again (see Fig. 1d for a 3-week-old animal and Fig. 1e for a 4-week-old animal). In adults, only a few cells contained detectable levels of janusin mRNA (Fig. 1f). With the exception of a few heavily labelled cells, signal intensity of janusin-expressing cells

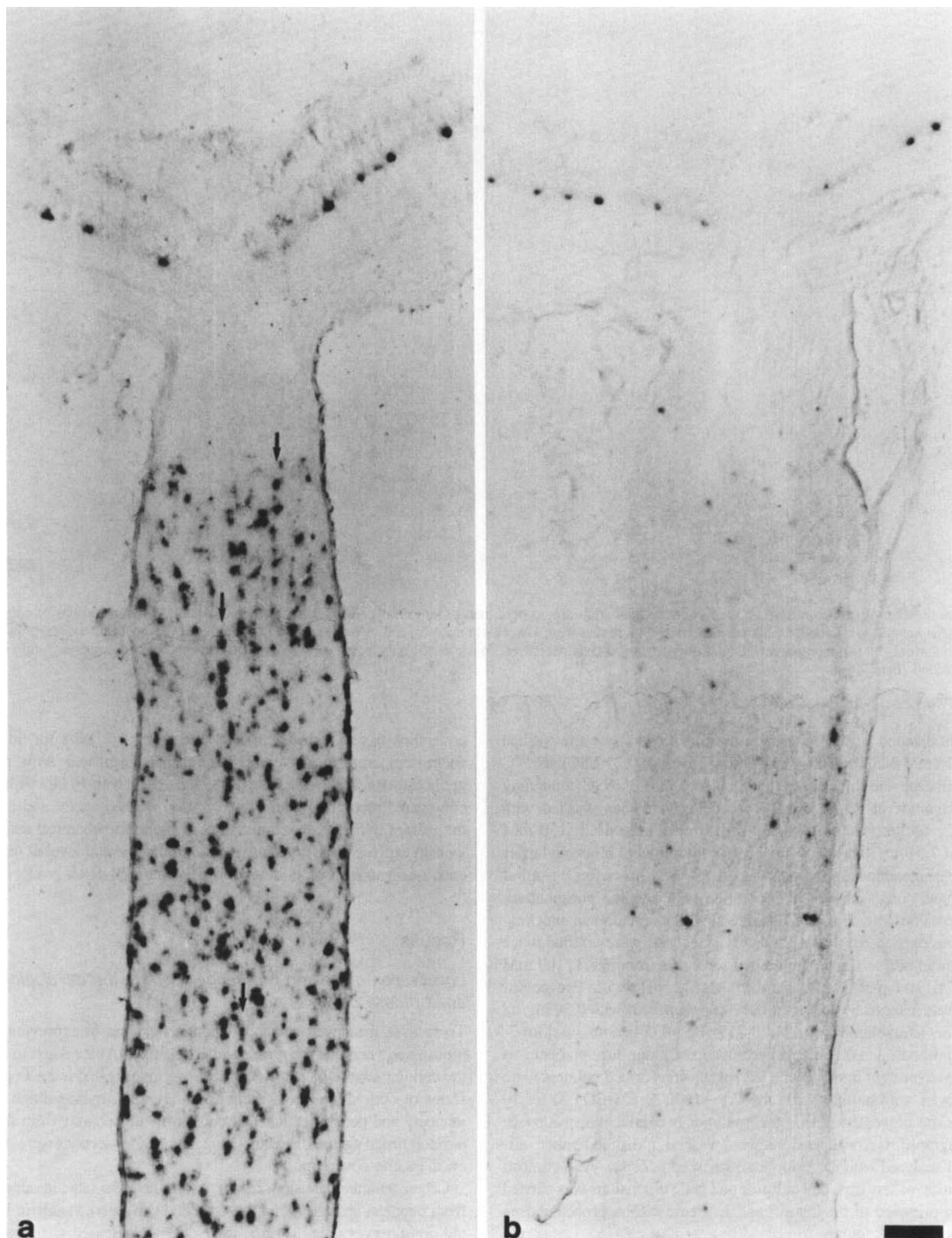


FIG. 2. Localization of janusin mRNA in longitudinally sectioned optic nerves with attached retinae from 2-week-old (a) and adult (b) mice. In the optic nerve of the 2-week-old animal, labelled cells are restricted to the myelinated part of the optic nerve (a). Here, they are usually aligned in longitudinally oriented rows of cells (arrows in a). In the retina, janusin-expressing cells are located at the outer margin of the inner nuclear layer. A similar distribution of labelled cells is detectable in the retina and optic nerve of adult mice (b), with the only exception that both the density and the labelling intensity of janusin-expressing cells in the myelinated distal part of the optic nerve are reduced. Bar: 100 μ m.

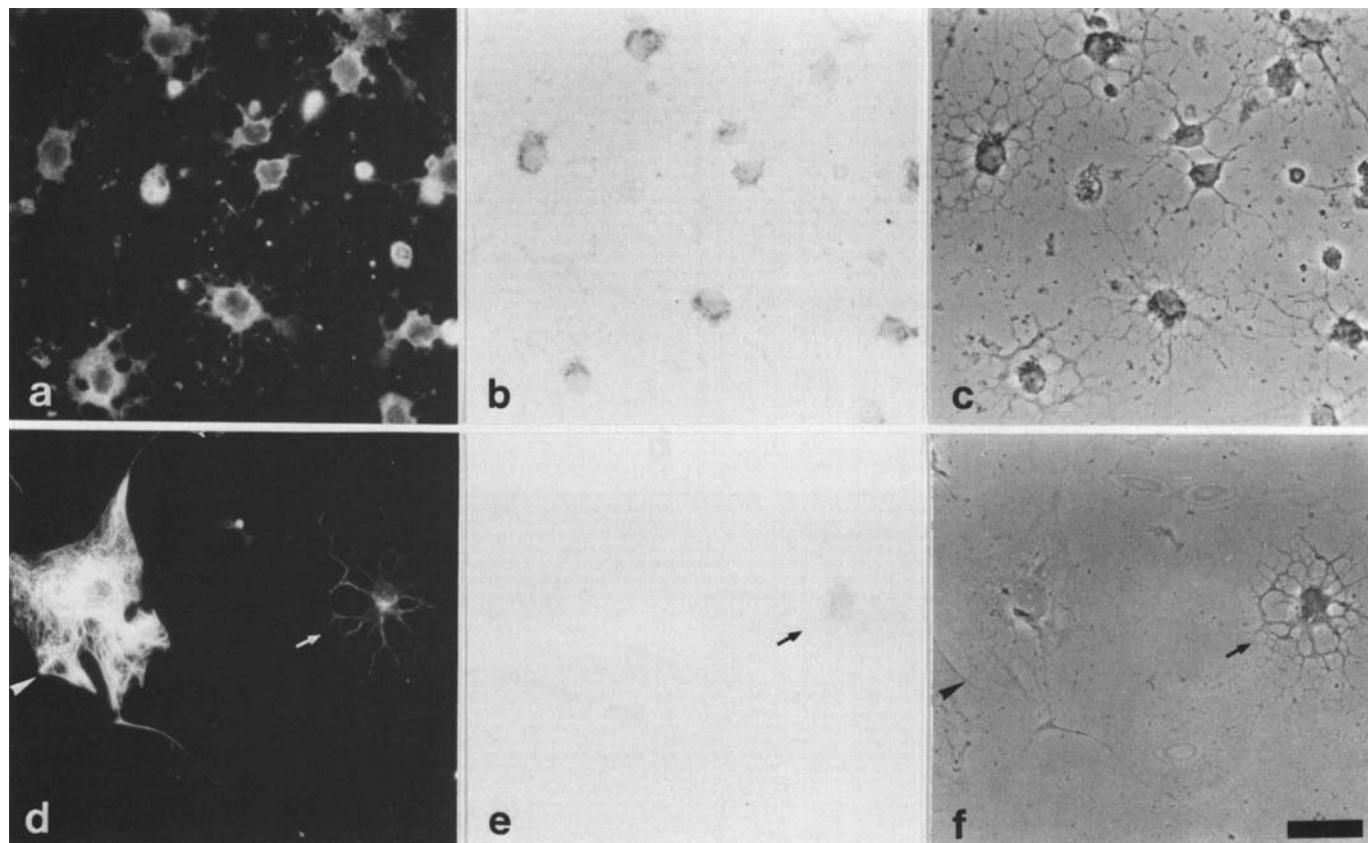


FIG. 3. Identification of janusin mRNA-containing cell types in optic nerve cell cultures prepared from 6–8-day-old mice and maintained for 3 days in modified SATO medium (a–c) or DMEM containing 10% FCS (d–f). O1-immunoreactive oligodendrocytes (a) are strongly labelled with the janusin anti-sense cRNA probe (b; c represents the phase-contrast micrograph of a and b). In addition, GFAP-positive cells with a stellate morphology (arrow in d and f), but not GFAP-positive cells with a fibroblast-like morphology (arrowhead in d and f), contain detectable levels of janusin mRNA (arrow in e; f represents the phase-contrast micrograph of d and e). Bar: 30 μ m.

in adults appeared less than in optic nerves from younger animals (compare Fig. 1f with Fig. 1b and c; see also Fig. 2a, b).

The analysis of longitudinally sectioned optic nerves from 2-week-old and adult animals revealed a characteristic and restricted distribution of janusin mRNA-expressing cells (Fig. 2a, b). Labelled cells were detectable in the distal (i.e. near chiasma) and myelinated part of the optic nerve, but were absent from the unmyelinated proximal (i.e. near retina) part of the nerve (Fig. 2a, b). The density of labelled glial cells in the myelinated part of the optic nerve and the signal intensity of most labelled cells decreased significantly with increasing age (compare Fig. 2a, b; see also Fig. 1). Usually, cells containing janusin mRNA were aligned in longitudinally oriented rows of cells (Fig. 2a; for cells containing janusin mRNA in the retina, see below).

Cell types expressing janusin in optic nerve cell cultures

We analysed optic nerve cell cultures prepared from 6- to 8-day-old mice for janusin expression. Cell cultures were maintained either in modified Sato medium to obtain oligodendrocytes or in DMEM with 10% FCS to obtain astrocytes (Raff, 1989). Oligodendrocytes, identified by morphological criteria (Fig. 3c) and their O1 immunoreactivity (Sommer and Schachner, 1981, 1982; Fig. 3a) were strongly labelled with the janusin anti-sense cRNA probe (Fig. 3b). Using polyclonal antibodies to GFAP (Fig. 3d) and morphological criteria (Fig. 3f), we distinguished between stellate type-2 astrocytes and fibroblast-like type-1 astrocytes.

Some of the type-2 astrocytes, but no type-1 astrocytes, contained detectable levels of janusin transcripts (Fig. 3e). However, the intensity of the *in situ* hybridization signal in type-2 astrocytes was significantly weaker than in oligodendrocytes (compare Fig. 3b and e).

Localization of janusin mRNA in the developing and adult mouse retina

As for the optic nerve, we analysed the developmental regulation of janusin expression and its cellular source in cryostat sections from a developmental series of mouse retinae (Fig. 4). A band of weakly labelled cells was detectable in the outer third of retinae from neonatal animals, the youngest animals analysed (Fig. 4a). The *in situ* hybridization signal increased in intensity in retinae of 1-week-old animals (Fig. 4b). Labelled cells were located at the outer margin of the inner nuclear layer (Fig. 4b), indicating that they are horizontal cells. Signal intensity, cell density and localization of janusin-expressing cells remained similar in retinae of 3-week-old (Fig. 4c) and 4-week-old (Fig. 4d) animals and in adults (Fig. 2b; for a 2-week-old retina see Fig. 2a). All other retinal cell types were unlabelled at all developmental ages analysed.

Cell types expressing janusin in the mouse retina

To further test whether janusin in the mouse retina is neuron-derived, we compared the localization of janusin mRNA (Fig. 5a) and L1 mRNA (Fig. 5b), a neuron-specific marker in the CNS of the postnatal mouse

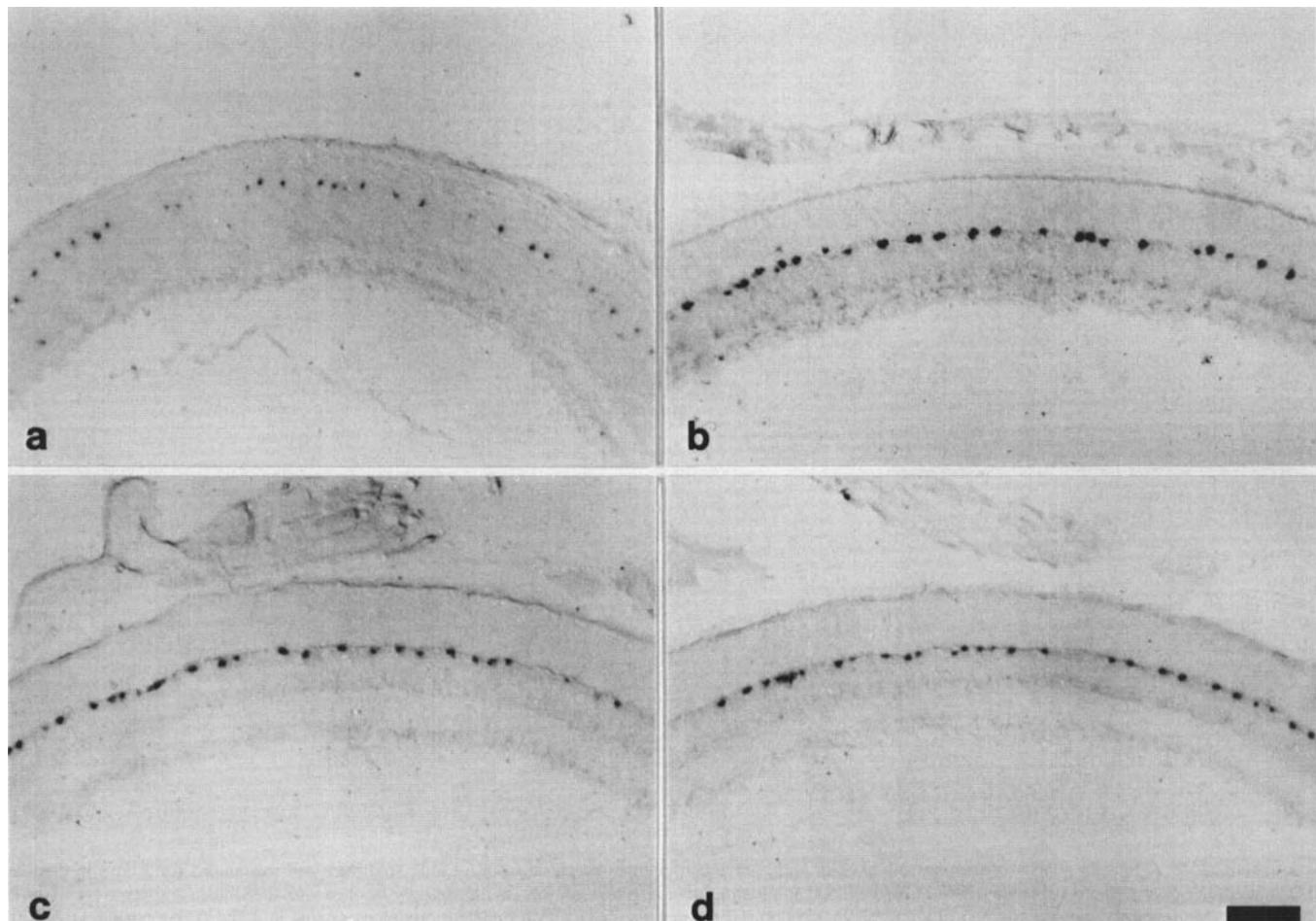


Fig. 4. Localization of janusin mRNA in the developing mouse retina. In the retina of newborn animals (a) weakly labelled cells are detectable in the outer part of the retina. In 1-week-old mice (b), cells are more intensely labelled and located beneath the outer plexiform layer, indicating that these cells correspond to horizontal cells. Note that the distribution of cells expressing janusin and the intensity of labelling is similar in retinas of 3-week-old (c) and 4-week-old (d) animals. Bar: 120 μ m.

(Rathjen and Schachner, 1984), in consecutive sections from 1-week-old mouse retinas. The L1 anti-sense cRNA probe strongly labelled the retinal ganglion cell layer (Fig. 5b). Weak labelling was detectable at the outer margin of the inner nuclear layer (Fig. 5b). Cells expressing L1 were also detectable in the outer part of the inner nuclear layer (Fig. 5b) with a location and cell density similar to those of cells containing janusin transcripts (compare Fig. 5a and b).

To further confirm the neuronal character of janusin-expressing cells, retinal cell suspensions prepared from 1-month-old mice were double-labelled with the janusin anti-sense cRNA probe and the monoclonal antibody D3 recognizing the neuron-specific 180-kD component of the neural cell adhesion molecule (N-CAM-180; Fig. 5c–e; Noble *et al.*, 1985; Murray *et al.*, 1986). Retinal cell suspensions contained a high number of N-CAM-180-immunoreactive cells (Fig. 5c). Some of these immunolabelled cells also contained detectable levels of janusin mRNA (Fig. 5d).

Localization of janusin mRNA in the developing and adult mouse spinal cord

In the upper cervical level of the spinal cord, a few cells expressing janusin were already detectable in neonatal animals, the earliest developmental age investigated (data not shown). The density of labelled

cells and the intensity of the *in situ* hybridization signal increased during the first postnatal week. In 1-week-old animals, most labelled cells were located in the developing white matter (Fig. 6a). In 2-week-old animals, the number of cells containing janusin mRNA also increased in the grey matter (Fig. 6b). In particular, multipolar and large-sized cells with a cell body diameter of ~30 μ m were labelled. These cells were located in aggregates in the ventral horns of the spinal cord and correspond to motor neurons (Fig. 6b–e). As in the optic nerve, the number of labelled cells and the intensity of the *in situ* hybridization signal decreased significantly after the second postnatal week, which was already obvious in spinal cords from 3-week-old animals (Fig. 6c). At this developmental age, however, an increased density of labelled cells was still detectable in the corticospinal tract (Fig. 6c). In adults, the density of labelled cells was significantly reduced in both the grey and the white matter (Fig. 6d). In addition, the labelling intensity of most cells, with the exception of motor neurons, was significantly reduced compared with that of younger animals (compare Fig. 6d, e with Fig. 6a, b).

Immunohistochemical localization of janusin in the developing and adult mouse spinal cord

In the spinal cord of neonatal animals, hardly any janusin immunoreactivity was detectable (Fig. 7a). The intensity of fluorescence

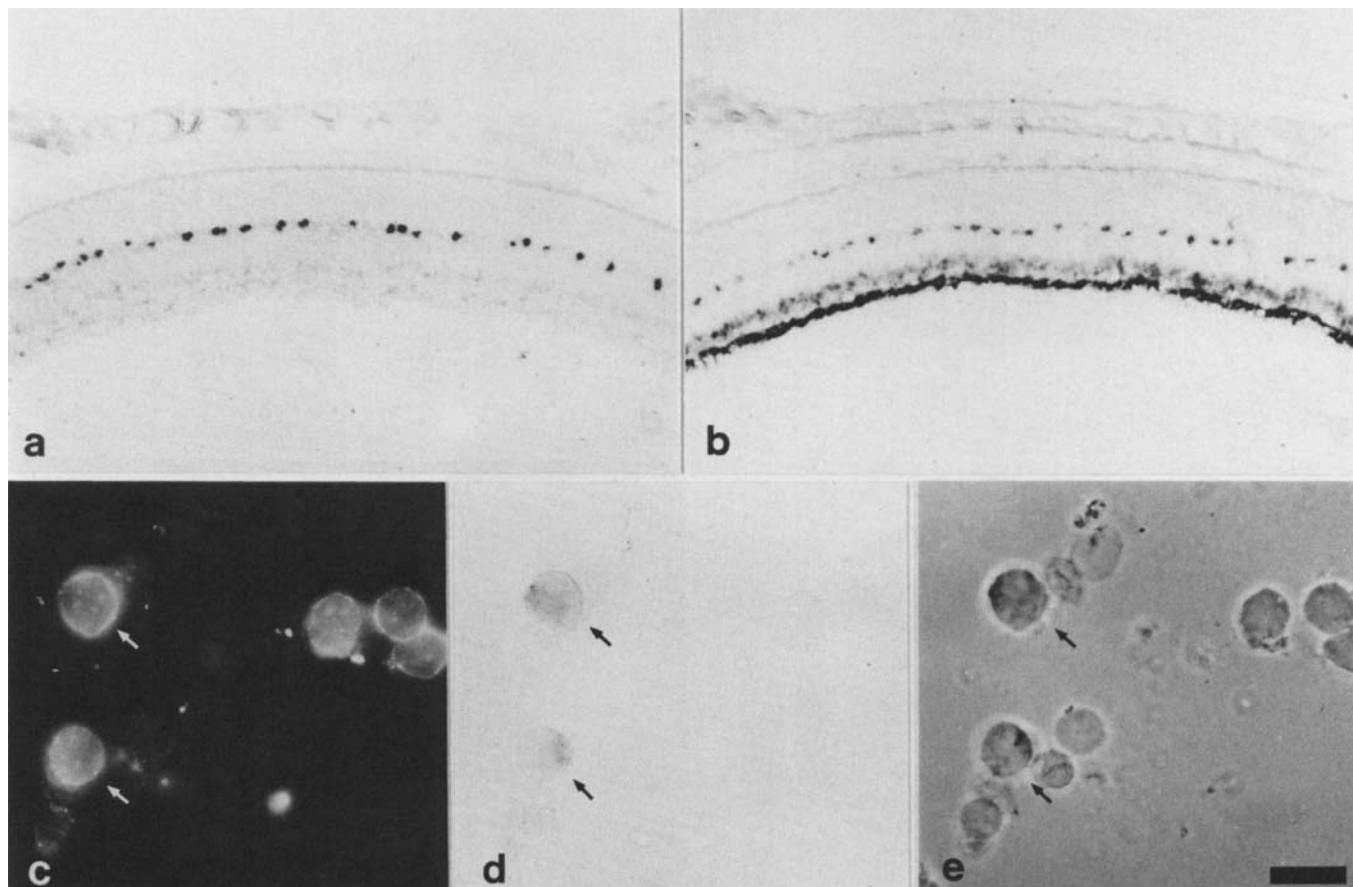


FIG. 5. Localization of janusin (a) and L1-specific (b) transcripts in consecutive sections of a 7-day-old mouse retina. Cells expressing janusin are located at the outer margin of the inner nuclear layer (a). A similar distribution of labelled cells is detectable with the L1 anti-sense cRNA probe (b). However, L1 mRNA is additionally detectable at the inner aspect of the inner nuclear layer and in the ganglion cell layer (b). In retinal cell suspensions from 1-month-old animals, several cells are N-CAM-180-immunoreactive (c). Some of these N-CAM-180-positive cells also contain janusin mRNA (marked with arrows in c and d; e represents the phase-contrast micrograph of c and d). Bar: a, b, 120 μ m; c–e, 15 μ m.

labelling increased during the first (Fig. 7b), second (Fig. 7c) and third (Fig. 7d) postnatal weeks, and then decreased with increasing age (for a 4-week-old and an adult animal see Fig. 7e and f, respectively). Intense fluorescence first became visible in the dorsal white matter of 7-day-old mice, in which the remaining white matter and grey matter was only weakly labelled (Fig. 7b). In older animals, both the white and the grey matter were labelled (Fig. 7c–f). Remarkably, the grey matter of animals older than 3 weeks showed more intense labelling than the white matter. In the white matter of adult animals, janusin was associated with myelinated axons (Fig. 7g). In longitudinal sections, increased amounts of janusin were found in association with nodes of Ranvier (Fig. 7h).

Discussion

Expression of the extracellular matrix molecule janusin has been reported to be restricted to the myelin-forming oligodendrocytes of the CNS (Pesheva *et al.*, 1989). It has also been demonstrated, however, that type-2 astrocytes present in cell cultures prepared from postnatal rat optic nerves are janusin-immunoreactive (ffrench-Constant and Raff, 1986a; Bartsch *et al.*, 1993). Moreover, immunocytochemical investigations have provided indirect evidence that janusin might be expressed not only by oligodendrocytes but also by other neural cell types, since the

oligodendrocyte-free retina of the developing and adult mouse is janusin-immunoreactive (Bartsch *et al.*, 1993). To identify the cellular source of this secreted glycoprotein, we analysed selected regions of the developing and adult CNS of the mouse using a non-radioactive *in situ* hybridization method.

The present results demonstrate that oligodendrocytes are the major cellular source of janusin *in vivo* since strong hybridization signals are present in myelinated regions of the developing optic nerve and spinal cord. These findings thus confirm previous immunocytochemical investigations performed on cell cultures prepared from young postnatal mouse cerebellum and spinal cord or from embryonic mouse brain (Pesheva *et al.*, 1989; Jung *et al.*, 1993). Astrocytes, in contrast, appear not to express janusin according to immunocytochemical *in vitro* investigations (Pesheva *et al.*, 1989) and according to our observation that the unmyelinated proximal part of the optic nerve and the astrocyte-containing retinal nerve fibre layer (Huxlin *et al.*, 1992) is devoid of cells expressing janusin. Consistent with this interpretation are our observations that janusin-specific transcripts are detectable in O1-immunoreactive oligodendrocytes (Sommer and Schachner, 1981, 1982; Trotter and Schachner, 1989), but not in fibroblasts or fibroblast-like GFAP-positive type-1 astrocytes in optic nerve cell cultures (Raff *et al.*, 1983; Raff, 1989). However, we cannot completely exclude the

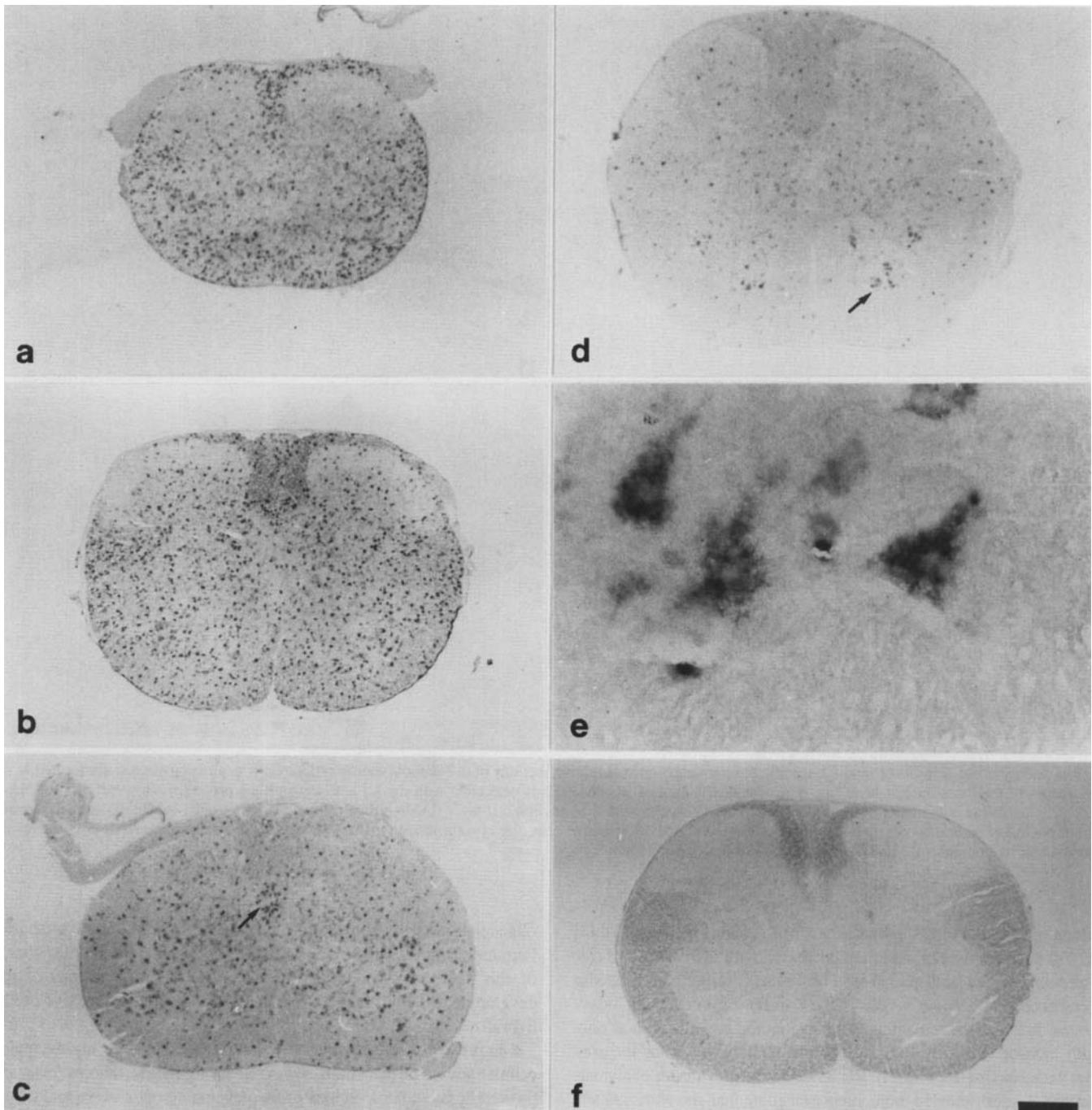


FIG. 6. Localization of janusin mRNA in the developing and adult spinal cord of the mouse at the cervical level. In 1-week-old animals (a), most labelled cells are located in the prospective white matter. In 2-week-old animals, the density of cells expressing janusin has reached its highest level (b), both in the white and grey matter. With the exception of the corticospinal tract (arrow in c), the number of janusin-expressing cells decreases in 3-week-old animals (c). In adults, all regions of the spinal cord reveal low numbers of weakly labelled cells (d). In addition to the numerous small cells, large motoneurons are labelled in the ventral horns of 3-week-old and adult (arrow in d) animals. (e: higher magnification of labelled cells in d). As a control, sections were hybridized with the sense cRNA probe and were unlabelled (f). Bar: a-d, f, 500 µm; e, 30 µm.

possibility that a distinct astrocyte subtype, restricted to myelinated CNS regions, expresses janusin. In fact, when analysing optic nerve cell cultures we found that some stellate GFAP-positive type-2 astrocytes (Raff *et al.*, 1983) indeed express janusin mRNA, although in considerably reduced amounts when compared to oligodendrocytes. Since increased janusin immunoreactivity is also associated with nodes of

Ranvier, it has been suggested that type-2 astrocytes identified *in vitro* correspond to perinodal astrocytes *in vivo* (ffrench-Constant *et al.*, 1986; ffrench-Constant and Raff, 1986a; Waxmann, 1986; Miller *et al.*, 1989). However, it is still a point of controversy whether type-2 astrocytes characterized *in vitro* actually exist *in vivo* (see e.g. Skoff, 1990; reviewed in Richardson *et al.*, 1990; Noble, 1991). In the future, the janusin cRNA

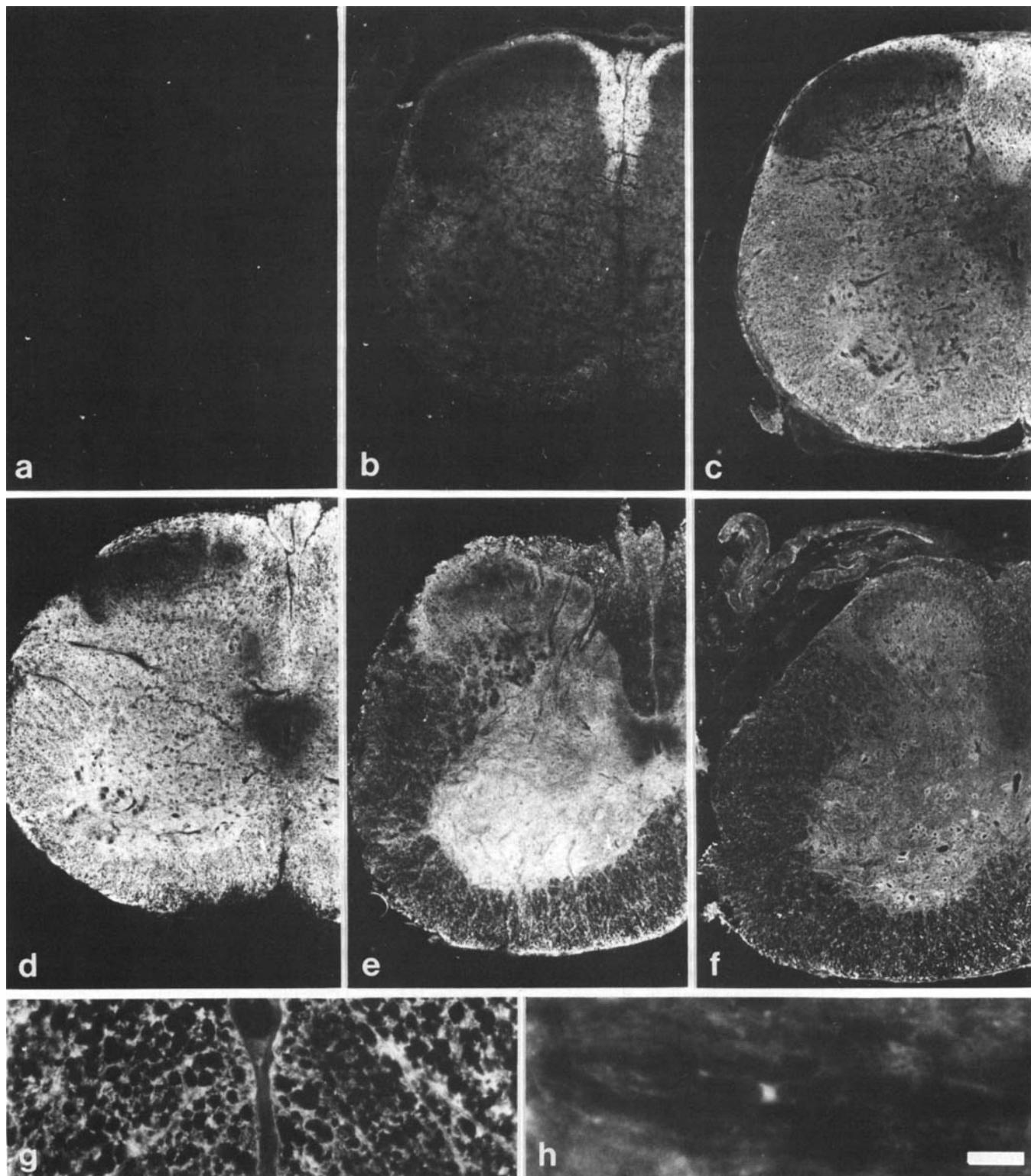


FIG. 7. Immunohistochemical localization of janusin in the developing (a–e) and adult (f–h) mouse spinal cord at the cervical level. Janusin is hardly detectable in the spinal cords of neonatal animals (a). In 1-week-old animals (b), intense labelling becomes visible in the dorsal white matter. Expression increases in 2-week-old (c) and 3-week-old (d) animals, and immunoreactivity is detectable in the white and grey matter. In older animals, expression decreases (e, a 4-week-old animal; f, an adult animal). In the white matter, janusin is associated with myelinated axons (g). Spots of increased janusin immunopositivity are present in the white matter (e, f), which correspond to an accumulation of the molecule at nodes of Ranvier (h). Bar: a–f, 300 µm; g 30 µm; h 15 µm.

probe will provide a tool to decide unequivocally whether a subpopulation of janusin-expressing glial cells in tissue sections is also positive for GFAP, either by combining differently labelled janusin- and GFAP-

specific cRNA probes or a janusin probe and GFAP immunocytochemistry.

Whereas expression of tenascin in the CNS is restricted to astroglial cells (Prieto *et al.*, 1990; Tsukamoto *et al.*, 1991; S. Bartsch *et al.*,

1992; U. Bartsch *et al.*, 1992), janusin is expressed not only by oligodendroglial cells but also by different neuronal cell types, including horizontal cells of the retina and motor neurons of the spinal cord. In addition, interneurons located in the molecular layer of the developing and adult cerebellar cortex contain janusin-specific transcripts (Fuss *et al.*, 1993). Neurons have not been identified as janusin-expressing cells in earlier studies (Pesheva *et al.*, 1989), possibly because only a limited number of distinct neuronal cell types, and thus only a minority of all CNS neurons, express these glycoproteins. The functional significance of janusin expression by a small number of neuronal cell types remains to be elucidated. We consider it unlikely, however, that expression of janusin by neurons results from the fact that, by analogy to oligodendrocytes and type-2 astrocytes, which derive from a common oligodendrocyte-type-2 astrocyte progenitor cell (Raff, 1989), oligodendrocytes and neurons have also been reported to originate from a common progenitor cell (Williams *et al.*, 1991). The progenitor cells of janusin-expressing retinal horizontal cells give rise to the different retinal neuronal cell types and to Müller cells but not to oligodendrocytes (Turner and Cepko, 1987). The expression of janusin by motor neurons in the spinal cord is worth mentioning, since these neurons project their axons into janusin-immunonegative peripheral nerves (Pesheva *et al.*, 1989; Wintergerst, Bartsch and Schachner unpublished observations). These findings suggest either that janusin is absent from the axonal surface of motor neurons or that it is differentially distributed on the axonal cell surface of this nerve cell type, being present only on the axonal segment restricted to the CNS.

Expression of janusin at the protein level is developmentally regulated in the neuron-free optic nerve, where levels of janusin protein increase up to the second or third postnatal week and then decrease again towards adulthood. In the oligodendrocyte-free retina, in contrast, levels of janusin expression are similar from the second postnatal week until adulthood. Similarly, down-regulation of janusin is more pronounced in white matter tracts of the spinal cord when compared to the grey matter, which contains the janusin-expressing motoneurons. Based on these observations, we have suggested that expression of glia-derived, but not neuron-derived, janusin is developmentally regulated (Bartsch *et al.*, 1993). These interpretations could be confirmed by our *in situ* hybridization analysis. Whereas the number and labelling intensity of labelled oligodendrocytes decreases significantly with increasing age, the number and labelling intensity of neurons remains similar throughout postnatal development and into adulthood. The developmental regulation of janusin expression by oligodendrocytes is also reflected in the fact that the late-forming and late-myelinating corticospinal tract (Jordan *et al.*, 1989) contains a high density of cells strongly labelled for janusin at times when the density of cells expressing janusin in other regions of the spinal cord is already reduced. By analogy, the analysis of janusin expression in cultures enriched in oligodendrocytes and their progenitor cells reveals that janusin expression by oligodendrocytes is dependent on the developmental stage of the cells, since it decreases with increasing maturity of oligodendrocytes (Jung *et al.*, 1993). Interestingly, expression of janusin by oligodendrocytes is susceptible to the influence of other neural cell types. Whereas the percentage of oligodendrocytes expressing janusin is increased when they are cocultured with astrocytes, it is reduced in cocultures with neurons, when compared to pure oligodendrocyte cultures (Jung *et al.*, submitted). Thus, it is tempting to speculate that down-regulation of janusin expression by oligodendrocytes *in vivo* may result from an increasing influence of neurons after the period of myelination, when oligodendrocytes and myelinated axons are in intimate cell-cell contact.

In the adult mouse optic nerve and spinal cord, only a few glial cells contain janusin mRNA. We suggest that the glial cells expressing janusin

might correspond to newly generated oligodendrocytes, derived from oligodendrocyte progenitor cells present in adult optic nerves (ffrench-Constant and Raff, 1986b; Wolswijk and Noble, 1989). This hypothesis is supported by preliminary experiments in which some janusin mRNA-expressing cells in optic nerves of adult mice incorporated 5-bromo-2 deoxyuridine (Gratzner *et al.*, 1982; Miller and Nowakowski, 1988) after cumulative intraperitoneal injections, indicating that these cells have been newly generated during the application period (Wintergerst, Bartsch and Schachner, unpublished observations).

Several properties of janusin show striking similarities to the recently described chick neural extracellular matrix molecule restrictin (Rathjen *et al.*, 1991; Nörenberg *et al.*, 1992). The two molecules have comparable molecular masses in reducing gels, exist in mixtures of monomers, dimers and trimers, have a similar structural architecture and 85% similarity in their amino acid sequence, indicating that restrictin represents a species homologue of janusin. Most interesting in the context of the present study are the following observations. First, restrictin immunoreactivity is mainly restricted to the white matter of the developing cerebellar cortex. Second, restrictin is expressed in the embryonic chick retina and is detectable on some, but not all, retinal neurons *in vitro*. Third, restrictin immunoreactivity in the developing chick spinal cord is visible around motor neurons and along motor axons (Rathjen *et al.*, 1991). A comparison between these immunocytochemical observations and our *in situ* hybridization data indicates that janusin and restrictin are similarly distributed in the CNS of mice and chickens respectively.

Purified janusin is non-adhesive for the attachment of small cerebellar neurons after some time in culture (Pesheva *et al.*, 1989; Morganti *et al.*, 1990). Janusin has not only non-permissive substrate properties for the attachment of neuronal cell bodies but also repulsive properties for advancing growth cones when offered as a sharp boundary. Moreover, it inhibits the elongation of neurites from CNS neurons (i.e. retinal ganglion cells), but not peripheral nervous system neurons (i.e. dorsal root ganglion cells) when offered as a homogeneous substrate in a mixture with the permissive substrate laminin (Taylor *et al.*, 1993). It is therefore tempting to speculate that the presence of janusin might be critical for the low regenerative capacity of differentiated CNS tissue. This hypothesis is strengthened by the following observations: (1) janusin is expressed late during development; (2) expression of janusin is restricted to the CNS and is not detectable in the regeneration-competent peripheral nervous system; (3) janusin is predominantly, although not exclusively, expressed by oligodendrocytes, glial cells known to be inhibitory for axonal growth (Schwab and Caroni, 1988; Fawcett *et al.*, 1989; Bandtlow *et al.*, 1990; Schwab, 1990). The analysis of janusin expression in lesioned CNS tissue might provide more information on whether this molecule is of functional relevance for cellular interactions following nervous tissue trauma.

Finally, janusin might be functionally involved in the formation of CNS myelin, since highest expression by oligodendrocytes is visible during the period of myelination, and since janusin is associated with myelinated axons and nodes of Ranvier and is detectable on the processes of myelinating oligodendrocytes before compaction of myelin occurs (Bartsch *et al.*, 1993).

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Abbreviations

BSA	bovine serum albumin
cDNA	complementary DNA
cRNA	complementary RNA
CNS	central nervous system
DMEM	Dulbecco's modified Eagle's medium
EDTA	ethylenediamine tetraacetic acid
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFAP	glial fibrillary acidic protein
HBSS ⁻	Hank's buffered salt solution
mRNA	messenger RNA
N-CAM	neural cell adhesion molecule
N-CAM-180	180-kD component of N-CAM
PBS	phosphate-buffered saline
TRITC	tetramethylrhodamine isothiocyanate

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