

# Tenascin-R Is Antiadhesive for Activated Microglia that Induce Downregulation of the Protein after Peripheral Nerve Injury: a New Role in Neuronal Protection

Doychin N. Angelov,<sup>1</sup> Michael Walther,<sup>1</sup> Michael Streppel,<sup>2</sup> Orlando Guntinas-Lichius,<sup>2</sup> Wolfram F. Neiss,<sup>1</sup> Rainer Probstmeier,<sup>4</sup> and Penka Pesheva<sup>3</sup>

Departments of <sup>1</sup>Anatomy and <sup>2</sup>Oto-Rhino-Laryngology, University of Cologne, 50924 Cologne, Germany, and Departments of <sup>3</sup>Physiology, Neurophysiology, and <sup>4</sup>Biochemistry, Institute of Animal Anatomy and Physiology, University of Bonn, 53111 Bonn, Germany

Microglial activation in response to pathological stimuli is characterized by increased migratory activity and potential cytotoxic action on injured neurons during later stages of neurodegeneration. The initial molecular changes in the CNS favoring neuronofugal migration of microglia remain, however, largely unknown. We report that the extracellular matrix protein tenascin-R (TN-R) present in the intact CNS is antiadhesive for activated microglia, and its downregulation after facial nerve axotomy may account for the loss of motoneuron protection and subsequent neurodegeneration. Studies on the protein expression in the facial and hypoglossal nucleus in rats demonstrate that TN-R is a constituent of the perineuronal net of motoneurons and 7 d after peripheral nerve injury becomes downregulated in the corresponding motor nucleus. This downregulation is reversible under regenerative (nerve suture) con-

ditions and irreversible under degenerative (nerve resection) conditions. In short-term adhesion assays, the unlesioned side of brainstem cryosections from unilaterally operated animals is nonpermissive for activated microglia, and this nonpermissiveness is almost abolished by a monoclonal antibody to TN-R. Microglia-conditioned media and tumor necrosis factor- $\alpha$  downregulate TN-R protein and mRNA synthesis by cultured oligodendrocytes, which are one of the sources for TN-R in the brainstem. Our findings suggest a new role for TN-R in neuronal protection against activated microglia and the participation of the latter in perineuronal net destruction, e.g., downregulation of TN-R.

**Key words:** axotomy; antiadhesive substrate; cell adhesion; extracellular matrix; facial nerve; hypoglossal nerve; microglia; oligodendrocyte; tenascin-R; TNF- $\alpha$

The appearance of activated microglia with high migratory and potentially cytotoxic capacity toward neurons and oligodendrocytes (OLs) has been observed in association with different CNS pathologies and is suggested to be one of the regeneration-inhibitory signals (Kreutzberg, 1996; Moore and Thanos, 1996). Although a number of studies has contributed to our understanding of microglial activation, the molecular changes in the CNS environment that favor the rapid migration and stable adhesion of microglia at the surface of injured neurons remain primarily unknown. During normal development, the establishment of complex communities of neural cells and their projections critically depends on the balance of adhesion-promoting or adhesion-inhibitory environmental signals present in the extracellular matrix (ECM) or at the membrane surface (Venstrom and Reichardt, 1993; Goodman, 1996). A typical example for such ECM molecules are the two members of the tenascin (TN) family, TN-C and TN-R (Erickson, 1993; Chiquet-Ehrismann, 1996). Depending on their topographical expression and the repertoire of cell surface receptors or intracellular signaling cascades, they can act as (1) adhesive or antiadhesive molecules and

(2) inhibitors or promoters of neurite outgrowth (Pesheva et al., 1993, 1997; Chiquet-Ehrismann, 1995; Götz et al., 1996; Nörenberg et al., 1996; Schumacher et al., 1997). In contrast to TN-C, TN-R is amply expressed in the adult CNS, in which it exists in two major isoforms of 160 (TN-R 160) and 180 (TN-R 180) kDa (Pesheva et al., 1989). TN-R is expressed by myelinating OLs and small subsets of CNS neurons (interneurons and motoneurons) during postnatal development and in adulthood (Pesheva et al., 1989; Fuss et al., 1993; Wintergerst et al., 1993). Mammalian TN-R is adhesive for macroglial cells and antiadhesive for various CNS neurons (Pesheva et al., 1989, 1993, 1997; Morganti et al., 1990; Taylor et al., 1993). The substrate-bound protein thus promotes OL cell adhesion and terminal differentiation by a sulfatide-mediated mechanism and inhibits neuron adhesion and axon outgrowth *in vitro* by its interaction with the neuronal protein F3/11.

Studies on TN-C during CNS pathology give evidence for the upregulation of protein expression in association with reactive gliosis and suggest its implication in the molecular control of cell migration, angiogenesis, and axon remodeling (Higuchi et al., 1993; Niquet et al., 1995; Zagzag et al., 1996; Scheffler et al., 1997). The expression of TN-R under pathological conditions remains, however, obscure. In the present study, we have examined the expression patterns of TN-R and TN-C in the adult rat brainstem and their functional relevance to the appearance of activated microglia after peripheral axotomy of the facial or hypoglossal nerve, because this animal model allows a precise analysis of (1) the cellular and molecular changes occurring in the

Received March 2, 1998; revised June 3, 1998; accepted June 8, 1998.

This work was supported by the BONFOR Research Program (P.P.), the Deutsche Forschungsgemeinschaft (R.P.), and the Jean Uhrmacher Foundation (M.S. and O.G.-L.). We thank Ilona Köpernick for expert technical help with the preparation of brainstem cryosections for cell adhesion assays.

Correspondence should be addressed to Dr. Penka Pesheva, Department of Physiology, Neurophysiology, University of Bonn, Wilhelmstrasse 31, 53111 Bonn, Germany.

Copyright © 1998 Society for Neuroscience 0270-6474/98/186218-12\$05.00/0

corresponding motor nucleus under experimental conditions allowing or not allowing subsequent regeneration and (2) microglial activation (Streit and Graeber, 1993; Angelov et al., 1995; Graeber, 1996).

We report that TN-R present in the perineuronal net of motoneurons becomes downregulated in the lesioned nucleus when activated microglia are found in direct contact with motoneurons. Functional studies *in vitro* imply the antiadhesive substrate properties of TN-R for activated microglia and a potential role of the latter in downregulation of the protein.

## MATERIALS AND METHODS

**Materials.** Mouse monoclonal Abs tn-R1 and tn-R2 (clones 597 and 596) to TN-R have been characterized (Pesheva et al., 1989). Monoclonal Ab tn-R6 was raised in BALB/c mice using an equimolar mixture of adult brain-derived chick and human TN-R immunoaffinity purified on a tn-R2 Ab column as antigen and for the screening of positive hybrid clones. The Ab belongs to the IgG1 subclass and recognizes a protein epitope of yet unknown location present on the 160 and 180 kDa isoforms of TN-R derived from chick, mouse, rat, bovine, and human brain. In Western blot analyses of brain extracts from a number of vertebrates, tn-R6 reacts with two single bands of 160 and 180 kDa corresponding to TN-R (P. Pesheva, E. Spiess, K. Winterhalter, and T. Peshev, unpublished observations). Polyclonal Abs to mouse brain-derived TN-R 160 (pTN-R), which react with TN-R 160 and TN-R 180 from rat brain but not with TN-C, were produced in rabbits. Polyclonal Abs to mouse brain-derived TN-C (recognizing TN-C in the rat) and laminin (LN) from Engelbreth-Holm-Swarm (EHS) mouse sarcoma were produced in rabbits (Pesheva et al., 1994). Rat monoclonal Ab J1/tn2 recognizes the fibronectin type III domain fnD of TN-C (Götz et al., 1996). Mouse monoclonal Abs O4 and O1 recognize glycosphingolipids expressed at the cell surface of OLs of different maturation stages (Sommer and Schachner, 1981; Bansal et al., 1989). Monoclonal Ab Ox-42 recognizing the rat complement receptor type 3 (CR 3) complex was purchased from Serotec (Oxford, England). Biotin-labeled Vicia villosa agglutinin was purchased from Sigma (Deisenhofen, Germany). TN-R was purified from brains of adult Wistar rats by immunoaffinity chromatography on a monoclonal tn-R2 Ab column (Pesheva et al., 1989). These TN-R preparations consisted of an approximately equimolar mixture of the 160 and 180 kDa isoforms and were recognized by all monoclonal Abs to TN-R used in this study when analyzed by Western blot and ELISA.

**Surgery.** For all surgical operations, 3-month-old female Wistar rats (strain HsdCpb:Wu) were used. Sixty-two animals underwent surgery, and two animals served as normal controls. The surgical operations on facial and hypoglossal nerves were performed under a microscope by trained microsurgeons. The animals were anesthetized with ether, and after an intraperitoneal injection of 1.4 ml of Avertin (2 gm of tribromethanol, 1 ml of 3-pentanol, 8 ml of absolute ethanol, and 90 ml of 0.9% NaCl), the main trunk of the facial or hypoglossal nerve was unilaterally exposed and immobilized. Nerves were axotomized under conditions allowing (transection and suture) or not allowing (resection) subsequent regeneration.

**Transection and suture.** Forty rats underwent an unilateral transection and immediate end-to-end suture of the facial (20 rats) or the hypoglossal (20 rats) nerve. The main trunk of the facial nerve was exposed and transected at its emergence from the foramen stylomastoideum distally to the posterior auricular branch. The proximal stump was then microsurgically sutured to the distal stump with two 11–0 atraumatic sutures (Ethicon), an operation known as facial-facial anastomosis (FFA) (Guntinas-Lichius et al., 1994). Animals undergoing FFA were divided into six groups (of 2 or 4 rats each) that were allowed to survive for 3, 5, 7, 14 (4 rats each), 56, and 112 postoperative days (PODs) (2 rats each). For unilateral transection of the hypoglossal nerve, the nerve trunk was exposed and transected between its medial and lateral branches, followed by an immediate end-to-end suture [(hypoglossal–hypoglossal anastomosis (HHA)). Animals undergoing HHA were also divided into six groups (of 2 or 4 rats each, as above) and allowed to survive for the same time periods as described above.

**Resection.** Sixteen rats underwent an unilateral resection of the motor nerve. In eight rats, a piece of 8–10 mm length of the right temporal, zygomatic, buccal, upper, and lower divisions of the marginal mandibular branch was removed surgically. In the other eight rats, a piece of 8–10 mm length of the hypoglossal nerve was removed (Neiss et al., 1992;

Guntinas-Lichius et al., 1994). Animals undergoing resection of the facial or hypoglossal nerve were divided into four groups each that were allowed to survive for 7, 14, 30, and 112 PODs (2 rats each).

At the end of each postoperation period, animals were anesthetized with ether, and their vascular systems were rinsed with 0.9% NaCl. Rats were then treated with fixative by transcardial perfusion with a periodate–lysine–paraformaldehyde fixative containing 4% paraformaldehyde (PA) and 10 mM sodium *meta*-periodate in 0.2 M lysine–HCl buffer, pH 7.4 (McLean and Nakane, 1974). The brainstems were subsequently removed and cut into 50- $\mu$ m-thick coronal vibratome sections in 0.1 M phosphate buffer, pH 7.4.

**Immunocytochemistry.** Immunostaining of tissue sections was performed as described previously (Angelov et al., 1995, 1996b). The immunoreaction product was visualized using a horseradish peroxidase–avidin–biotin complex and diaminobenzidine tetrahydrochloride (DAB). In control experiments, the omission of primary or biotinylated secondary Abs resulted in a complete lack of immunoreaction product.

Perineuronal nets around motoneurons were identified by biotinylated Vicia villosa agglutinin (Sigma). Tissue sections were incubated with biotinylated Vicia villosa agglutinin (1:200) for 3 hr, and lectin binding was detected by Cy2-conjugated streptavidin (1:100) (Sigma).

The expression of specific cell surface markers by cultured microglial cells and oligodendrocytes from early postnatal rat brain was analyzed by indirect immunofluorescence (Pesheva et al., 1998) using Cy3-conjugated secondary Abs (Jackson ImmunoResearch, West Grove, PA).

**Electron microscopy.** After HRP-DAB reaction, sections were rinsed in 0.1 M cacodylate buffer, pH 7.2, incubated for 2 hr in 1% OsO<sub>4</sub> and 1.5% K<sub>3</sub>Fe<sup>III</sup>(CN)<sub>6</sub> in the same buffer, and dehydrated in graded acetones. Sections were then embedded in araldite (Durcupan ACM; Fluka, Buchs, Switzerland) and further processed for electron microscopy.

**Glial cell cultures.** Microglial cells were obtained from cerebral glial cultures of 1- or 2-d-old rat pups (from Wistar or Lewis rats) as described previously (Pesheva et al., 1998). After 11–14 d in culture, microglial cells were shaken off the astrocytic monolayer and immediately used for cell adhesion assays (see below) or maintained for several days in culture in DMEM containing 10% or 5% fetal calf serum (FCS). This procedure yielded a homogeneous population of microglial cells expressing marker molecules for activated microglia, such as CR 3 and galectin-3 (Graeber et al., 1988; Pesheva et al., 1998).

OLs were obtained from cerebral glial cultures of neonatal rat pups (McCarthy and de Vellis, 1980). After 12 d in culture in DMEM containing 10% FCS, OLs were shaken off the astrocytic monolayer, collected by centrifugation (600  $\times$  g for 10 min at 4°C), and resuspended in the same medium. Contaminating microglial cells were removed from the cell suspensions by preadhesion to untreated culture dishes (twice for 30 min at 37°C). Nonadherent cells were then collected by centrifugation, resuspended in DMEM containing 5% FCS, plated onto poly-L-lysine (PLL)-coated (0.1 mg/ml in water) tissue culture dishes at a density of 1–2  $\times$  10<sup>6</sup> cells/ml, and maintained for several days in culture. Under these conditions, 100% of the cells were O4-positive, and the majority of them were O1-positive, defining them as mature OLs (R. Probstmeier and P. Pesheva, unpublished observations).

Microglia-conditioned media (MCM) were obtained after 2 or 3 d in culture in DMEM containing 5% FCS, cleared by centrifugation (100,000  $\times$  g for 20 min at 4°C), and immediately frozen at –70°C until use or directly applied to cultured OLs. After 2 d in culture on PLL-treated multiwell plates (4- or 6-well plates; Nunc, Roskilde, Denmark), OLs were cultured for 2 d in MCM or DMEM containing 5% FCS in the absence or presence of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (200 U/ml; PeproTech, Rocky Hill, NJ) or interleukin-1 $\beta$  (IL-1 $\beta$ ) (10 U/ml; PeproTech). The resulting OL-conditioned media were cleared by centrifugation (100,000  $\times$  g for 20 min at 4°C) and stored at –70°C until use.

**Cell adhesion assays.** For cell adhesion assays on purified protein substrates, tissue culture dishes were coated with PLL (concentration of 0.01%), washed three times with water, and air-dried. TN-R and LN from EHS sarcoma (20  $\mu$ g/ml in PBS) were coated onto the PLL layer as 2  $\mu$ l droplets for 60 min at 37°C, and dishes were then washed twice with PBS. After 60 min of incubation at 37°C with PBS containing 2% heat-inactivated bovine serum albumin (BSA), Abs to TN-R (final concentration of 100  $\mu$ g/ml) were added to the solution, and dishes were incubated overnight at 4°C and subsequently washed three times with PBS. Microglial cells derived from mixed glial cultures of 1- or 2-d-old rat pups (see above, Glial cell cultures) were plated onto the substrates at a density of 1  $\times$  10<sup>6</sup> cells/ml in DMEM containing 10% FCS. After 30 min

of incubation at 37°C, nonadherent cells were washed away with PBS, and dishes were incubated for 30 min at room temperature with 4% PA in PBS. Adherent cells were then stained for 20 min with 0.5% toluidine blue in 2.5% Na<sub>2</sub>CO<sub>3</sub>, washed once with water, and air-dried. The substrate spots were visualized by ELISA using monoclonal tn-R2 and polyclonal Abs to LN, and the respective secondary alkaline phosphatase-conjugated Abs (Promega, Madison, WI) or in Ab perturbation experiments, the secondary Abs only. For estimation of the number of cells adhering to the different substrates, cells from micrographs were counted in microscopic fields corresponding to 800  $\mu$ m<sup>2</sup>. Mean  $\pm$  SD of number of adherent cells in five different microscopic fields are represented as percentages.

For cell adhesion assays on brainstem cryosections, two rats underwent unilateral resection of the facial nerve, and another two underwent resection of the hypoglossal nerve. After a postoperative survival period of 10 d, the brainstems were quickly removed and immediately frozen at  $-159^{\circ}\text{C}$  in melting isopentane precooled in liquid nitrogen. The brainstem regions containing the facial or hypoglossal nuclei were then cut into 10- $\mu$ m-thick cryosections, mounted onto sterile coverslips (11 mm in diameter), placed into wells of 24-well plates, and kept at  $-70^{\circ}\text{C}$  until use. Microglial cells shaken off the astrocytic monolayer (see above) were labeled for 10 min at room temperature with bisbenzimidazole (20  $\mu$ g/ml in Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free HBSS). Cells were subsequently washed three times (600  $\times$  g for 5 min at 4°C) with cold DMEM containing 10% FCS (culture medium) and resuspended in culture medium. Frozen 24-well plates containing brainstem cryosections were kept for 10 min at room temperature and incubated for 90 min at 37°C with 200  $\mu$ l/well culture medium containing or not containing monoclonal and polyclonal Abs to TN-R (100  $\mu$ g of IgG/ml culture medium). For each individual Ab, five equidistant cryosections through the nuclei (with an interval between the sections of  $\sim 200$   $\mu$ m) were preincubated as described above and used as a substrate for microglial adhesion. The medium was then carefully removed and single-cell suspensions of labeled microglial cells (1  $\times 10^6$  cells/ml culture medium; 200  $\mu$ l/well) were added to the wells. After 30 min of incubation at 37°C, unbound cells were gently washed away, once with 500  $\mu$ l of culture medium and twice with 500  $\mu$ l of PBS containing 1% BSA. Cryosections were subsequently treated for 30–45 min at room temperature with 4% PA containing 5% sucrose prewarmed at 37°C, washed twice with PBS, and embedded. For quantitative analysis, the boundaries of the nuclei on each section were delineated in the bright-field image using a CCD video camera system (Optronics Engineering) and image analyzing software Optimas 6.1 (Optimas) and were superimposed onto the fluorescence image of bisbenzimidazole-labeled cells (filter set 01; Zeiss, Jena, Germany). The number of labeled cells adherent to individual nuclei of the operated and unoperated side was counted and related to the surface area of the nucleus. The data obtained are represented as mean  $\pm$  SD of the number of cells per area (500  $\mu$ m<sup>2</sup>) of a nucleus from the unoperated (control) and operated side, respectively. To measure statistically significant Ab effects, the Student's *t* test was applied.

**Western blot analysis.** Two rats underwent unilateral resection of the facial nerve, and at POD 7, the animals were anesthetized and decapitated. The regions of the brainstem containing the facial nuclei were quickly removed and separated along the midline into an operated and unoperated portion. The tissue pieces were subsequently homogenized in 10 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, pH 7.4, containing 1 mM spermidine, 1  $\mu$ M aprotinin, 5  $\mu$ M soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, and 1 mM type III egg-white trypsin inhibitor (extraction buffer) extracted for 90 min at 37°C, and insoluble material was cleared by centrifugation (100,000  $\times$  g for 30 min at 4°C). TN-R in tissue extracts of equal protein concentration was selectively immunoprecipitated by using pTN-R and pansorbin cells (Calbiochem, La Jolla, CA) or monoclonal Ab tn-R2 and rabbit anti-mouse IgG (Jackson ImmunoResearch) linked to pansorbin cells as a carrier. After an end-to-end rotation overnight at 4°C, pansorbin cells were washed three times with 1 ml of extraction buffer (100,000  $\times$  g for 2 min at 4°C), resuspended in 40  $\mu$ l of SDS sample buffer (Laemmli, 1970), and boiled for 4 min. Pansorbin cells were removed by centrifugation, and protein samples were subjected to SDS-PAGE using 7% polyacrylamide slab gels and further analyzed by Western blot using monoclonal TN-R Abs or pTN-R, secondary HRP-conjugated Abs (Boehringer Mannheim, Mannheim, Germany), and the ECL method for detection (Pierce, Rockford, IL).

**Sandwich ELISA.** Media conditioned by OLs (see above, Glial cell cultures) were analyzed by sandwich ELISA using monoclonal Abs to

TN-R as a linker (tn-R1 or tn-R2 at a coating concentration of 20  $\mu$ g/ml) and pTN-R immunoaffinity purified on a TN-R column for detection. After incubation of OL-conditioned media (overnight at 4°C, 100  $\mu$ l/well) with the immobilized monoclonal Abs, polyclonal Ab binding (for 2 hr at 37°C) was detected by biotinylated Fab fragments of sheep anti-rabbit IgG and streptavidin-HRP conjugate (Boehringer Mannheim). Values for the TN-R content under different culture conditions were plotted onto a standard curve prepared in parallel from purified rat TN-R (2  $\mu$ g/ml to 3 pg/ml) and represented as mean  $\pm$  SD of triplicate measurements.

**RT-PCR.** Total RNA was isolated from 2–4  $\times 10^6$  OLs cultured under different conditions by phenolchloroform extraction (Chomczynski and Sacchi, 1987), and 3  $\mu$ g of RNA was used for oligo-dT-primed single-strand cDNA synthesis with superscript reverse transcriptase using the SuperScript preamplification system (Life Technologies, Eggenstein, Germany) in 20  $\mu$ l of reaction volume. Single-strand cDNA (4  $\mu$ l) was amplified in 20  $\mu$ l of reaction volume using the rat TN-R-specific primers 5'-GACATACAAGTCCACCGAT-3' and 5'-CTGTGAGACGATG-GATGTA-3' in 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>, 0.1% Triton X-100, 0.2  $\mu$ M dNTP, and 0.26  $\mu$ M primer each. This amplification leads to a 827 bp product. As a control, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH)-specific primers were used with 1  $\mu$ l of template cDNA. Products were amplified through 39 cycles of 45 sec at 94°C, 45 sec at 50°C, and 90 sec at 72°C. PCR products (8  $\mu$ l) were analyzed on 1% agarose gels.

## RESULTS

### TN-R is a constituent of the perineuronal net of motoneurons

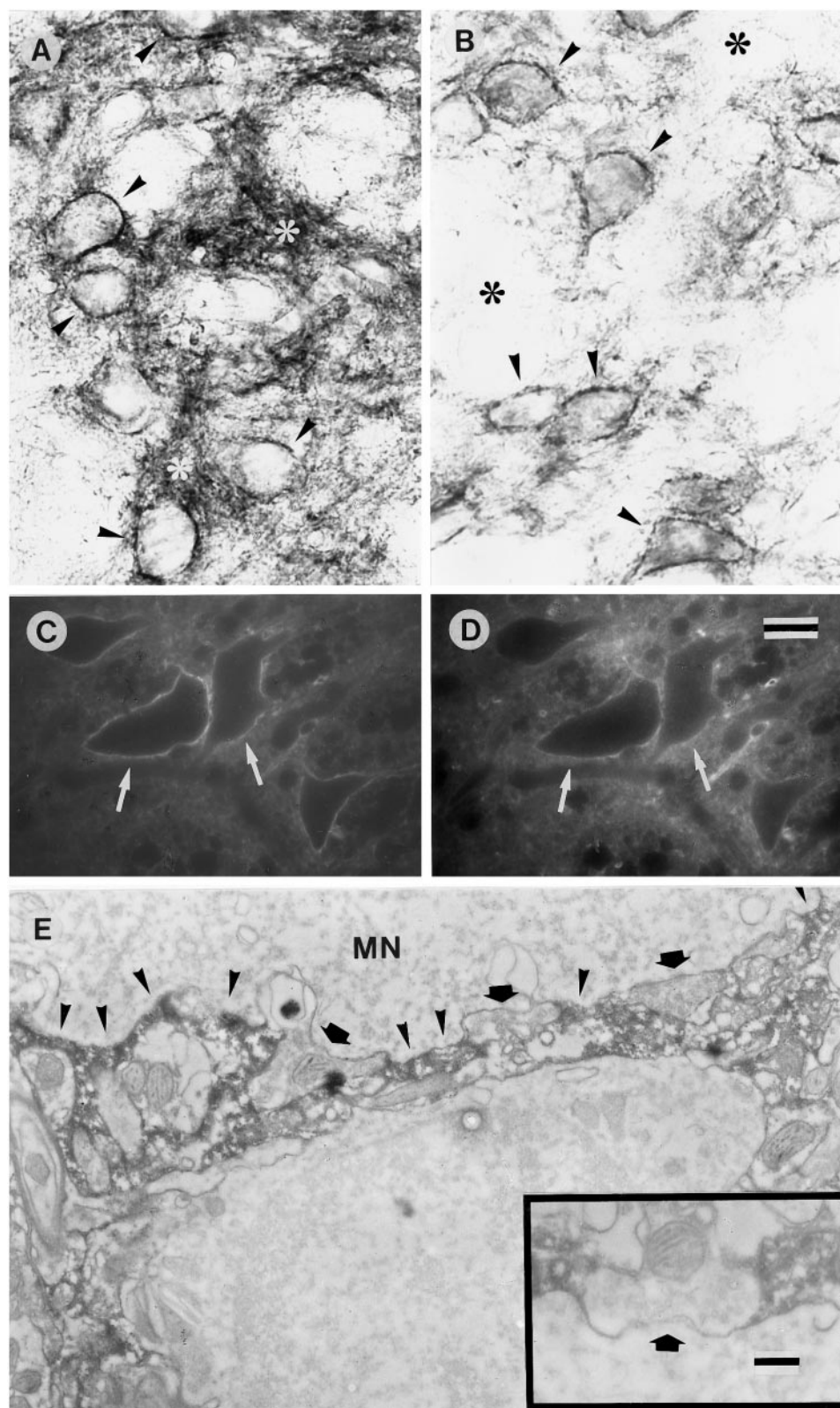
In the adult CNS, TN-R appears in association with the surface of interneurons, motoneurons, OLs, and myelinated axons in the white matter (Pesheva et al., 1989; Rathjen et al., 1991; Bartsch et al., 1993). In the OL-free rodent retina, the protein is detectable in the outer and inner plexiform layers in association with neuronal cell bodies and processes. Immunocytochemical analysis of TN-R expression in the facial or hypoglossal nucleus of the adult rat brainstem revealed two main locations: (1) diffusely spread throughout the neuropil and (2) associated with the surface of motoneuron somata as a well defined band (Fig. 1A). The latter staining pattern was also observed when brainstem sections were analyzed by indirect double immunofluorescence using Vicia villosa agglutinin, a marker for perineuronal nets (Fig. 1C) (Celio and Blümke, 1994), and TN-R-specific Abs (Fig. 1D). Immunostaining of adjacent sections of the facial nucleus with Abs to the structurally related ECM protein TN-C revealed a similar expression pattern but of much weaker intensity (Fig. 1B).

At the ultrastructural level, TN-R-specific immunoreaction product was observed as fine granular and homogeneously distributed deposits in closest contact with the outer surface of neuronal cell membranes and diffusely filling the extracellular space between neighboring glial cells (Fig. 1E). Immunoreaction product outlining the neuronal cell membrane was absent at axosomatic synaptic sites (Fig. 1E, inset). Immunostaining for TN-R was not found in the cytoplasm of motoneurons, in their axonal projections and glial cells (macroglia and microglia), or in the basement membrane of blood vessels; endothelial and perivascular cells were also devoid of immunoreaction product (D. Angelov and M. Walther, unpublished observations).

### TN-R is downregulated in the brainstem after facial nerve axotomy

To study the possible functional implication of TN-R under pathological conditions, we next examined the protein expression in the facial or hypoglossal nucleus after peripheral nerve axo-

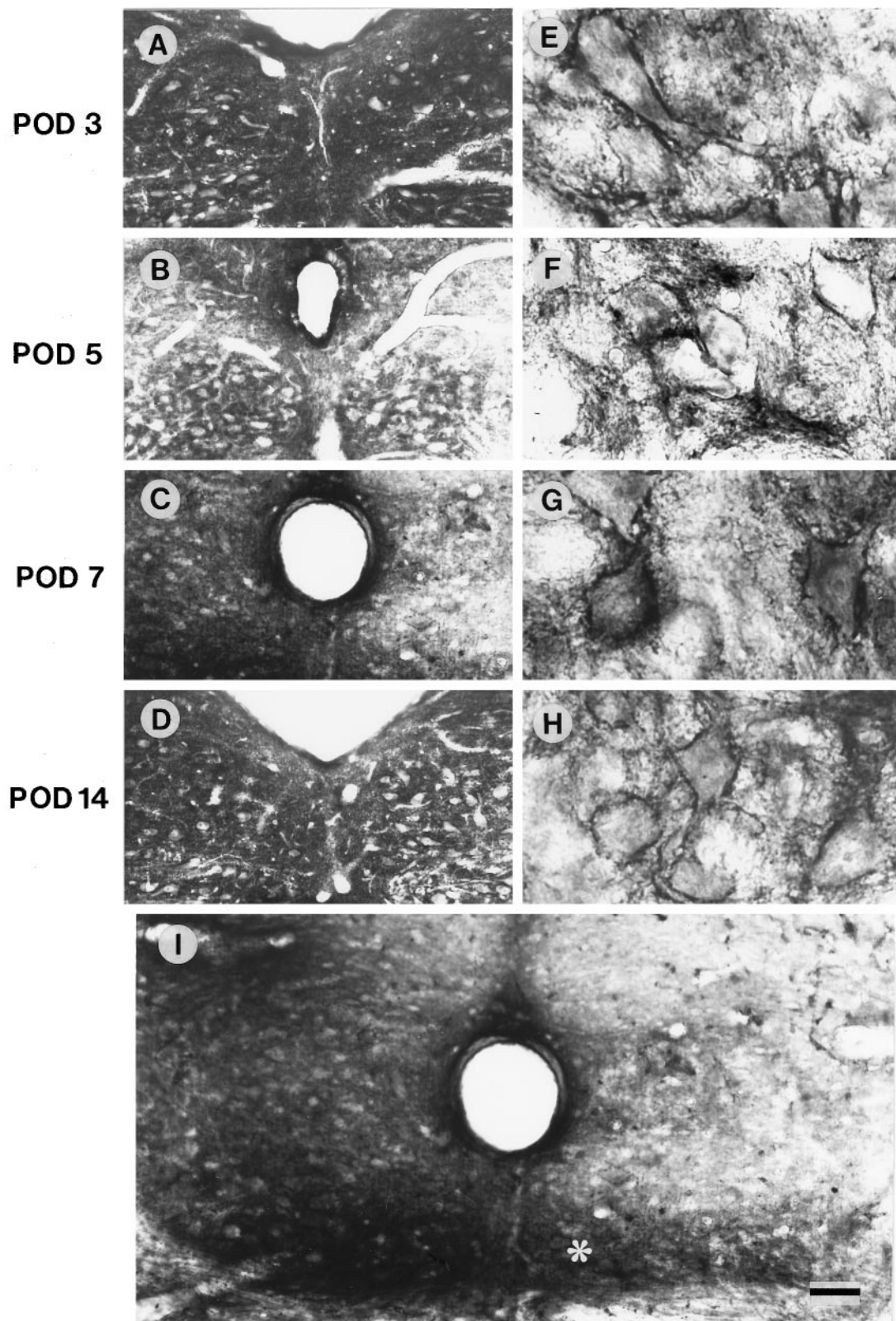




**Figure 1.** Expression pattern of TN-R (*A, D, E*) and TN-C (*B*) in the facial nucleus of the adult rat. *A, B*, Low-power photomicrographs of nuclei immunostained with Ab tn-R1 to TN-R and polyclonal Abs to TN-C. Note the dense (for TN-R) and faint (for TN-C) deposits of immunoreaction product outlining the cell bodies of motoneurons (arrowheads) and the diffuse immunostaining throughout the neuropil (asterisks). *C, D*, Double-immunofluorescent labeling of a nucleus with Vicia villosa agglutinin (*C*) and Ab tn-R4 to TN-R (*D*). Arrows indicate the overlapping staining pattern for TN-R and the lectin at perineuronal nets of motoneurons. Scale bar (in *D*): *A–D*, 50  $\mu$ m. *E*, Immunoelectron micrograph of a peripheral portion of a facial motoneuron (MN). The immunoreaction product (arrowheads) outlines the neuronal cell membrane and fills homogeneously the perineuronal ECM. Note that the axosomatic synaptic clefts (arrows) are devoid of immunoreactivity. Inset shows an axosomatic synapse lacking TN-R at a higher magnification. Scale bar (in inset): *E*, 1  $\mu$ m; inset, 0.3  $\mu$ m.

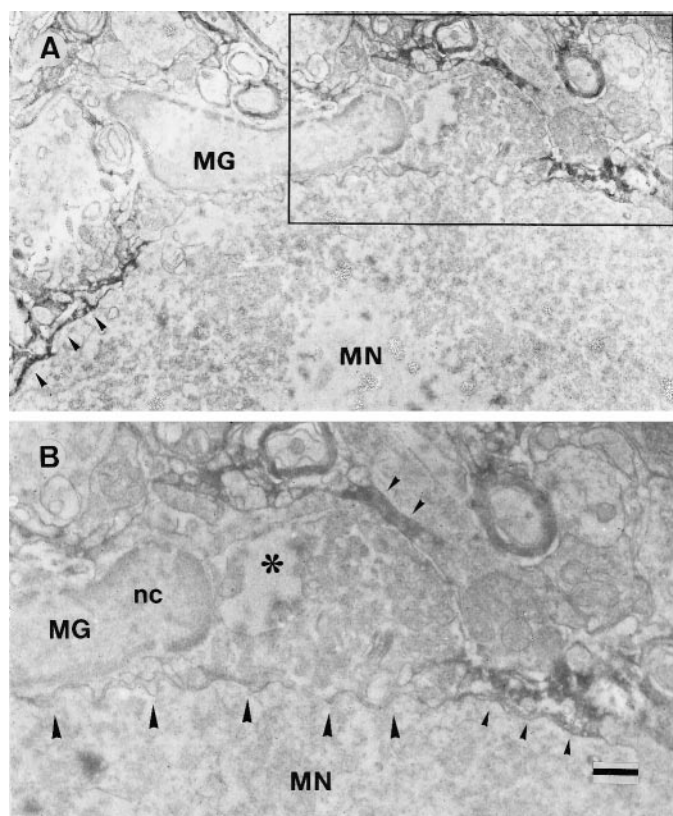
tomy at POD 3–112 in Wistar rats under conditions allowing (transection and immediate suture) or not allowing (nerve resection) subsequent regeneration. Under both conditions, the blood–brain barrier remains intact, thus allowing the selective examination of brain-intrinsic factors (Shelper and Adrian, 1980; Streit and Kreutzberg, 1988).

After FFA or HHA, the axons regenerate successfully within 28–42 PODs (Aldskogius and Thomander, 1986; Angelov et al., 1996a). At POD 3, no obvious changes in the distribution of TN-R were detected (Fig. 2*A*, hypoglossal nucleus). Starting with POD 5, a slight decrease in TN-R expression in the axotomized nucleus could be observed (Fig. 2*B*). Between POD 7 and POD



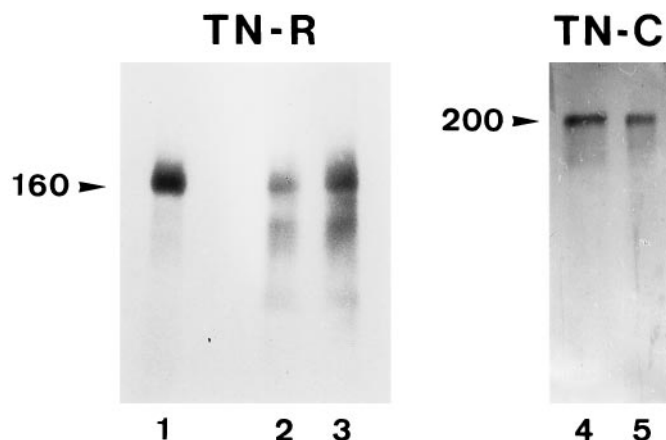
**Figure 2.** Time course of TN-R and TN-C expression in the hypoglossal (TN-R) and facial nucleus (TN-C) after unilateral transection of the corresponding motor nerve. Brainstem sections derived from animals at PODs 3, 5, 7, and 14 and containing control (*left*) and axotomized (*right*) hypoglossal (*A–D, I*) or facial nuclei (*E–H*) were immunostained in parallel with Ab tn-R1 to TN-R (*A–D*) and polyclonal Abs to TN-C (*E–H*), respectively. *I*, Extended photomicrograph of the hypoglossal nucleus shown in *C*. Note the marked decrease in TN-R immunostaining in the axotomized hypoglossal nucleus with the exception of the ventromedial hypoglossal subnucleus (*asterisk*) whose axons have not been transected. Scale bar (in *I*): *A–D*, 100  $\mu$ m; *E–H*, 50  $\mu$ m; *I*, 80  $\mu$ m.





**Figure 3.** Immunoelectron microscopic analysis of TN-R expression in the hypoglossal nucleus 7 d after unilateral transection of the hypoglossal nerve. *A, B*, Immunoelectron micrographs of the periphery of a hypoglossal motoneuron demonstrating that the axosomatic synapses have been replaced by activated microglial cells (*B* corresponds to *inset* in *A*). The contact side (*large arrowheads*) between a microglial cell (*MG*), containing an elongated nucleus (*nc*) and phagolysosome (*asterisk*), and a motoneuron (*MN*) is visible. Note the faint immunoreaction product for TN-R in the neuropil (*small arrowheads*) and the virtual lack of TN-R immunostaining in the tortuous cleft between the motoneuron and the microglial cell. Scale bar (in *B*): *A*, 0.9  $\mu$ m; *B*, 0.5  $\mu$ m.

10, a marked decrease in the expression of TN-R occurred in the neuropil and around motoneurons, although less pronounced (Fig. 2*C,I*, POD 7). During later survival periods (PODs 14, 56, and 112), the expression pattern of the protein did not differ any more from that in the control nucleus or in unoperated animals (Fig. 2*D*, POD 14). In contrast to the downregulation of TN-R, there were no detectable changes in the expression of TN-C at PODs 3 and 5 (Fig. 2*E,F*) and a slight increase in the TN-C-specific immunostaining between PODs 7 and 10 (Fig. 2*G*). From POD 14 on, the immunostaining pattern was similar to that in unoperated animals (Fig. 2*H*, facial nerve). The decreased TN-R immunoreactivity was also evident at the ultrastructural level (Fig. 3). At this time, the well known “synaptic stripping” (i.e., postlesional withdrawal of presynaptic boutons from the motoneuron surface membrane) has occurred (Blinzinger and Kreutzberg, 1968). Notably, microglial cells were observed to contact the lesioned motoneurons exclusively at sites that were virtually devoid of immunoreaction product (Fig. 3*A,B*, *large arrowheads*). Electron microscopic examination of such motoneurons revealed that at 49 of 50 examined contact sites between motoneurons and microglia, TN-R immunoreaction product was completely absent.



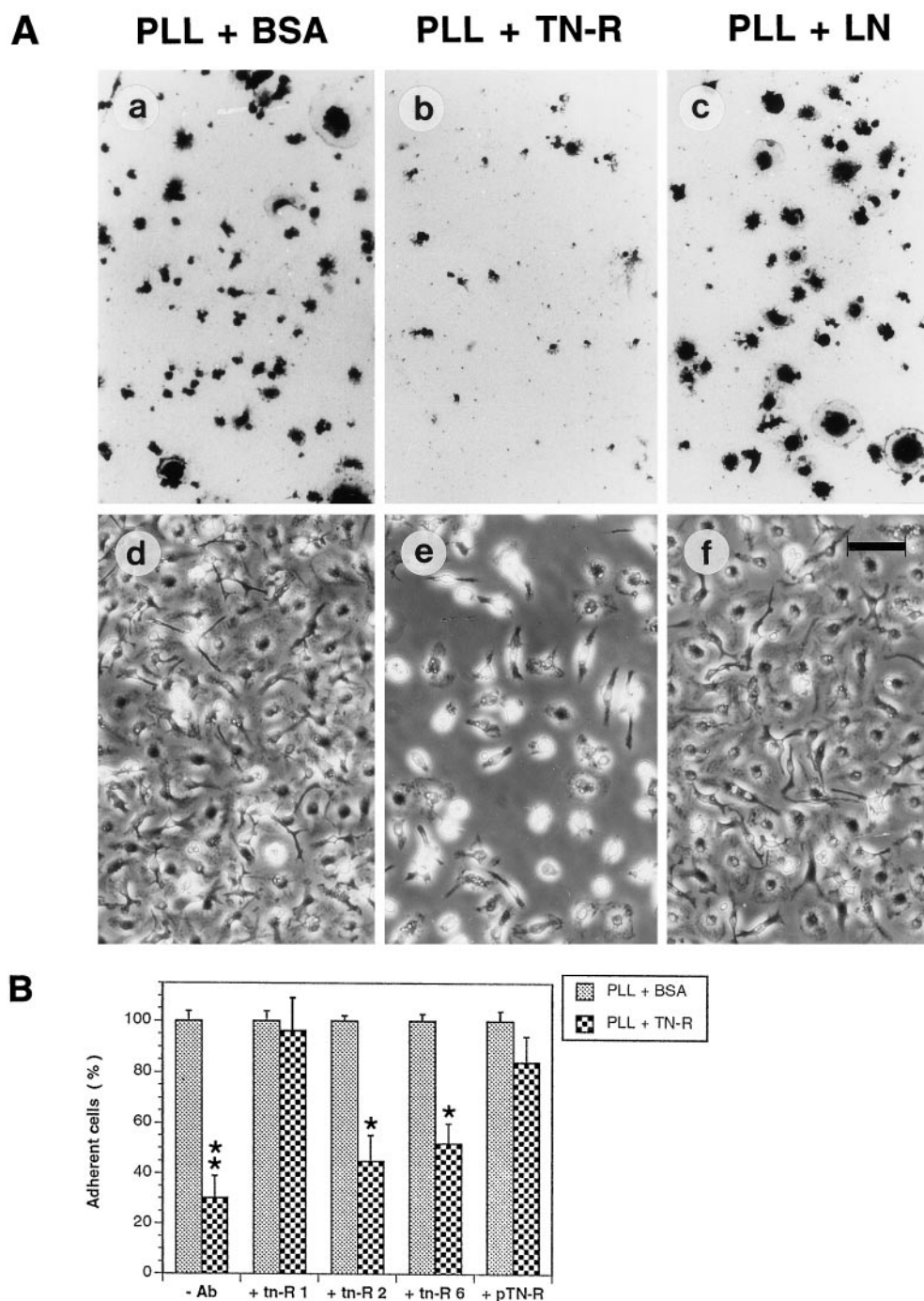
**Figure 4.** Western blot analysis of the expression of TN-R and TN-C in the facial nucleus 7 d after unilateral resection of the facial nerve. TN-R and TN-C were isolated from tissue extracts derived from the operated (*lanes 2 and 4*) and unoperated (*lanes 3 and 5*) sides of the brainstem by sequential immunoprecipitation. Immunoprecipitates and TN-R 160 immunoaffinity purified from adult mouse brain (*lane 1*) were analyzed by Western blot using monoclonal Abs tn-R2 to TN-R (*lanes 1–3*) and J1/tn2 to TN-C (*lanes 4 and 5*). The apparent molecular weights (in kilodaltons) are shown at the left.

In contrast to transection or crush, nerve resection causes a permanent separation of motoneurons from their target musculature, accompanied by a slowly occurring neuron degeneration (Borke et al., 1993; Guntinas-Lichius et al., 1994; Angelov et al., 1995). On resection of the motor nerve, a significant change in the number of motoneurons is first detectable at POD 56 when it comprises ~84% of that in the unlesioned contralateral nucleus (Guntinas-Lichius et al., 1994; Angelov and Neiss, 1996). After resection of the hypoglossal nerve, the decrease in TN-R expression in the respective nucleus at PODs 7 and 14 was similar to that observed at POD 7 after HHA (Fig. 2*C,I*). In the degenerating facial or hypoglossal nucleus, the expression of TN-R further decreased until POD 30, and this downregulation was not followed by a recovery of the normal immunostaining pattern (studied until POD 112). After 30 and 112 PODs, the immunostaining pattern of TN-C in the lesioned facial nucleus was indistinguishable from that in the unlesioned one (Fig. 1*B*) or that observed at POD 14 after FFA (Fig. 2*H*).

Western blot analyses of tissue extracts prepared from lesioned and unlesioned facial nuclei 7 d after unilateral resection of the nerve confirmed the downregulation of TN-R observed by immunohistochemistry (Fig. 4). The amount of TN-R, which in the brainstem is mainly represented by the 160 kDa isoform, was strongly reduced in the lesioned side (Fig. 4, *lane 2*) compared with the unlesioned side (Fig. 4, *lane 3*) of the nucleus. In contrast, there were no significant changes in the amount of TN-C (Fig. 4, *lanes 4 and 5*).

### TN-R is antiadhesive for activated microglia

The downregulation of TN-R expression after motor nerve axotomy coincides with the appearance and neuronopetal migration of activated microglia in the lesioned nucleus (Blinzinger and Kreutzberg, 1968). Molecular cues enabling their clustering around injured neurons may be provided by microglia themselves, as suggested by the elevated expression of cell adhesion molecules



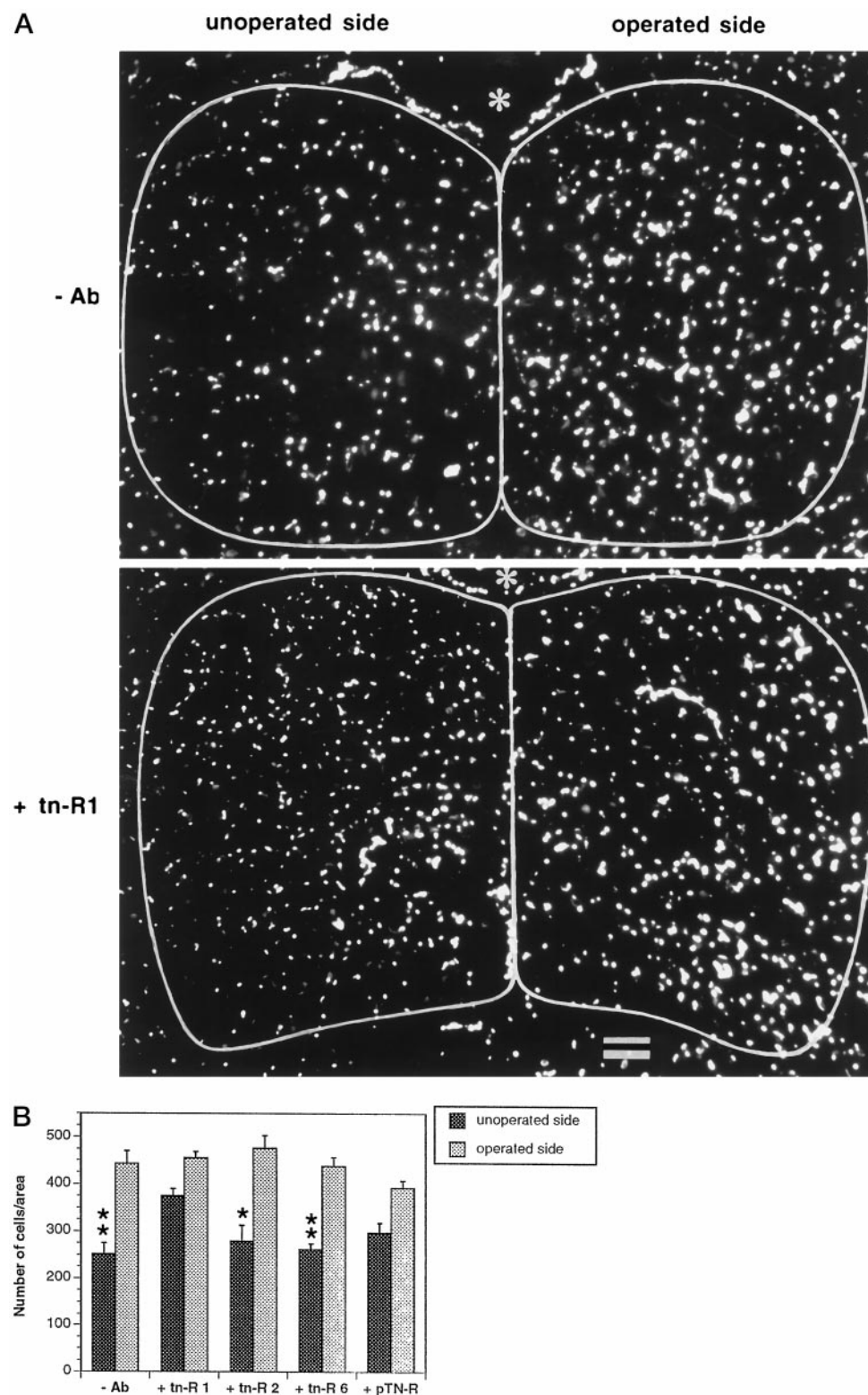
**Figure 5.** Effect of TN-R on microglial adhesion to PLL. **A**, Adhesion pattern of microglial cells on BSA-containing (PLL + BSA), TN-R-containing (PLL + TN-R), and LN-containing (PLL + LN) PLL substrates after 30 min (**a–c**) and 24 hr (**d–f**) in culture. **a–c**, Cells were stained with toluidine blue. Scale bar, 75  $\mu$ m. **B**, Effect of monoclonal (*tn-R1*, *tn-R2*, and *tn-R6*) and polyclonal (*pTN-R*) Abs to TN-R on microglial adhesion to PLL + BSA and PLL + TN-R substrates. Protein substrates were incubated in the absence (*-Ab*) or presence of Abs to TN-R before plating the cells. The number of adherent cells after 30 min of incubation on PLL + BSA (control substrates) was set as 100%. Values represent the mean  $\pm$  SD of one representative of three to five independent experiments performed in triplicate. Asterisks indicate statistically significant differences in the number of adherent cells. \*  $p < 0.1$ ; \*\*  $p < 0.01$ .

(Moneta et al., 1993). Another intriguing possibility is the down-regulation of molecular components present in the ECM or at the neuronal surface membrane that are nonpermissive for microglial adhesion. To address the latter possibility, we first examined the substrate properties of TN-R for microglial adhesion by short- (30 min) and long-term (24 hr) adhesion assays (Fig. 5*A*). For this purpose, BSA, TN-R isolated from adult rat brain, and LN were immobilized as substrates on PLL-treated culture dishes, and single-cell suspensions of microglial cells were plated onto these substrates. After 30 min, microglial cells readily adhered to BSA- or LN-containing substrates and acquired flat morphology, whereas the number of cells adherent to substrate-bound TN-R was strongly reduced by  $\sim 70\%$  (Fig. 5*A,B*). The inhibitory effect

of TN-R persisted even after longer culture periods when only few cells with loose contact with the substrate remained attached (Fig. 5*A*, after 24 hr). Similar results were obtained when TN-R was directly immobilized as a substrate on tissue culture plastic or in a mixture with LN, suggesting that the microglia–TN-R interaction induces a potent antiadhesive mechanism that acts independently of the presence of adhesive molecules. The antiadhesive action of TN-R on microglial cells was strongly inhibited by polyclonal Abs (*pTN-R*) and completely neutralized by monoclonal Ab *tn-R1* to TN-R (Fig. 5*B*).

To prove whether this mechanism is operable also *in situ*, we next studied the adhesion of bisbenzimidazole-labeled microglial cells to brainstem cryosections containing the facial or hypoglossal





**Figure 6.** TN-R present in the intact CNS is antiadhesive for activated microglial cells. **A**, Adhesion pattern of bisbenzimidazole-labeled microglial cells on brainstem cryosections containing lesioned (*operated side*) and control (*unoperated side*) hypoglossal nucleus (*outlined*) in the absence (*-Ab*) or presence of monoclonal Ab tn-R1 (*+tn-R1*). The central canal is marked by an *asterisk*. Scale bar, 75  $\mu$ m. **B**, Effect of Abs to TN-R on microglial adhesion to brainstem cryosections. Microglial cells were plated onto cryosections preincubated in the absence (*-Ab*) or presence of Abs to TN-R, and the number of cells adherent on each hypoglossal nucleus (unoperated vs operated side) was estimated microscopically. Values for each Ab represent the mean  $\pm$  SD of the cell number per 500  $\mu$ m<sup>2</sup> surface area from five equidistant sections. One representative of three independent experiments is shown. *Asterisks* indicate statistically significant differences in the number of adherent cells. \*  $p < 0.1$ ; \*\*  $p < 0.01$ .

nuclei of rats who underwent unilateral resection of the peripheral nerve and were allowed to survive for 10 d, a time at which TN-R expression is downregulated in the lesioned nucleus (Fig. 6, hypoglossal nucleus). After 30 min of incubation, there was a striking difference in the number of cells adherent to the unoperated versus the operated side of the brainstem (Fig. 6A). In the absence of Abs or in the presence of monoclonal Abs tn-R2 or

tn-R6, much less microglial cells (comprising 55–60% of the cell number found on the lesioned nucleus) adhered to the unoperated side (Fig. 6A,B). The nonpermissive substrate properties of the intact hypoglossal nucleus were almost neutralized in the presence of monoclonal Ab tn-R1. These results demonstrate that the intact CNS tissue is nonpermissive for microglial adhesion and that TN-R accounts for this nonpermissiveness.



### Activated microglia and TNF- $\alpha$ downregulate TN-R expression by oligodendrocytes

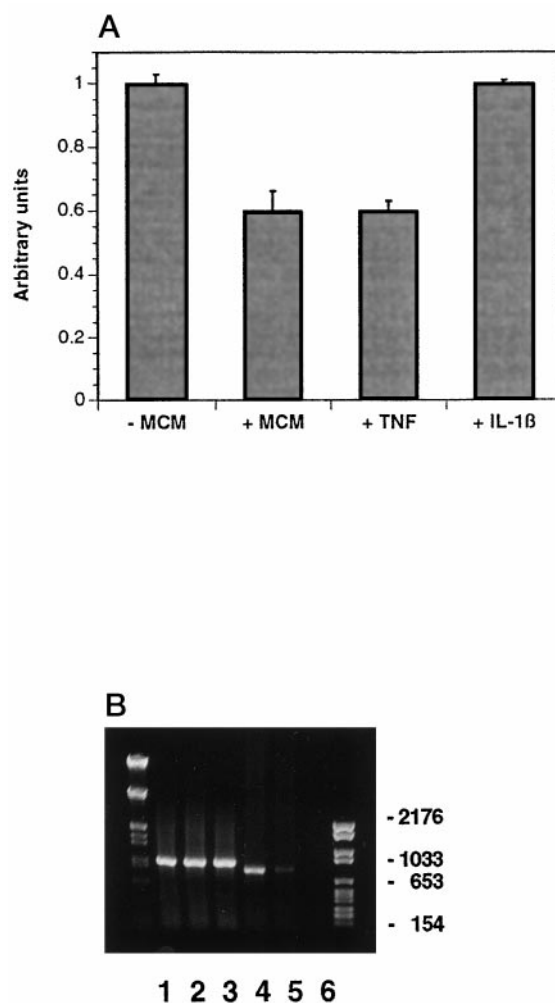
To address the question whether injury-associated factors secreted by activated microglia could account for the downregulation of TN-R expression in the lesioned facial nucleus, we examined the effect of MCM and cytokines observed in association with facial nerve axotomy (Raivich et al., 1997) on TN-R synthesis by cultured OLs, which are one of the cellular sources for TN-R in the brainstem (Wintergerst et al., 1993). OLs were maintained for 2 d in culture in MCM or in media containing or not containing defined cytokines, (i.e., TNF- $\alpha$  and IL-1 $\beta$ ), and the amount of TN-R protein released into the medium was measured by sandwich ELISA (Fig. 7A). Cultured OLs produce substantial amounts of TN-R, most of which are released into the culture medium (Jung et al., 1993; Pesheva et al., 1997). In the presence of MCM or TNF- $\alpha$ , TN-R expression decreased two-fold compared with that in the absence of additives, whereas IL-1 $\beta$  did not alter the protein expression by cultured cells (Fig. 7A).

To determine whether a downregulation of TN-R expression occurs at the transcriptional level, we further analyzed the expression of TN-R mRNA by RT-PCR (Fig. 7B). After 2 d in culture, MCM and TNF- $\alpha$  were not found to evoke cell death in cultured OLs (Probstmeier and Pesheva, unpublished observations). The TN-R-specific mRNA expression in cells maintained in the presence of MCM decreased significantly (Fig. 7B, lane 5) compared with that in culture medium only (Fig. 7B, lane 4), and this effect was not dependent on the rat strain from which microglial cells were obtained (Lewis or Wistar). TNF- $\alpha$  alone was a potent inhibitor of TN-R mRNA synthesis in cultured OLs (Fig. 7B, lane 6). In contrast, the levels of expression of the housekeeping enzyme GAPDH remained constant under all conditions tested (Fig. 7B, lanes 1–3).

### DISCUSSION

Our findings demonstrate that TN-R present in the perineuronal net of motoneurons is antiadhesive for activated microglia and after peripheral nerve axotomy becomes downregulated in the lesioned nucleus by a mechanism likely to involve injury-associated cytokines released by activated microglia. Hence, the loss of ECM components with nonpermissive properties for activated microglia may contribute to a loss of neuronal protection under pathological conditions.

The downregulation of TN-R expression in the lesioned facial or hypoglossal nucleus and its persistence under degenerative conditions coincides with the appearance and permanent presence (during neurodegeneration) of activated microglia around injured motoneurons (Streit and Kreutzberg, 1988; Streit and Graeber, 1993; Angelov et al., 1995). The role of microglial activation in motoneuron regeneration is not completely understood. On the one hand, its negative influence is suggested by studies on microglial deactivation after optic nerve lesion, demonstrating that the application of substances suppressing the microglial metabolism retards axotomy-induced neurodegeneration and enhances axon regeneration (Thanos et al., 1993). Furthermore, cultured (i.e., activated) microglial cells have been shown to secrete different, potentially neurotoxic agents, such as reactive oxygen intermediates (Colton and Gilbert, 1987), TNF- $\alpha$  (Frei et al., 1987), glutamate (Piani et al., 1991), and nitric oxide (Boje and Arora, 1992; Chao et al., 1992). On the other hand, microglia may exert beneficial effects on neuron survival via the



**Figure 7.** Activated microglia downregulate TN-R expression by cultured OLs. *A*, Effect of MCM and cytokines on TN-R protein expression. Media conditioned by OLs maintained for 2 d in MCM, TNF- $\alpha$  (+TNF), or IL-1 $\beta$  (+IL-1 $\beta$ ) were analyzed by sandwich ELISA using monoclonal Ab tn-R1 as a linker. Values for the TN-R content represent mean  $\pm$  SD of triplicate measurements of OL-conditioned media obtained from three (for MCM and TNF- $\alpha$ ) and two (for IL-1 $\beta$ ) independent experiments. In each experimental set, values for TN-R from OLs maintained in culture medium only (-MCM) were set as 1.0. *B*, Effect of MCM and TNF- $\alpha$  on TN-R mRNA expression by cultured OLs. Total RNA isolated from OLs maintained for 2 d in culture medium (lanes 1 and 4), MCM (lanes 2 and 5), or in culture medium containing TNF- $\alpha$  (lanes 3 and 6) was analyzed by RT-PCR using GAPDH-specific (lanes 1–3) and TN-R-specific primers (lanes 4–6). The apparent molecular weights of the DNA marker (in base pairs) are shown at the right.

secretion of various trophic factors (Streit, 1993; Banati and Graeber, 1994; Barron, 1995). During early stages of CNS response to peripheral nerve injury, microglial activation does not seem to be necessary for synaptic stripping or motoneuron regeneration (Reisert et al., 1984; Svensson and Aldskogius, 1993a,b,c), again suggesting that their devastating effect on neurons, if any, occurs during degeneration. Whatever the role of activated microglia in neuron regeneration, the downregulation of TN-R in perineuronal nets seems to make neurons accessible to the stable adhesion of microglia and could thus affect the reestablishment of normal connectivity required for neuron survival.

In contrast to the general upregulation of TN-C expression by reactive astrocytes in different parts of the injured CNS after

disruption of the blood–brain barrier (for review, see Faissner et al., 1996), no significant changes in the low protein levels occur during motoneuron regeneration and/or degeneration in the lesioned facial nucleus in which the blood–brain barrier remains intact. Hence, this member of the TN family does not appear to substitute for the downregulation of TN-R in the lesioned nucleus, i.e., the suggested functional implication of TN-R in neuron protection. Moreover, polyclonal Abs to TN-C are not found to interfere with the nonpermissive substrate properties of intact motor nuclei for microglial adhesion (Probstmeier and Pesheva, unpublished observations). Because the regulation of astrocytic TN-C appears to be mediated by the synergistic action of transforming growth factor- $\beta$ 1 and basic fibroblast growth factor *in vitro* and after injury *in vivo* (Smith and Hale, 1997) and thus differs from that of OL-derived and presumably motoneuron-derived TN-R (see below), the levels of expression of these growth factors in the axotomized motor nucleus seem to be insufficient to induce a stable upregulation of the molecule.

The molecular mechanisms of TN-R downregulation need further clarification. There are, in fact, two main possibilities: (1) a decrease in synthesis by TN-R producing cells in the motor nucleus, i.e., OLs and motoneurons, and/or (2) an increased proteolysis. At least for OLs, the first possibility is given by our present findings on the effect of MCM and TNF- $\alpha$ , a proinflammatory cytokine released by activated microglia and observed in CNS pathology or after facial nerve injury (Dickson et al., 1993; Raivich et al., 1997), on TN-R expression by these cells. In addition to the proposed microglia-mediated mechanism, TN-R synthesis by lesioned motoneurons, which could also contribute to TN-R production and perineuronal net formation, might depend on reinnervation of the peripheral target. While TN-R expression in OLs seems to depend on platelet-derived growth factor (Jung et al., 1993), thyroid hormone, and/or TN-R itself (Pesheva et al., 1997), the regulation of its expression by motoneurons is presently unknown. Furthermore, proteolytic activity could account for the observed downregulation of TN-R, because the release of proteases by activated microglia has been suggested to contribute to ECM degradation and to favor microglial migration (Nakajima et al., 1992).

The molecular basis of TN-R-mediated microglial repulsion is presently unknown. Microglia do not express the presently known cell surface receptors for TN-R, such as F3/11, CALEB, and sulfatides, expressed by neurons and/or OLs (Brümmendorf et al., 1993; Pesheva et al., 1993, 1997; Koch et al., 1997; Schumacher et al., 1997). In analogy to its antiadhesive action on CNS neurons (Pesheva et al., 1989, 1993), TN-R inhibits microglial adhesion independently of the adhesive molecular cues present *in vitro* or *in situ*. Microglial detachment, however, takes place within 30 min in culture, a time at which neurons are still attached to TN-R substrates (Morganti et al., 1990; Pesheva et al., 1993), suggesting the activation of signaling cascade(s) different from that in neurons. Furthermore, Ab tn-R1, which completely neutralizes the antiadhesive effect of TN-R on microglia *in vitro*, does not interfere with the interaction of F3/11-expressing neurons or chinese hamster ovary cell transfectants with TN-R and ensuing detachment of neurons (Pesheva et al., 1989, 1993). The protein epitope recognized by tn-R1 is presently unknown, but it is also present in human TN-R, and current studies demonstrate that human brain-derived TN-R displays similar antiadhesive properties toward microglia, suggesting the relevance of such mechanisms to human CNS pathologies (Pesheva, Spiess, Winterhalter, and Peshev, unpublished observations). During initial stages of micro-

glial activation, TN-R present in the neuropil could enhance microglial motility, i.e., facilitate migration by preventing stable adhesion to cells in the lesioned nucleus. This is supported by the observations that microglial rings surrounding injured motoneurons in the facial nucleus are established at POD 7–10, a period characterized by (1) a dramatic decrease of TN-R in the neuropil and the perineuronal net and (2) a reduced microglial motility (Leong and Ling, 1992; Streit and Graeber, 1993; present study).

The expression of TN-R in perineuronal nets appears to be a common feature of interneurons and motoneurons in different parts of the mammalian CNS, such as cortex, hippocampus, cerebellum, retina, brainstem, and spinal cord (Bartsch et al., 1993; Celio and Rathjen, 1993; Wintergerst et al., 1993, 1996; present study). TN-R may thus participate in the macromolecular organization of the perineuronal net by assembling complexes of ECM proteins and proteoglycans based on its divalent cation-dependent homophilic binding properties (Pesheva et al., 1991) and heterophilic interactions with chondroitin sulfate proteoglycans, such as versican (Celio and Blümcke, 1994; Aspberg et al., 1997). As shown previously for cortical and hippocampal interneurons (Celio and Blümcke, 1994; Scheffler et al., 1997), TN-C is also a constituent of the perineuronal net of motoneurons in the brainstem in which the expression of TN-R, however, is much more pronounced. Perineuronal nets are supposed to be involved in neuron–glia recognition mechanisms and in maintaining neuronal homeostasis by concentrating different growth factors, proteases, and ions (Brückner et al., 1993; Celio and Blümcke, 1994). During postnatal life, the extrasynaptic appearance of ECM constituents with nonpermissive properties for axon outgrowth, such as TN-R and TN-C, is likely to provide a molecular barrier to the formation of new synaptic contacts. Supporting such functional implication is the fact that the expression of these molecules is preceded by the accomplishment of experience-dependent plasticity (Hockfield et al., 1990; Wintergerst et al., 1993, 1996). In the developing rat neocortex, TN-R expression in the perineuronal net of interneurons coincides with the appearance and maturation of the neuron membrane-associated cytoskeleton, which may participate in the organization of perineuronal ECM molecules via association with their cell surface receptors (Wintergerst et al., 1996). In light of our present findings, an intriguing speculation is that peripheral nerve axotomy evokes derangement of the motoneuron cytoskeleton, resulting in impaired structural integrity of the perineuronal net, thus facilitating the destruction of the latter by proteases secreted by activated microglia. Together, these findings suggest that downregulation of TN-R and ensuing perineuronal net destruction in the lesioned motor nucleus might be a prerequisite for abnormal or lacking connectivity, the latter attributable in part to the stable adhesion of microglia at the TN-R-free neuronal cell membrane. Notably, perineuronal nets are found to disappear around cortical neurons of HIV-infected brains (Celio et al., 1993) and in Alzheimer's-type dementia (Kobayashi et al., 1989). Downregulation of TN-R expression in perineuronal nets in brain diseases has not yet been reported, but as our study suggests, it could affect neuronal function and/or survival.

## REFERENCES

- Aldskogius H, Thomander L (1986) Selective reinnervation of somatotopically appropriate muscles after facial nerve transection and regeneration in the neonatal rat. *Brain Res* 375:126–134.
- Angelov DN, Neiss WF (1996) Phagocytic microglia in the intracerebral presentation of antigen to the immune system. Morphological correlates. In: Topical issues in microglia research. (Ling EA, Tan CK, Tan



- CBC, eds), pp 165–188. Singapore: Goh Brothers Enterprise Humanities.
- Angelov DN, Gunkel A, Stennert E, Neiss WF (1995) Phagocytic microglia during delayed neuronal loss in the facial nucleus of the rat: time course of the neuronofugal migration of brain macrophages. *Glia* 13:113–129.
- Angelov DN, Neiss WF, Streppel M, Andermahr J, Mader K, Stennert E (1996a) Nimodipine accelerates axonal sprouting after surgical repair of rat facial nerve. *J Neurosci* 16:1041–1048.
- Angelov DN, Neiss WF, Streppel M, Walther M, Guntinas-Lichius O, Stennert E (1996b) ED2-positive perivascular cells act as neuronophages during delayed neuronal loss in the facial nucleus of the rat. *Glia* 16:129–139.
- Aspberg A, Miura R, Bourdoulous S, Shimonaka M, Heinegard D, Schachner M, Ruoslahti E, Yamaguchi Y (1997) The C-type lectin domains of lecticans, a family of aggregating chondroitin sulfate proteoglycans, bind tenascin-R by protein-protein interactions independent of carbohydrate moiety. *Proc Natl Acad Sci USA* 94:10116–10121.
- Banati R, Graeber M (1994) Surveillance, intervention and cytotoxicity: is there a protective role of microglia? *Dev Neurosci* 16:114–127.
- Bansal R, Warrington AE, Gard AL, Ranscht B, Pfeiffer SE (1989) Multiple and novel specificities of monoclonal antibodies O1, O4, R-mAB used in the analysis of oligodendrocyte development. *J Neurosci Res* 24:548–557.
- Barron KD (1995) The microglial cell. A historical review. *J Neurol Sci [Suppl]* 134:57–68.
- Bartsch U, Pesheva P, Raff M, Schachner M (1993) Expression of janusin (J1–160/180) in the retina and optic nerve of the developing and adult mouse. *Glia* 9:57–69.
- Blinzinger K, Kreutzberg GW (1968) Displacement of synaptic terminals from regenerating motoneurons by microglial cells. *Z Zellforsch Mikrosk Anat* 85:145–157.
- Boje KM, Arora PK (1992) Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. *Brain Res* 587:250–256.
- Borke RC, Curtis M, Ginsberg C (1993) Choline acetyltransferase and calcitonin gene-related peptide immunoreactivity in motoneurons after different types of nerve injury. *J Neurocytol* 22:141–153.
- Brückner G, Brauer K, Härtig W, Wolff JR, Rickmann MJ, Derouiche A, Delpech B, Girard N, Oertel WH, Reichenbach A (1993) Perineuronal nets provide a polyanionic, glia-associated form of microenvironment around certain neurons in many parts of the rat brain. *Glia* 8:183–200.
- Brümmendorf T, Hubert M, Treubert U, Leuschner R, Tarnok A, Rathjen FG (1993) The axonal recognition molecule F11 is a multifunctional protein: specific domains mediate interactions with Ng-CAM and restrictin. *Neuron* 10:711–727.
- Celio MR, Blümcke I (1994) Perineuronal nets—a specialized form of extracellular matrix in the adult nervous system. *Brain Res Rev* 19:128–145.
- Celio MR, Rathjen FG (1993) Restrictin occurs in “perineuronal nets” of the adult rat brain. *Soc Neurosci Abstr* 19:689.
- Celio MR, Dumas C, Myklosky J (1993) Disappearance of perineuronal nets around parvalbumin-neurons of HIV-infected brains. *Experientia* 49:A72.
- Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK (1992) Activated microglia mediate neuronal cell injury via a nitric oxide mechanism. *J Immunol* 149:2736–2741.
- Chiquet-Ehrismann R (1995) Inhibition of cell adhesion by anti-adhesive molecules. *Curr Opin Cell Biol* 7:715–719.
- Chiquet-Ehrismann R (1996) Tenascins, a growing family of extracellular matrix proteins. *Experientia* 51:853–862.
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159.
- Colton CA, Gilbert DL (1987) Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Lett* 223:284–288.
- Dickson DW, Lee SC, Mattiace LA, Yen SHC, Brosnan C (1993) Microglia and cytokines in neurological disease, with special reference to AIDS and Alzheimer's disease. *Glia* 7:75–83.
- Erickson HP (1993) Tenascin-C, tenascin-R and tenascin-X: a family of talented proteins in search of functions. *Curr Opin Cell Biol* 5:869–876.
- Faissner A, Götz B, Joester A, Wigger F, Scholze A, Schütte K (1996) Tenascin-C glycoproteins in neural pattern formation and plasticity. *Semin Neurosci* 8:347–356.
- Frei K, Siepl C, Groscurth P, Bodmer S, Scherdel C, Fontana A (1987) Antigen presentation and tumor cytotoxicity by interferon-gamma treated microglial cells. *Eur J Immunol* 17:1271–1278.
- Fuss B, Wintergerst ES, Bartsch U, Schachner M (1993) Molecular characterization and *in situ* messenger RNA localization of the neural recognition molecule J1–160/180—a modular structure similar to tenascin. *J Cell Biol* 120:1237–1249.
- Goodman CS (1996) Mechanisms and molecules that control growth cone guidance. *Annu Rev Neurosci* 19:341–377.
- Götz B, Scholze A, Clement A, Joester A, Schütte K, Wigger F, Frank R, Spiess E, Ekblom P, Faissner A (1996) Tenascin-C contains distinct adhesive, anti-adhesive, and neurite outgrowth promoting sites for neurons. *J Cell Biol* 132:681–699.
- Graeber MB (1996) The microglial sensor of pathology. In: *Topical issues in microglia research*. (Ling EA, Tan CK, Tan CBC, eds), pp. 203–218. Singapore: Goh Brothers Enterprise Humanities.
- Graeber MB, Streit WJ, Kreutzberg GW (1988) Axotomy of the rat facial nerve leads to increased CR3 complement receptor expression by activated microglial cells. *J Neurosci Res* 21:18–24.
- Guntinas-Lichius O, Neiss WF, Gunkel A, Stennert E (1994) Differences in glial, synaptic and motoneuron responses in the facial nucleus of the rat brainstem following facial nerve resection and nerve suture reanastomosis. *Eur Arch Oto-rhino-laryngol* 251:410–417.
- Higuchi M, Ohnishi T, Arita N, Hiraga S, Hayakawa T (1993) Expression of tenascin in human gliomas: its relation to histological malignancy, tumor dedifferentiation and angiogenesis. *Acta Neuropathol (Berl)* 85:481–487.
- Hockfield S, Kalb RG, Zaremba S, Fryer H (1990) Expression of neural proteoglycans correlates with the acquisition of mature neuronal properties in the mammalian brain. *Cold Spring Harb Symp Quant Biol* 55:505–514.
- Jung M, Pesheva P, Schachner M, Trotter J (1993) Astrocytes and neurons regulate the expression of the neural recognition molecule janusin by cultured oligodendrocytes. *Glia* 9:163–175.
- Kobayashi K, Emson PC, Mountjoy CQ (1989) Vicia villosa lectin-positive neurones in human cerebral cortex. Loss in Alzheimer-type dementia. *Brain Res* 498:170–174.
- Koch T, Brugger T, Bach A, Gennarini G, Trotter J (1997) Expression of the immunoglobulin superfamily cell adhesion molecule F3 by oligodendrocyte lineage cells. *Glia* 19:199–212.
- Kreutzberg GW (1996) Microglia: a sensor for pathological events in the CNS. *Trends Neurosci* 19:312–318.
- Laemmli UK (1970) Cleavage of structural proteins during assembly of the head of the bacteriophage T4. *Nature* 227:680–685.
- Leong SK, Ling EA (1992) Amoeboid and ramified microglia: their interrelationship and response to brain injury. *Glia* 6:39–47.
- McCarthy KD, DeVellis J (1980) Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol* 85:890–902.
- McLean IW, Nakane PK (1974) Periodate-lysine-paraformaldehyde fixative: a new fixative for immunoelectron microscopy. *J Histochem Cytochem* 22:1077–1087.
- Moneta M, Gehrman J, Töpfer R, Banati RB, Kreutzberg GW (1993) Cell adhesion molecule expression in the regenerating facial nucleus. *J Neuroimmunol* 45:203–206.
- Moore S, Thanos S (1996) The concept of microglia in relation to central nervous system disease and regeneration. *Prog Neurobiol* 48:441–460.
- Morganti MC, Taylor J, Pesheva P, Schachner M (1990) Oligodendrocyte-derived J1–160/180 extracellular matrix glycoproteins are adhesive or repulsive depending on the partner cell type and time of interaction. *Exp Neurol* 109:98–110.
- Nakajima K, Tsuzaki N, Shimojo M, Hamanoue M, Kohsaka SH (1992) Microglia isolated from rat brain secrete a urokinase-type plasminogen activator. *Brain Res* 577:285–292.
- Neiss WF, Guntinas-Lichius O, Angelov DN, Gunkel A, Stennert E (1992) The hypoglossal-facial anastomosis as model for neuronal plasticity in the rat. *Ann Anat* 174:419–433.
- Niquet J, Jorquera I, Faissner A, Ben-Ari Y, Repressa A (1995) Gliosis and axonal sprouting in the hippocampus of epileptic rats are associated with an increase of tenascin-C immunoreactivity. *J Neurocytol* 24:611–624.
- Nörenberg U, Hubert M, Rathjen FG (1996) Structural and functional characterization of tenascin-R (restrictin), an extracellular matrix glycoprotein of glial cells and neurons. *Int J Dev Neurosci* 14:217–231.

- Pesheva P, Spiess E, Schachner M (1989) J1–160 and J1–180 are oligodendrocyte-secreted nonpermissive substrates for cell adhesion. *J Cell Biol* 109:1765–1778.
- Pesheva P, Probstmeier R, Spiess E, Schachner M (1991) Divalent cations modulate the inhibitory substrate properties of murine glia-derived J1–160 and J1–180 extracellular matrix glycoproteins for neuronal adhesion. *Eur J Neurosci* 3:356–365.
- Pesheva P, Gennarini G, Goridis C, Schachner M (1993) The F3/11 cell adhesion molecule mediates the repulsion of neurons by the extracellular matrix glycoprotein J1–160/180. *Neuron* 10:69–82.
- Pesheva P, Probstmeier R, Skubitz APN, McCarthy JB, Furcht LT, Schachner M (1994) Tenascin-R (J1 160/180) inhibits fibronectin-mediated adhesion—functional relatedness to tenascin-C. *J Cell Sci* 107:2323–2333.
- Pesheva P, Gloor S, Schachner M, Probstmeier R (1997) Tenascin-R is an intrinsic autocrine factor for oligodendrocyte differentiation and promotes cell adhesion by a sulfatide-mediated mechanism. *J Neurosci* 17:4642–4651.
- Pesheva P, Urschel S, Frei K, Probstmeier R (1998) Murine microglial cells express functionally active galectin-3 *in vitro*. *J Neurosci Res* 51:49–57.
- Piani D, Frei K, Do KQ, Cuenod M, Fontana A (1991) Murine brain macrophages induce NMDA receptor mediated neurotoxicity *in vitro* by secreting glutamate. *Neurosci Lett* 133:159–162.
- Raivich G, Jones LL, Kloss C, Werner A, Neumann H, Wekerle H, Kreutzberg GW (1997) Immune surveillance in the injured nervous system: T-lymphocytes invade the axotomized mouse facial motor nucleus and aggregate around sites of neuronal degeneration. In: *Functions of glial cells: final symposium of the DFG study group*, p 125. Berlin: Blankenburg, Bernau.
- Rathjen FG, Wolff JM, Chiquet-Ehrismann R (1991) Restrictin: an extracellular matrix glycoprotein involved in cell attachment co-purifies with the cell recognition molecule F11. *Development* 113:151–164.
- Reisert I, Wildemann G, Grab D, Pilgrim C (1984) The glial reaction in the course of axon regeneration: a stereological study of the rat hypoglossal nucleus. *J Comp Neurol* 229:121–128.
- Scheffler B, Faissner A, Beck H, Behle K, Wolf HK, Wiestler OD, Blümcke I (1997) Hippocampal loss of tenascin boundaries in Ammon's horn sclerosis. *Glia* 19:35–46.
- Schumacher S, Volkmer H, Buck F, Otto A, Tärnok A, Roth S, Rathjen FG (1997) Chicken acidic leucine-rich EGF-like domain containing brain protein (CALEB), a neural member of the EGF family of differentiation factors, is implicated in neurite formation. *J Cell Biol* 136:895–906.
- Shelper RL, Adrian EK (1980) Non-specific esterase activity in reactive cells in injured nervous tissue labeled with  $^3\text{H}$ -thymidine or  $^{125}\text{I}$ -iododeoxyuridine injected before injury. *J Comp Neurol* 194:829–844.
- Smith GM, Hale JH (1997) Macrophage/microglia regulation of astrocytic tenascin: synergistic action of transforming growth factor-beta and basic fibroblast growth factor. *J Neurosci* 17:9624–9633.
- Sommer I, Schachner M (1981) Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytochemical study in the central nervous system. *Dev Biol* 83:311–327.
- Streit WJ (1993) Microglial-neuronal interactions. *J Chem Neuroanat* 6:261–266.
- Streit WJ, Graeber MB (1993) Heterogeneity of microglial and perivascular cell populations: insights gained from the facial nucleus paradigm. *Glia* 7:68–74.
- Streit WJ, Kreutzberg GW (1988) Response of endogenous glial cells to motor neuron degeneration induced by toxic ricin. *J Comp Neurol* 268:248–263.
- Svensson M, Aldskogius H (1993a) Infusion of cytosine-arabinoside into the cerebrospinal fluid of the rat inhibits microglial cell proliferation after hypoglossal nerve injury. *Glia* 7:286–298.
- Svensson M, Aldskogius H (1993b) Synaptic density of axotomized motoneurons following pharmacological blockade of the microglial cell proliferation. *Exp Neurol* 120:123–131.
- Svensson M, Aldskogius H (1993c) Regeneration of hypoglossal nerve axons following blockade of the axotomy-induced microglial cell reaction in the rat. *Eur J Neurosci* 5:85–94.
- Taylor J, Pesheva P, Schachner M (1993) Influence of janusin and tenascin on growth cone behavior *in vitro*. *J Neurosci Res* 35:347–362.
- Thanos S, Mey J, Wild M (1993) Treatment of the adult retina with microglia-suppressing factors retards axotomy-induced neuronal degradation and enhances axonal regeneration *in vivo* and *in vitro*. *J Neurosci* 13:555–565.
- Venstrom KA, Reichardt LF (1993) Extracellular matrix 2: role of extracellular matrix molecules and their receptors in the nervous system. *FASEB J* 7:737–743.
- Wintergerst ES, Fuss B, Bartsch U (1993) Localization of janusin mRNA in the central nervous system of the developing and adult mouse. *Eur J Neurosci* 5:299–309.
- Wintergerst ES, Vogt Weisenhorn DM, Rathjen FG, Riederer BM, Lambert S, Celio MR (1996) Temporal and spatial appearance of the membrane cytoskeleton and perineuronal nets in the rat neocortex. *Neurosci Lett* 209:173–176.
- Zagzag D, Friedlander DR, Dosik J, Chikramane S, Chan W, Greco MA, Allen JC, Dorovini-Zis K, Grumet M (1996) Tenascin-C expression by angiogenic vessels in human astrocytomas and by human brain endothelial cells *in vitro*. *Cancer Res* 56:182–189.