Tenascin-C Contains Distinct Adhesive, Anti-Adhesive, and Neurite Outgrowth Promoting Sites for Neurons

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Abstract. The glia-derived extracellular matrix glycoprotein tenascin-C (TN-C) is transiently expressed in the developing CNS and may mediate neuron-glia interactions. Perturbation experiments with specific monoclonal antibodies suggested that TN-C functions for neural cells are encoded by distinct sites of the glycoprotein (Faissner, A., A. Scholze, and B. Götz. 1994. Tenascin glycoproteins in developing neural tissues—only decoration? *Persp. Dev. Neurobiol.* 2:53–66). To characterize these further, bacterially expressed recombinant domains were generated and used for functional studies. Several short-term–binding sites for mouse CNS neurons could be assigned to the fibronectin type III (FNIII) domains. Of these, the alternatively spliced insert TNfnA1,2,4,B,D supported initial attachment for both embryonic day 18 (E18) rat and postnatal day 6 (P6) mouse neurons. Only TNfn1-3 supported binding and growth of P6 mouse cerebellar neurons after 24 h, whereas attachment to the other domains proved reversible and resulted in cell detachment or aggregation. In choice assays on patterned substrates, repulsive properties could be attributed to the EGF-type repeats TNegf, and to TNfnA1,2,4. Finally, neurite outgrowth promoting properties for E18 rat hippocampal neurons and P0 mouse DRG explants could be assigned to TNfnB,D, TNfnD,6, and TNfn6. The epitope of mAb J1/tn2 which abolishes the neurite outgrowth inducing effect of intact TN-C could be allocated to TNfnD. These observations suggest that TN-C harbors distinct cell-binding, repulsive, and neurite outgrowth promoting sites for neurons. Furthermore, the properties of isoform-specific TN-C domains suggest functional significance of the alternative splicing of TN-C glycoproteins.

THE development of the central nervous system of vertebrates evolves in a well-defined temporal sequence of events which includes proliferation of epithelial stem cells, migration of neuronal precursors from the ventricular zone to target areas, arrangement of connections, and neuronal cell death (Jacobson, 1991). Neuron-glia interactions play a crucial role in several of these processes. For example, immature astrocytes are believed to guide neuronal precursors and extending fiber projections or to segregate forming neuronal nuclei and fiber tracts (Rakic, 1988; Steindler, 1993). In this context, cell adhesion molecules (CAMs)[1] of the immunoglobulin and cadherin superfamilies constitute important regulatory components (Rathjen and Jessel, 1991; Brümmendorf and Rathjen, 1993). On the other hand, inhibitory and/or anti-adhesive molecules have also been implicated in growth cone guidance and neural pattern formation, e.g., the NI-antigens, oligodendroglia-derived glycoproteins which induce growth cone collapse and interfere with the regeneration of transected CNS fiber tracts (Keynes and Cook, 1992; Schwab et al., 1993; Luo et al., 1993; Luo and Raper, 1994; Faissner and Steindler, 1995; Dodd and Schuchardt, 1995; Tessier-Lavigne, 1995). In addition, glycoproteins of the extracellular matrix (ECM) and their receptors, the integrins, are involved in the control of neuronal migration and axon elongation (Sanes, 1989; Reichardt and Tomaselli, 1991; Hynes and Lander, 1992; Letourneau et al., 1994).

Among the ECM proteins, tenascin (TN) glycoproteins have attracted particular attention because they embody both stimulatory and anti-adhesive properties for various cell types (Erickson, 1993; Tucker, 1994; Faissner and Steindler, 1995). The TN gene family currently comprises three members, namely TN-C, formerly also designated tenascin, cytotactin or J1-200/220, TN-R, also known as restrictin or janusin and TN-X (Erickson, 1993). Structur-

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1. *Abbreviations used in this paper*: aa, amino acid; CAM, cell adhesion molecule; CNS, central nervous system; DRG, dorsal root ganglia; ECM, extracellular matrix; fbg, fibrinogen; FNIII, fibronectin type III; PORN, poly-ornithine; TN-C, tenascin.

ally, TN-C exhibits a modular organization with a cysteine-rich amino terminus followed by $14^1/_2$ EGF-like and 8 fibronectin type III (FNIII) repeats in mouse. The sequence is terminated by homologies to fibrinogen (fbg) β and γ at the carboxy terminus. Several isoforms are created by insertion of alternatively spliced FNIII motives between FNIII repeats 5 and 6 of the basic structure. TN-C monomers are linked to hexamers (hexabrachia) at their amino terminus (Erickson, 1993).

In the CNS, TN-C is transiently expressed by immature astrocytes, and, in a minority of cases, by subpopulations of neurons, e.g., retinal ganglion cells. In some areas, TN-C is distributed in discrete boundaries which delineate emerging functional processing units, for example, in the barrel field of the developing somatosensory cortex, in the patch-matrix compartments of the nigrostriatal projection, or around glomeruli of the olfactory bulb (Steindler et al., 1989*a,b*; Crossin et al., 1989; Steindler, 1993; Gonzalez and Silver, 1994). A possible role of TN-C in neuron-glia interactions had originally been inferred from the observation that antibodies to the glycoprotein reduce the adhesion of neurons to astrocyte surfaces in short-term assays (Kruse et al., 1985; Grumet et al., 1985). Subsequently, antibody perturbation experiments have shown that TN-C is involved in regulating migration of cerebellar neurons from the external to the internal granule cell layer of the developing mouse (Chuong et al., 1987; Husmann et al., 1992). Moreover, purified TN-C promotes neurite outgrowth of peripheral and central nervous system neurons (Wehrle and Chiquet, 1990; Crossin et al., 1990; Lochter et al., 1991; Lochter and Schachner, 1993; Taylor et al., 1993) and the motility of neuroblastoma cells (Halfter et al., 1989). Recent observations suggest that neuronal responses to the glycoprotein are partially regulated by cell lineage (Perez and Halfter, 1993; Taylor et al., 1993). On the other hand, inhibitory, anti-adhesive properties of TN-C have also been described. Thus, both neuronal cell bodies and growth cones are deflected from sharp TN-C boundaries on patterned substrates consisting of conducive components such as laminin-1 or poly-DL-ornithine which alternate with areas additionally containing the glycoprotein (Faissner and Kruse, 1990; Crossin et al., 1990; Taylor et al., 1993). TN-C boundaries observed in some regions of the developing CNS may thus serve to conceal neurons. TN-C is downregulated in most regions of the CNS after neurohistogenesis has proceeded, but is upregulated in CNS and PNS lesions (Laywell et al., 1992; Martini et al., 1990; Daniloff et al., 1989; Brodkey et al., 1995; Irintchev et al., 1993). Polyclonal antibodies specific for TN-C have recently been shown to delay reinnervation of neuromuscular junctions after cryolesion of the peripheral nerve, which suggests that the glycoprotein is involved in the regeneration process (Langenfeld-Oster et al., 1994). The use of monoclonal antibodies to distinct regions of TN-C in bioassays in vitro suggested that the diverse effects of the multifunctional glycoprotein are encoded by separate domains. Thus, influence on neuronal migration was allocated to FNIII domains around the proximal and neurite outgrowth promoting effects were assigned to FNIII repeats around the distal splice site (for reviews see Faissner et al., 1994*a*, 1995; Faissner and Steindler, 1995). Distinct binding sites for several nonneural cell types have been identified in the third FNIII domain (TNfn3) and the carboxy-terminal fibrinogen homology region TNfbg using recombinant proteins (Spring et al., 1989; Prieto et al., 1992, 1993; Aukhil et al., 1993; Joshi et al., 1993). In contrast, anti-adhesive activities for fibroblasts have been mapped to the EGF-type repeats (TNegf) and TNfn7-8 (Spring et al., 1989; Prieto et al., 1992). By comparison, not very much is known about specific recognition sites in TN-C for neurons and their processes. To analyze the structure-function relationships of TN-C with regard to neuronal differentiation, recombinant proteins which span the coding region of TN-C were generated and used in bioassays in vitro. We show here that distinct sites in TN-C are responsible for neuron binding, the promotion of neurite outgrowth, and the repulsion of neuronal cell bodies and their processes.

Materials and Methods

Animals

For the preparation of cell cultures from embryonic or postnatal brains, SD-rats or NMRI mice were used. The day a vaginal plug was found was designated embryonic day 0 (E0). New Zealand rabbits were used for immunization experiments. All animals were kept at the local facility (Versuchstieranlage des Zentralbereichs Theoretikum, Im Neuenheimer Feld, Heidelberg, Germany).

ECM Proteins

Human serum fibronectin (FN) and laminin-1 (LN) isolated from Engelbreth-Holm-Swarm mouse sarcoma cells were purchased from Boehringer Mannheim GmbH (Mannheim, Germany) or acquired from GIBCO BRL (Eggenstein, Germany). TN-C from P7-P14 mouse brains was obtained by immunoaffinity chromatography, as described elsewhere (Faissner and Kruse, 1990).

Poly- and Monoclonal Antibodies

Fluorescein isothiocyanate (FITC)-, tetramethylrhodamine isothiocyanate (TRITC)-, or peroxidase-derivatized polyclonal anti-rat, anti-rabbit, and anti-mouse antibodies were purchased from Dianova (Hamburg, Germany). Anti-T7 tag mouse monoclonal antibody (mAb) was purchased from Novagen (obtained through AGS, Heidelberg, Germany). mAbs J1/tn1 (clone 576), J1/tn2 (clone 578), J1/tn3 (clone 630), and J1/tn5 (clone 635) against TN-C were purified and concentrated from hybridomas growing in serum-free medium (Faissner and Kruse, 1990; Husmann et al., 1992). Polyclonal antibodies (pAbs) to the cell adhesion molecule L1 and to TN-C (KAF[9/2]) have been described elsewhere (Faissner et al., 1985; Faissner and Kruse, 1990). The antisera against the bacterially expressed proteins (see below) TNegf and TNfnA1,2,4 were raised in rabbits using standard procedures. Briefly, 50–100 μg of purified recombinant domains emulsified in Freund's adjuvans were injected subcutaneously at multiple sites into the back of white New Zealand rabbits. The animals were boosted at 4-wk intervals with the same amount of antigen emulsified in incomplete Freund's adjuvans. One week after the third boost a first bleed was collected. 50-ml serum against TNfnA1,2,4 was adsorbed against 5 mg TNfn6 (see below) coupled to a Ni^{++}-chelate column and the nonbound IgG-fraction was purified from the run-through using protein A affinity chromatography after standard protocols (Faissner and Kruse, 1990). Serum against TNegf was cross-adsorbed against 5 mg TNfn1-3 (see below) and processed as described above. The domain specificity of the IgG fractions was assessed by Western blotting and ELISA using a panel of recombinant TN-C proteins (see below).

Analytical Procedures

Protein concentrations were measured using the protein assay (Bio Rad Laboratories, Munich, Germany) or the Micro BCA reagent (Pierce Chem. Co., Rockford, IL). SDS-PAGE was performed with 17.5% acrylamide gels under reducing conditions (Laemmli, 1970). Gels were stained

with Coomassie brilliant blue (Serva, Heidelberg, Germany). Western blots were carried out as described and developed with peroxidase-derivatized secondary antibodies or ^{125}I-protein A (Faissner et al., 1988). For detection of HRP-coupled antibodies the ECL kit, which is based on the detection of light emission from oxidized luminol on photosensitive films (Amersham Buchler GmbH & Co. KG, Braunschweig, Germany), was used according to the supplier's instructions.

Construction of Expression Vectors for TN-C Domains

The constructs encoding the cDNA of mouse TN-C have been described (Weller et al., 1991). cDNA inserts corresponding to TNfn1-3, TNfn4,5, TNfn6, TNfnD,6, TNfn7,8, and TNfbg were generated by polymerase chain reaction (PCR) using up- and downstream primers as documented in Table I. The primers were designed to match modular boundaries of the protein and contained restriction sites for directional cloning into the pTrc-His expression vector system (Invitrogen BV, NV Leek, The Netherlands). For PCR amplification, 2.5 U of replitherm (Biozym, Oldendorf, Germany) were used in a total volume of 100 μl. The reaction was performed 20 times using the cycle (94°C, 1 min, 65°C, 1 min, 72°C, 2 min). The reaction mixture contained 5 μl of 20× incubation buffer (supplied by Biozym), 0.25 mM of each dNTP, 500 pmol of each primer and up to 500 ng plasmid. TNegf was subcloned from clone r5′-19 (nucleotides 177–2156, Weller et al., 1991) into pTrcHisA using the EcoRI linker at both ends of the insert. TNfnA1,2,4,B,D was subcloned from clone 03′-23 (nucleotides 3368–4657, Weller et al., 1991) into pTrcHisB using the EcoRI sites at both ends of the insert. The AatII site (nucleotides 4147-4152, Weller et al., 1991) was used to create TNfnA1,2,4 and TNfnB,D expression constructs. TNfnA1,2,4,B,D was digested with AatII and HindIII with subsequent *E. coli* DNA polymerase I treatment and the purified vector was blunt-end religated to yield the construct TNfnA1,2,4. TNfnB,D was generated by digesting TNfnA1,2,4,B,D with PstI and AatII, subsequent *E. coli* DNA polymerase treatment and blunt-end religation. The constructs were partially sequenced for proper reading frame and termination using the dideoxynucleotide chain termination method with T7 DNA polymerase (USB, Amersham, Braunschweig, Germany). The nucleotide sequence of TNfnA1,2,4 ranges from nucleotides 3368 to 4147 (Weller et al., 1991). The resulting amino acid (aa) sequence includes positions 1082–1340. On the cDNA level, TNfnB,D comprises the nucleotide sequence 4152–4657. The derived amino acid sequence of TNfnB,D encompasses aa 1343–1510. The fragment TNfnD,6 was obtained by PCR amplification of reverse transcribed total RNA from P6 mouse cerebellum. Total RNA was isolated by the cesium chloride step gradient method (Chirgwin et al., 1979). First strand cDNA synthesis was performed on 5 μg of total RNA, primed with 100 pmol of TNfnD,6 reverse primer (see Table I). Reverse transcription was carried out at 52°C for 1 h, using 4 U of AMV-reverse transcriptase (Promega, Madison, WI) /20-μl reaction volume with 1 mM dNTPs (MBI Fermentas), 5 mM $MgCl_2$, 1 mM DTT, and 10 U of RNAsin (Promega). PCR amplification was performed on the entire reverse transcription reaction, by adding 1 U of taq-polymerase (AGS), 8 μl of 10× reaction buffer, 100 pmol of reverse primer TNfnD,6, 200 pmol of forward primer TNfnD,6 and $MgCl_2$ to a final concentration of 2 mM in a total volume of 100 μl. Amplification was accomplished by repeating the cycle (95°C for 1 min, 57°C for 1 min, and 72°C for 3 min) 35 times. The resulting fragment of 550 base pairs was digested with EcoRI and BamHI and cloned into pTrcHisA (Invitrogen, San Diego, CA) with T4-DNA-ligase (MBI Fermentas). The cloned fragment was confirmed to represent the FNIII-domains D and 6 of TN-C by sequencing (Sequenase Version 2.0, Amersham).

Production, Purification, and Characterization of Recombinant Proteins

A 20-ml overnight culture in L-broth/amp (100 μg/ml) of bacteria (TOP 10, Invitrogen) transformed with the expression construct was diluted 1:50 and induced with 1 mM IPTG when the optical density (OD_{660}) reached 0.6. After 4 h of culture the cells were collected by centrifugation (20 min at 5,000 rpm, 4°C, Sorvall GSA rotor). The cell pellet was resuspended in 50 ml PBS (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) containing 1 mg/ml lysozyme, 0.1 mM PMSF, 5 μg/ml DNAse and 10 μg/ml RNAse, briefly sonicated and incubated at 37°C for 1 h. The sample was then centrifuged at 20,000 *g*, 4°C for 30 min. By this treatment, TNfn1-3, TNfn4,5, TNfn6, and TNfn7,8 were released into the supernatant and subsequently purified on nickel-chelate columns under physiological conditions. The columns were washed with 10-bed volumes PBS, pH 5.7, and eluted with 2-bed volumes 0.3 M imidazole in PBS, pH 6.3. The eluates were extensively dialyzed against PBS. TNegf, TNfnA1,2,4,B,D, TNfnA1,2,4, TNfnB,D, TNfnD,6, and TNfbg were pelleted with 20,000 *g* after lysis and

Table I. PCR Primers for Cloning TN-C Expression Proteins

Recombinant protein name	Template‡	Forward primer / Reverse primer	(and translation) (sense strand, and translation)§
TNfn1-3	R14/2	Fwd:	ACA **CTC GAG** ATG GAG GTG TCC CCT CCC AAA GAC C M E V S P P K D
		Rev:	CCT GCC AAA GAG ACC TTC ATC ACA TA**G AAT TC**C AA P A K E T F I T *
TNfn4,5	R14/2	Fwd:	ACA **CTC GAG** ATG GGC CTG GAT GCT CCC AGG AAT CTC M G L D A P R N L
		Rev:	GCA CGT GTG AAG GCA TCC ACG TAG **GAA TTC** CAA A R V K A S T *
TNfnD,6	RNA‖	Fwd:	CG **GGA TCC** GAA GCT GAA CCG GAA GTT GA E A E P E V
		Rev:	TC TCG GGG ACT CTA ATC ACA TA**G AAT TC**C G S G T L I T *
TNfn6	O3′-22	Fwd:	CG **CTC GAG** GCC ATG GGT TCT CCG AAG G A M G S P K
		Rev:	TC TCG GGG ACT CTA ATC ACA TA**G AAT TC**C G S G T L I T *
TNfn7,8	O3′-22	Fwd:	CG**G GAT CC**G GCT CTG GAT GGT CCA TCT A L D G P S
		Rev:	C CAA ACC ATC TTC ACA ACA TA**G AAT TC**C AA Q T I F T T *
TNfbg	O3′-22	Fwd:	CG**G GAT CC**G ATT GGA CTC CTG TAC CCA TTC C I G L L Y P F
		Rev:	GGC AGG CGT AAG CGG GCA TAA **GAA TTC** CAA G R R K R A *

*A stop codon was introduced between the coding sequence of the reverse primer and the restriction site.

‡The cDNA templates used for PCR have been described elsewhere (Weller et al., 1991).

§The reverse primer is listed as sense strand (with translation), but the antisense strand sequence was actually used. A 2–4 base overhang at the free 5′ or 3′ end of the restriction sites (bold and underlined) for facilitating binding of the corresponding restriction enzyme was included in the primer sequence.

‖Total RNA of P6 mouse cerebellum was reverse transcribed and TNfnD,6 was amplified by subsequent PCR.

dissolved in 50 ml of 8 M urea, 0.1 M Tris-HCl, pH 8.0 containing 10 mM β-mercaptoethanol. The resulting protein solution was cleared by centrifugation (20,000 *g*, 30 min, 4°C) and loaded on the nickel-chelate affinity-column. Thereafter, recombinant proteins were washed with 10-bed volumes 8 M urea, 0.1 M Tris-HCl, pH 6.2, and eluted with 2-bed volumes 0.3 M imidazole in 8 M urea, 0.1 M Tris-HCl, pH 7.0. For further purification, TNfnA1,2,4,B,D containing eluates were dialyzed against 25 mM Tris-HCl, 50 mM KCl, 1 mM dithioerythritol (Sigma Chem. Co., Deisenhofen bei München, Germany), 8 M urea and 10% (vol/vol) glycerol, pH 7.9 (KDTG-buffer), and circulated over a Q-Sepharose HP 16:10 column (Pharmacia LKB, Freiburg, Germany). The column was washed with 15-bed volumes KDTG-buffer and the bound protein recovered in a linear KCl gradient (150 mM—1 M in KDTG-buffer) at 350 mM KCl. The urea-containing eluates were renatured by dialysis against renaturation buffer (Rudolph et al., 1979) consisting of 200 mM L-arginine, 10 mM cystamine, 100 mM Tris-HCl, 2 mM EDTA, pH 8.0, thereafter dialyzed against PBS and finally cleared by centrifugation at 20,000 *g*, 4°C, 30 min. The fusion proteins appeared up to 95% pure as estimated by densitometry of Coomassie-stained SDS-PAGE gels. For storage, the recombinant proteins were snap-frozen in liquid nitrogen, stored at −70°C, and thawed on ice shortly before use to prevent functional inactivation. To examine whether these storage conditions favor aggregate formation, the recombinant proteins were analyzed by size exclusion chromatography. To this end, 1 mg of protein at a concentration of 1 mg/ml in PBS, pH 7.4, was loaded on a Hiload™ 16:60 column furnished with Superdex™ 200 matrix (Pharmacia LKB). 1.0-ml fractions were collected at a flow rate of 60 ml/h at room temperature in the elution volume range 40.0–95.0 ml and designated according to their relative V_e/V_o positions (V_o: 40 ml). Protein peaks were localized by extinction at 280 nm and further analyzed by SDS-PAGE and Western blot with specific antibodies. Apparent molecular weights were calculated using the markers blue dextran (2×10^3 kD), alcohol dehydrogenase (150 kD), BSA (66 kD), egg white albumin (45 kD), and carbonic anhydrase (29 kD, all from Sigma). Some batches of recombinant proteins were additionally characterized by inspection of rotary shadowed preparations with electron microscopy after established protocols (Pesheva et al., 1989).

Radioactive Labeling of Proteins with $Na^{125}I$

For radiolabeling with $Na^{125}I$ after the iodogen procedure (Salacinski et al., 1981), TN-C and the recombinant proteins TNegf, TNfn1-3, TNfn4,5, TNfnA1,2,4, TNfnA1,2,4,B,D, TNfnB,D, TNfnD,6, TNfn6, TNfn7,8, and TNfbg (20 μg each in 100 μl PBS) were preincubated with 200 μCi $Na^{125}I$ for 5 min at room temperature. Iodination was started by adding a polysterene bead coupled with the oxidizing reagent *N*-chloro-benzenesulfonamide (IODO-BEADS, Pierce, BA Oud-Beijerland, The Netherlands). After 15 min incubation at room temperature the reaction was ended by separating the solution from the bead. Labeled protein was loaded onto a Sephadex G10 column (Pharmacia LKB) equilibrated with PBS, 0.1% wt/vol gelatine, 0.1% wt/vol azide, pH 7.4, and nonincorporated $Na^{125}I$ was removed by gel filtration. Peak fractions were monitored by γ-counting, pooled, and stored at 4°C. The integrity of the radioiodinated proteins was checked by SDS-PAGE on 12% or 17.5% gels and subsequent autoradiography. Specific activity was determined as described earlier (Faissner et al., 1990) and yielded for TN-C: 3.6 μCi/μg of protein; TNegf: 3.2 μCi/μg; TNfn1-3: 2.7 μCi/μg; TNfn4,5: 0.68 μCi/μg; TNfnA1,2,4: 0.36 μCi/μg; TNfnB,D: 1.59 μCi/μg; TNfnA1,2,4,B,D: 0.17 μCi/μg; TNfnD,6: 2.38 μCi/μg; TNfn6: 2.24 μCi/μg; TNfn7,8: 1.23 μCi/μg; and TNfbg: 0.5 μCi/μg.

Enzyme-linked Immunosorbent Assay Screening Procedures

Microtest flexible assay plates (Falcon, Becton Dickinson GmbH, Heidelberg, Germany) were coated overnight with 50 μl per well of recombinant TN-C domains diluted to 20 μg/ml protein in PBS. For titration of protein substrates, dilutions of TN-C and derived recombinant domains ranging from 0.2 μM to 0.1 nM and 5 μM and 1 nM, respectively, were applied to the wells. After coating, the plates were washed five times with PBS and blocked for 1 h with 2 mg/ml BSA in PBS. The antibodies J1/tn1, J1/tn2, J1/tn3, and J1/tn5 were applied following published procedures (Faissner and Kruse, 1990; Husmann et al., 1992); subsequently, the ELISA plates were kept for 1 h at 37°C, washed three times with PBS, 0.05% (wt/vol) Tween 20 (PBS-Tween), incubated for 1 h at 37°C with HRP-derivatized secondary anti-rat antibodies (0.16 μg/ml), washed three times with PBS-Tween and developed with 2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS). The reaction was stopped by addition of SDS to a final concentration of 0.2% (wt/vol). The colored reaction product was quantified with an ELISA reader at 405 nm (Titertek multiskan, Flow Laboratories, Meckenheim, Germany). In some cases, binding of mAbs to immobilized TN-C was competed by addition of recombinant TN-C proteins. Standard concentrations of mAb were preincubated with various dilutions of recombinant proteins containing the respective antibody-binding site, or control proteins taken from distinct segments of TN-C, and subsequently used for ELISA as described above.

Cell Culture and Immunocytology

Cultures of cerebellar neurons from postnatal day 6 (P6) mice were established by fractionation of cell suspensions on a Percoll gradient (Pharmacia) as detailed elsewhere and kept in BME 10% horse serum (HS) (Keilhauer et al., 1985; Faissner and Kruse, 1990). Rat hippocampal neurons were prepared according to standard procedures (Banker and Cowan, 1977) except for some modifications. In brief, hippocampi were dissected from brains of embryonic day 18 (E18) rat fetuses in 37°C Ca-Mg-free Hanks balanced salt solution (HBSS) plus 0.6% (wt/vol) glucose and 7 mM Hepes, pH 7.4, treated with 0.25% trypsin for 15 min at 37°C, washed three times with HBSS, and dissociated by repeated passages through a fire polished Pasteur pipette. The cells were cultivated in MEM containing the N2 supplements of Bottenstein and Sato (1979) plus ovalbumin (0.1% wt/vol) and pyruvate (0.1 mM). Cerebellar microexplants were prepared from P6 mouse cerebella according to published protocols (Fischer et al., 1986) and maintained in modified SATO medium containing 1% (vol/vol) HS (Trotter et al., 1989). Analysis of the cultures using double immunofluorescence–labeling techniques with mono- and polyclonal antibodies followed standard procedures (Faissner and Kruse, 1990). For staining of E18 hippocampal neurons in microtiter plates, cultures were fixed with 2.5% (vol/vol) glutaraldehyde in ddH_2O, washed once with PBS (200 μl/well) for 12 min and blocked with 0.1 M glycine in PBS (100 μl/well) for 20 min. This and the following steps were all carried out at room temperature. Thereafter, neurons were permeabilized by addition of 0.2% (vol/vol) Triton X-100 in PBS for 90 s and washed five times with 1% (wt/vol) BSA in PBS (200 μl/well, 2 min). Subsequently, a monoclonal anti-tubulin antibody (T-9026, Sigma) was added at 1:4,000 in 1% (wt/vol) BSA, 0.1% (vol/vol) Tween-20 in PBS for 30 min. After five washes with PBS containing 1% (wt/vol) BSA, HRP-coupled secondary antibody diluted 1:5,000 in PBS, 1% (wt/vol) BSA was added for 30 min. Incubation was ended by five washes with PBS, 1% (wt/vol) BSA and the color reaction was developed with diamino-benzidine (DAB). The reaction was blocked by washing with ddH_2O and the culture was dried for morphological inspection. Dorsal root ganglia from P0 or P1 mice were prepared by opening decapitated mice at their dorsal side, cutting through the spinal cord and backbone with a scalpel and peeling out the cord using forceps to bend back the vertebrae. The ganglia were placed in HBSS on ice and tissue and nerve fibers sticking to the explants were removed (Seilheimer and Schachner, 1988). Whole ganglia were transferred into culture medium consisting of DMEM medium supplemented with 2 mM L-glutamine, 15 ng/ml nerve growth factor (Sigma), and 2×10^{-5} M Arabinosylcytosine (AraC, Sigma). The properties and culture conditions of the astroglial cell line neu-7 have recently been discussed (Smith-Thomas et al., 1994).

Cell-binding Assay

Nitrocellulose supports for cell culture were produced after established procedures (Lagenaur and Lemmon, 1987; Faissner and Kruse, 1990). In brief, 25 cm^2 of BA 85 nitrocellulose (Schleicher and Schuell, Dassel, Germany) were solubilized in 50 ml methanol, 1-ml aliquots were dispensed to 3-cm plastic petri dishes (Nunc) and dried under sterile conditions. For adhesion assays, 5-μl droplets containing recombinant proteins or ECM-molecules at 50 μg/ml were applied overnight in triplicate to nitrocellulose-coated surfaces in a humidified atmosphere at 37°C, after established procedures (Nörenberg et al., 1995). Thereafter, the petri dishes were washed three times with PBS and blocked by incubation with 2% (wt/vol) heat-inactivated (5 min, 70°C) fatty acid-free bovine serum albumin (BSA; Boehringer Mannheim) for 1 h. Subsequently, the dishes were washed with HBSS, and freshly dissociated P6 cerebellar or E18 hippocampal neurons were plated in their culture media (see above) at a density of 3×10^6 and 10^6 cells per dish, respectively. After 1 h or 24 h of incuba-

tion, the dishes containing cerebellar neurons were gently washed three times with PBS and the cultures fixed by flooding with PBS containing 4% (wt/vol) paraformaldehyde (PA). Dishes with hippocampal neurons were fixed by direct addition of glutaraldehyde to 2.5% (vol/vol) final concentration and washed by gentle flooding with PBS. The number of substrate-attached cells was determined by counting the cell bodies in nine visual fields (~0.3 mm^2 per visual field). For perturbation assays, mono- and polyclonal TN-C antibodies were incubated at various concentrations in PBS with the culture substrate for 1 h at 37°C after blocking with BSA and were thereafter added to the culture medium at the same concentrations.

Repulsion Assay

The assay for the detection of repulsive properties of TN-C or recombinant TN-C domains was carried out as described earlier (Faissner and Kruse, 1990). 11-mm-diam glass coverslips were washed in concentrated HNO_3 for 3 h, rinsed in ddH_2O until the pH reached 7.0, dried on sheets of filter paper and heat sterilized in a glass bottle. Thereafter, coverslips were placed into 24-well plates (Falcon) and incubated for 1–2 h in 15 μg/ml poly-DL-ornithine (PORN; Sigma) in 0.1 M Na_2HBO_3, pH 8.2 (borate buffer). After the incubation, the coverslips were washed three times with ddH_2O and air dried. Specified concentrations of protein in 50 μl PBS were incubated overnight on the coverslips at 37°C in a humidified atmosphere containing 5% CO_2. Subsequently, coverslips were washed twice with PBS. For creation of patterned substrates, the coverslips were covered with 50 μl of HBSS and bound protein was removed by gentle scraping with the tip of a plastic pipette. The HBSS was removed and replaced with the appropriate culture medium. Freshly dissociated neurons were plated in their respective culture media at a density of 1.5×10^5 cells per coverslip. The cultures were fixed with 4% (wt/vol) PA in PBS after 72 h. To assess repulsive substrate properties of recombinant TN-C domains for growing neurites, cerebellar explants instead of single cell suspensions (see cell culture) were plated on patterned coverslips. Cerebellar explants obtained from one cerebellum were seeded on four glass coverslips (15 mm diam). The explants were cultured for 72 h and fixed with 4% (wt/vol) PA in PBS.

Neurite Outgrowth Assays

Neurite outgrowth of E18 hippocampal neurons plated on PORN-conditioned supports coated with various TN-C recombinant proteins was determined as published elsewhere (Lochter et al., 1991; Faissner et al., 1994*b*). In brief, chamber slides (Nunc) were treated with 1.5 μg/ml PORN in 0.1 M borate buffer, pH 8.2, for 1 h at 37°C in a humidified atmosphere. ECM glycoproteins and recombinant proteins were coated at concentrations ranging from 10–50 μg/ml in 100 μl PBS per well for 1 h or overnight at 37°C in the incubator. The chambers were washed twice with HBSS before plating E18 hippocampal neurons at 8,000 cells/cm^2. After 24 h of culture, neurons were fixed by addition of glutaraldehyde to 2.5% (vol/vol), gently washed with PBS and stained for 15 min with 0.5% (wt/vol) toluidine blue in 2.5% (wt/vol) Na_2CO_3. Alternatively, wells of 96-microtiter plates (Nunc) were conditioned with poly-DL-ornithine (1.5 μg/ml, 50 μl/well) for 1 h at 37°C and washed twice with PBS. Thereafter, fusion proteins were coated at 50 μg/ml in PBS (50 μl/well) overnight at 37°C and washed twice with PBS immediately before cell plating. E18 hippocampal neurons were seeded at 7,000 cells/cm^2 (2,000 neurons/well) and fixed in defined culture medium with 2.5% (vol/vol) glutaraldehyde in ddH_2O (50 μl/well) for 15 min at room temperature. After fixation, cells were washed three times with PBS (200 μl/well), stained with toluidine blue (50 μl/well) for 1 h at room temperature or stained for tubulin. Wells were air dried after two final washes with ddH_2O (200 μl/well). For quantitative analysis, the fraction of cells bearing neurites was quantified by counting at least 200 cells per well. The morphometric analysis of neurite lengths was performed with the system Quantimet 500 MC (LEICA, Bensheim, Germany) by measuring 50 randomly drawn neurons with a process longer than one neuronal cell body diameter. At least three independent experiments were analyzed. The data were evaluated using nonparametric statistics, as detailed recently (Faissner et al., 1994*b*). Distribution of the longest neurites were compared using the nonparametric Mann-Whitney U-test. The mean values of sums of the longest neurites were compared by *t*-test. Stimulation of neurite outgrowth from PNS tissues was determined by growing P0/P1 mouse DRGs in tissue culture plastic petri dishes (3-cm diam, Nunc) coated with TN-C or recombinant proteins at 20 μg/ml overnight at 37°C. The substrates were blocked with 0.5% (wt/vol) BSA for 2 h. At least 10 explants were plated per dish and test protein substrate. In some cases, the substrates were preincubated after BSA treatment with mAbs against TN-C at a concentration of 60 μg/ml in PBS for an additional 2 h. Thereafter, the substrates were washed twice with HBSS and the mAbs were added to the culture medium at 60 μg/ml for the duration of the experiment (Lochter et al., 1991). After 48 h the DRG cultures were fixed for 1 h by the addition of glutaraldehyde to a final concentration of 2.5% (vol/vol).

Determination of Substrate Coating Efficiency

Microtiter plates (96 wells, Nunc) were coated with nitrocellulose (solubilized in methanol) or polyornithine (1.5 μg/ml in 0.1 M borate buffer, pH 8.2) and incubated with recombinant proteins. ELISAs were carried out with an mAb (0.01–0.1 μg/ml) specific for the T7-tag of the fusion proteins (50 μl per well) used at several concentrations (0.1–50 μg/ml) and goat-anti-mouse-HRP secondary antibodies (0.2 μg/ml). ELISAs were developed with ABTS and soluble color reaction products were quantified at OD_{405}. To quantify the amounts of protein adsorbed to the various substrates used for cell culture or ELISA procedures, coating solutions were replenished with the corresponding ^{125}I-labeled proteins as radioactive tracers. 2×10^4 cpm (9 nCi) of radioactive TN-C or recombinant protein corresponding to 1–5 ng of protein (depending on the specific activity) were added to the coating solution and substrates were created as described earlier. Subsequently, substrates were washed and bound radioactivity was detached by incubation with 0.1 N NaOH, 1% wt/vol SDS for 20 min at room temperature. Both the unbound radioactivity after the coating step and the bound radioactivity were measured by γ-counting. The percent-fraction, the molarity, and, where appropriate, the density of adsorbed protein were calculated for the different recombinant proteins and substrates used in this study. In some cases, proteins with tracers were subjected to doubling dilution series and the dependence of coating efficiency from the protein concentration was determined.

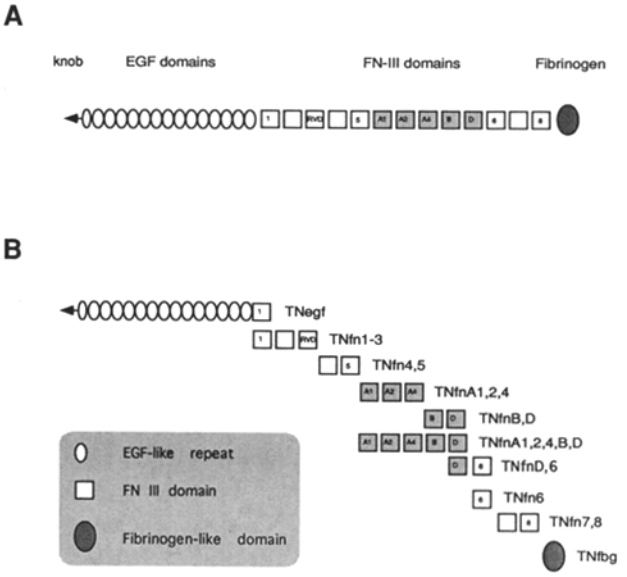


Figure 1. Domain structure of TN-C. TN-C consists of a serial arrangement of amino-terminal EGF-type repeats followed by FNIII repeats and carboxy-terminal homologies to fibrinogen β and γ. (*A*) Several isoforms have been described for TN-C which are generated by alternative splicing of varying numbers of repeats (*shaded boxes*) between FNIII domains 5 and 6. The largest isoform described in mouse fibroblasts is shown (Weller et al., 1991). The meaning of the geometrical symbols is indicated. (*B*) The library of recombinant domains of TN-C cloned into the pTrc-His vectors is illustrated. The nomenclature noted at the carboxy terminus follows a recent suggestion (Aukhil et al., 1993). The sequence RVD is designated in the third FNIII domain TNfn3 to emphasize that the RGD motive present at this position in chicken and human TN-C has been substituted in the mouse gene.

Results

Construction of a Library of Recombinant TN-C Domains

To advance the understanding of the structure-function relationships of TN-C for neural cell types expression vectors which contain defined domains were constructed on the basis of available cDNA clones for mouse TN-C (Fig. 1 *A*; Weller et al., 1991). cDNA segments, amplified by PCR or based on natural restriction sites (Table I), were cloned into a polyhistidine-tagged expression vector. The nomenclature of the resulting expression proteins follows earlier suggestions of the literature (see Aukhil et al., 1993, Fig. 1 *B*). After induction and lysis of transformed bacteria TNfn1-3, -fn4,5, -fn6, and -fn7,8 were soluble in PBS. These recombinant proteins were purified in a single step by affinity chromatography on Ni^{2+}-chelate ProBond™ resin (Fig. 2 *A*). After this schedule, the purification of the soluble expression proteins yielded between 5 mg/l (TNfn6) and up to 25 mg/l of bacterial culture (TNfn1-3). In contrast to these domains, TNegf, TNfnA1,2,4,B,D, TNfnA1,2,4, TNfnB,D, TNfnD,6, and TNfbg required 8.0 M urea for solubilization and were enriched as described above, with the exception that urea-containing buffers were used throughout the procedure. TNfnD,6 was purified to the same degree as the other fusion proteins and comigrated with TNfn4,5 or TNfn7,8 in SDS-PAGE (not shown). In some cases, an additional step was necessary to remove contaminating proteins. Thus, TNfnA1,2,4,B,D was further purified by anion exchange chromatography in the presence of 8.0 M urea. Fractions were monitored by SDS-PAGE and those containing the recombinant protein were pooled (Fig. 2 *B*). Proteins which had been processed in the presence of urea were renatured by dialysis against cystamine-supplemented buffer and, finally, against PBS. All recombinant proteins stayed in solution and only minor degradation could occasionally be observed (Fig. 2 *C*). Degradation bands represented <5% of the recombinant protein as judged by densitometry of Coomassie-stained gels or Western blots. These components were derived from the fusion constructs because they were specifically recognized by the anti-T7 tag mAb which reacts with an 11–amino acid motif contained in the T7 phage capsid that follows the polyhistidine stretch of the expression vector (not shown). When available, the structural relationship to TN-C was further confirmed with adequate mAbs, as shown for J1/tn1 (Figs. 2 *B* and 4 *A*). TNfbg behaved differently from these proteins in that it partially formed precipitating aggregates in physiological salt solutions which had to be removed by centrifugation before use in bioassays (see below). To estimate the degree of aggregation, the recombinant proteins were examined by gel filtration in physiological salt buffer. The proteins migrated at their appropriate, predicted apparent M_r positions, as deduced from monitoring the collected fractions by OD_{280}, SDS-PAGE and Western blot with appropriate antibodies (Fig. 3). The conclusion that the recombinant proteins consisted of

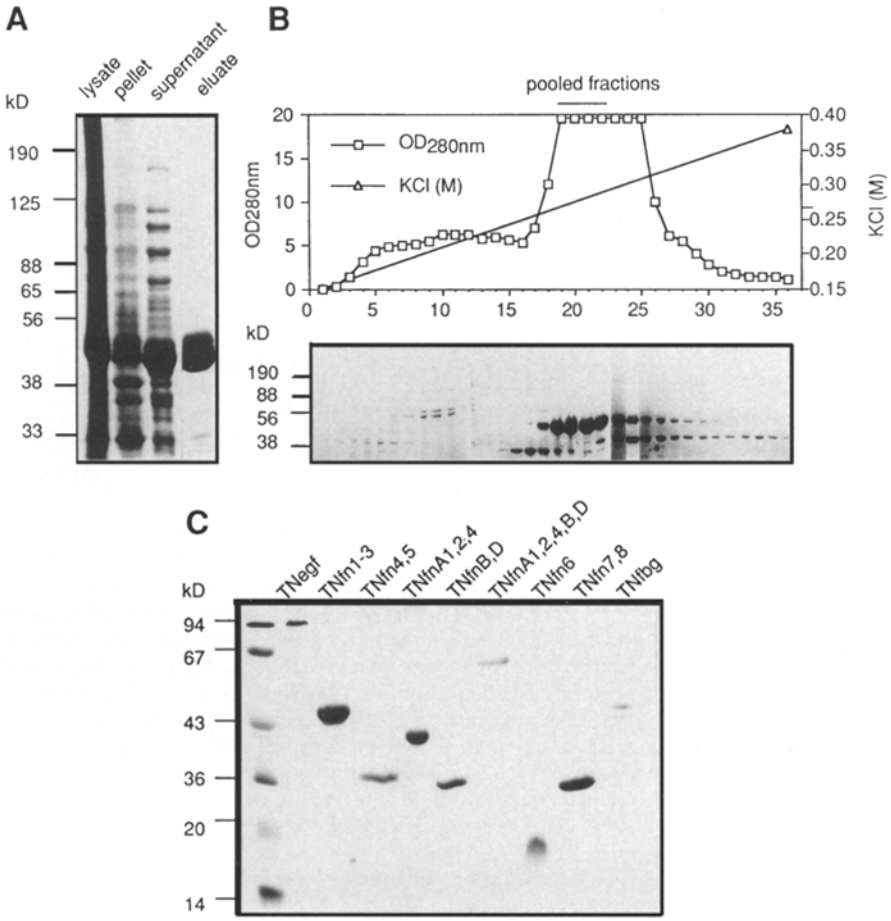


Figure 2. Purification of recombinant TN-C domains. An example of purification under physiological conditions is given in *A*. After induction and lysis of bacteria, pellet and supernatant were separated by centrifugation. The final lane displays the enrichment of TNfn1-3 obtained by one-step affinity chromatography on Ni^{2+}-chelate resin. *B* shows further purification of TNfnA1,2,4,B,D by anion-exchange chromatography in the presence of urea. SDS-PAGE under reducing conditions of fractions monitored by OD_{280} is shown. The pooled fractions contain a minor degradation band which is specifically recognized by mAb J1/tn1 (Fig. 4 *A*). A survey of recombinant TN-C domains is shown in *C*. In all cases, SDS-PAGE was performed in 17.5% gels which were stained with Coomassie brilliant blue.

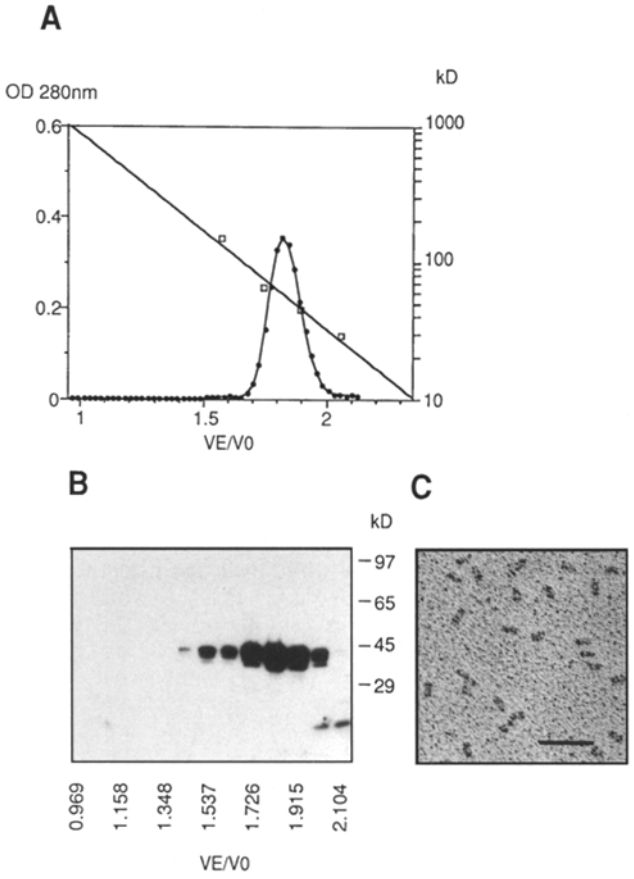


Figure 3. Characterization of recombinant TN-C proteins by gel filtration. To assess the degree of protein aggregation, TNfn1-3 was analyzed by size exclusion chromatography. (*A*) As shown by OD_{280} measurement (*left ordinate*), the majority of the protein was recovered as a single peak. The ratios of elution (V_E) vs exclusion volume (V_0) are indicated on the abscissa. M_r markers (*right ordinate*) were alcohol dehydrogenase (150 kD), BSA (66 kD), egg white albumin (45 kD), and carbonic anhydrase (29 kD). (*B*) Fractions obtained in *A* were resolved by SDS-PAGE on 17.5% gels and the identity of the protein was confirmed by Western blotting with polyclonal pTN antibodies. An autoradiogram of a blot developed with ^{125}I-protein A is shown. The lower molecular weight bands represent degradation products which elute at later positions because they are also recognized by mAb T7 which is specific for a protein motif encoded by the expression vector. (*C*) Recovered TNfn1-3 protein was inspected by electron microscopy of a rotary-shadowed sample. No obvious aggregates were visible in the homogeneous preparation. Comparable results with regard to gel filtration, Western blot, and rotary shadowing electron microscopy were obtained for all recombinant proteins containing FNIII-repeats. Bar, (*C*) 50 nm.

homogeneous, singular proteins comprising TN-C domains was further supported by electron microscopy of rotary shadowed preparations which yielded results comparable to those published in the literature (Aukhil et al., 1993), e.g., rodlike shapes for FNIII-repeats and a globular appearance of TNfbg (Fig. 3, and not shown).

Mapping the Epitopes of mAbs Specific for TN-C

The integrity of the fusion proteins was confirmed by immunochemical studies with a panel of defined mAbs to mouse TN-C. Some of these are known to interfere with functional properties of the intact glycoprotein (Lochter et al., 1991; Husmann et al., 1992). The epitopes of these mAbs were mapped by ELISA techniques which expose the recombinant domains in a nondenatured way. In all cases the mAbs attached to their binding sites, which renders misfolding of the corresponding recombinant proteins unlikely. These experiments confirmed and refined conclusions which were obtained earlier on the basis of electron microscopy of rotary shadowed mAb/TN-C complexes (Lochter et al., 1991; Husmann et al., 1992). In particular, the epitope for mAb J1/tn1 was located on TNfnA1,2,4, that is on the alternatively spliced region of the glycoprotein. This finding was confirmed by Western blot (Fig. 4 *A*). J1/tn2 bound to TNfnB,D and TNfnD,6 but not to TNfn6 using ELISA techniques (Fig. 4 *B*). Therefore, the epitope for J1/tn2 has been allocated to TNfnD. J1/tn3 bound to TNegf and J1/tn5 to TNfn4,5 using ELISA techniques, as predicted (not shown). Western blots carried out with the library of recombinant proteins confirmed these attributions. Only in case of J1/tn2 an additional, although weak reaction with TNfn6 could occasionally be disclosed (not shown). In these experiments, TNegf seemed partially degraded in some cases, because several bands immunoreactive with J1/tn3 were detected in lower molecular weight regions (not shown). As negative control, lysates of *E. coli* transformed with pTrc-HisA without cDNA insert were used. In a second series of experiments, the recombinant proteins were immunologically compared to native TN-C isolated from postnatal mouse brains. To this end, TN-C and recombinant domains were adsorbed onto microtiter plates at various concentrations and probed by ELISA. When compared at equal coating molarities, both intact TN-C and domains which are recognized by specific mAbs yielded comparable OD-values, while control recombinant proteins which did not harbor the mAb epitopes resulted in background signals (Fig. 4 *C*). These values reflected equal numbers of mAb binding sites because the molar amounts of protein bound to the wells of the assay plates were in the same range at equivalent signal intensities (Fig. 4 *D*). These results suggest that four available mAbs to distinct epitopes recognized their respective recombinant proteins with the same affinity as intact TN-C, and, therefore, the preparations of recombinant domains contained similar fractions with correctly folded antigenic sites than the native glycoprotein. For these reasons, it seems plausible to assume that the relative activities of the proteins are comparable.

Adhesion and Growth of CNS Neurons on Recombinant Domains

To identify potential binding sites for neurons, the recombinant TN-C domains were used in short (1 h) or medium term (24 h) adhesion assays using normal gravity (1 g). To this end, TN-C and the derived recombinant proteins were adsorbed to translucent nitrocellulose (Lagenaur and Lemmon, 1987; Faissner and Kruse, 1990). For screening purposes, a standard coating concentration of 50 μg/ml was chosen, which resulted in similar protein contents, but different molar concentrations in the culture substrate in dependence of the protein mass (Table II). This should

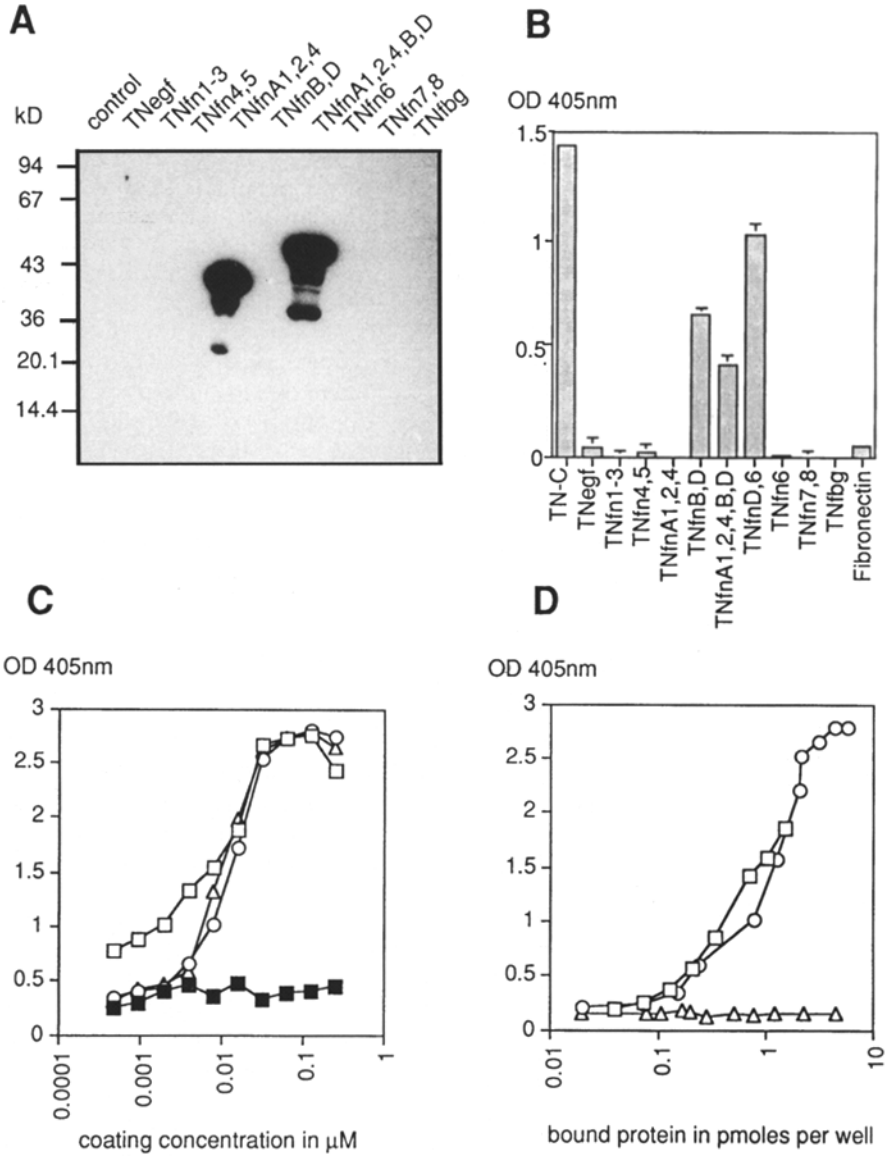


Figure 4. Epitope mapping of mAbs specific for mouse TN-C. The library of recombinant TN-C domains was used to map the binding sites of defined mAbs to TN-C. Assignments were based on ELISA and Western blots. (*A*) Whole cell lysate of bacteria transformed with the poly-histidine expression vector without insert (50 μg) was loaded in the first lane as negative control. 1 μg recombinant protein per lane as indicated were resolved by SDS-PAGE and the corresponding Western blot was developed with mAb J1/tn1. Note that only TNfnA1,2,4 and TNfnA1,2,4,B,D are reactive, which locates the epitope to the amino-terminal part of the alternatively spliced domains. The minor bands are degradation products of the recombinant protein because they reacted specifically with J1/tn1 and the mAb T7 to the T7 tag, but not with mAb J1/tn2 (*A*, and not shown). (*B*) The recombinant proteins were coated to PVP plates and ELISA was carried out with J1/tn2. Note that TNfnA1,2,4, B,D, TNfnB,D, TNfnD,6, but not TNfn6 are immunoreactive, which places the epitope to the alternatively spliced FN III repeat D. (*C*) To titrate the mAb-binding sites of the recombinant domains, the proteins were coated at various concentrations as indicated. The mAbs J1/tn1 (*open circles*), J1/tn2 (*open triangles*), and J1/tn5 (*open squares*) yielded similar dose response curves on their epitopes TNfnA1,2,4, TNfnD,6, and TNfn4,5, respectively. The mAbs did not react with the control protein TNfn1-3, as shown for J1/tn5 (*filled squares*) only, for the sake of clarity. Note that specific signals are still obtained at 0.01 μM coating concentration. (*D*) The amount of protein bound to the wells was determined by using ^{125}I-labeled tracer proteins and plotted against the OD-values obtained in the ELISA. Note that TNegf (*open circles*) and TN-C (*open squares*) result in similar binding curves for mAb J1/tn3, suggesting that equivalent OD values reflect equal numbers of mAb-binding sites. No signal was observed on the control TNfn1-3 (*open triangles*). Analogous results were obtained for the proteins used in *C*. Values in *C* and *D* were determined in triplicate and three experiments were carried out with similar results. Standard deviations were <10% of the corresponding OD values (not shown).

not be critical for the results of the cell culture assays, because earlier studies had already documented that recombinant TN-C proteins yield maximal cell biological responses at significantly lower coating concentrations, e.g., 10–15 μg/ml, or less (Joshi et al., 1993; Aukhil et al., 1993). In view of the possible differences in the dependence of developmental stage and/or lineage, E18 hippocampal were compared to P6 cerebellar neurons. After 1 h, hippocampal neurons were substantially enriched on the recombinant proteins which span the alternatively spliced region, and these domains were also adhesive after 24 h (Fig. 5 *A*). The binding was more pronounced than on laminin, which by itself showed weak, although statistically significant cellular attachment within 1 h. The slight differences between the recombinant proteins spanning the alternative splice site might reflect variances of their respective coating efficiencies (Table II). Yet, hippocampal neurons did not form monolayers on these substrates. Instead, individual cell bodies assembled to small clusters which were interconnected by fasciculating fibers (Fig. 6). Similar networks of small hippocampal neuron aggregates also emerged on TN-C, TNfn1-3, TNfn6, TNfn7,8, and TNfbg after 24 h of incubation, which documents that the cells are able to respond to these proteins. In all of these cases, adherence to the substrates was weak and aggregates could be dislodged by thorough washing. In this series of experiments, only TNfn4,5 behaved exceptionally because it was not adhesive at all. In contrast, hippocampal neurons developed a monolayer on the control substrate laminin-1 within 24 h, as expected (Fig. 6). TN-C is known to medi-

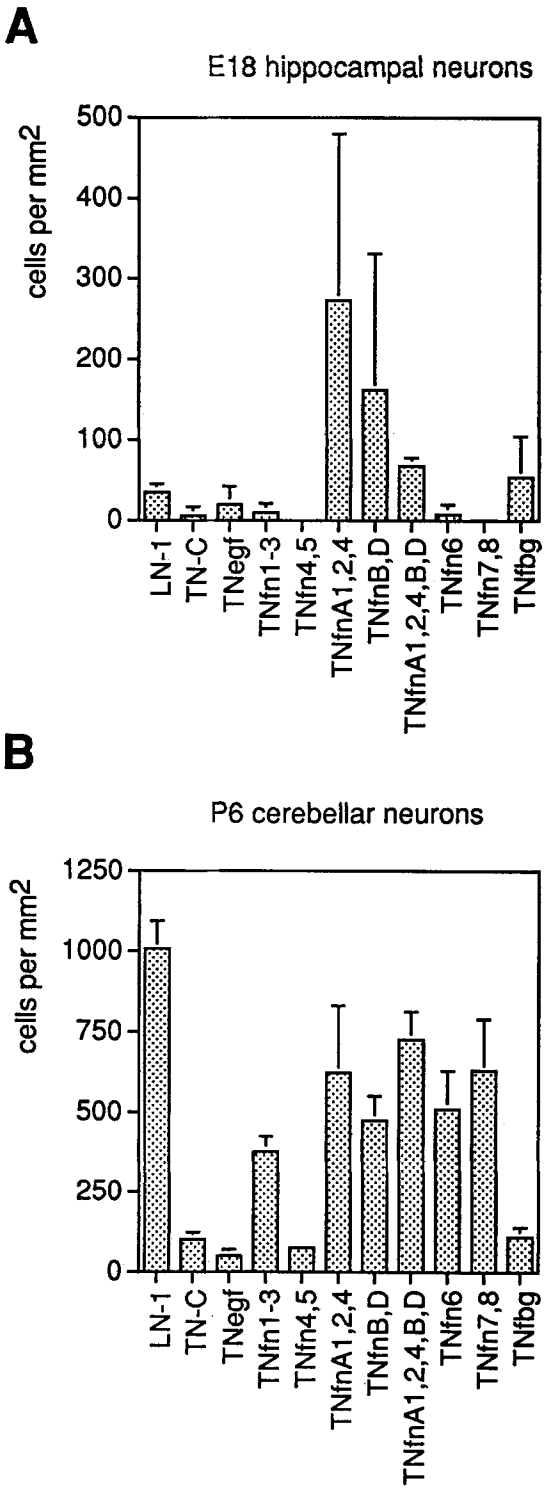


Figure 5. Short-term binding of CNS neurons to TN-C domains. E18 hippocampal (*A*, 500 cells/mm^2) and P6 cerebellar (*B*, 1,500 cells/mm^2) neurons were plated on spots of laminin-1, TN-C, and TN-C–derived recombinant proteins adsorbed to nitrocellulose (5 μl of protein per spot, 50 μg/ml), incubated for 60 min at 37°C, gently washed, and fixed. The columns give the number of cells per mm^2 binding to the proteins, three spots per protein were evaluated and at least three independent experiments were carried out. Note that distinct proteins contain short-term cell-binding sites, and these differ for hippocampal and cerebellar neurons, which suggests lineage-dependent interaction modes. Overall, the attachment of hippocampal proved weaker than the one of cerebellar neurons. This, in conjunction with lower plating densities, resulted in experimental variance with high standard deviations. Statistical analysis using the Mann-Whitney U-test confirms, however, that binding of E18 hippocampal neurons to TNfnA1,2,4 ($P < 0.005$), TNfnB,D ($P < 0.005$), TNfnA1,2,4,B,D ($P < 0.005$), TNfbg ($P < 0.05$), and laminin-1 ($P < 0.005$) was significantly different from attachment to TN-C. The protein concentrations in the spots are detailed in Table II and do not account for the distinct binding properties. For example, TNfn4,5 (4.40 pmol/cm^2) does not exhibit cell attachment, although its molar concentration in the substrate is twice that of TNfn7,8 (2.03 pmol/cm^2), which displays short-term binding of cerebellar neurons (*B*).

ate the attachment of cerebellar neurons to astrocyte surfaces in short-term assays (Kruse et al., 1985; Faissner et al., 1988). Within 1 h of incubation, attachment of P6 cerebellar neurons was observed on TNfn1-3, TNfnA1,2,4, TNfnB,D, TNfnA1,2,4,B,D, TNfn6, and TNfn7,8. These neurons adhered only weakly, or not at all to TNegf, TNfn4,5, TNfbg, or intact TN-C (Figs. 5 *B* and 7). Interestingly, the picture was different after 24 h of incubation in that a developing monolayer culture was visible on TNfn1-3, while the other recombinant proteins did not support further neuronal growth. Instead, the cells detached from the protein spots or formed weakly adherent aggregates, in some cases in particular, on TNfnA1,2,4 and TNfnA1,2,4,B,D (Figs. 5 and 7). As expected, laminin-1 proved an excellent substrate for cerebellar neurons both after 1 and 24 h of incubation (Figs. 5 and 7). In contrast, the astrocytic cell line neu-7 preferentially adhered to TNfn1-3 and TNfbg, which indicates lineage-dependent cellular binding specificities for TN-C (not shown). Interestingly, the distinct responses of E18 hippocampal and P6 cerebellar neurons to different components of the library of recombinant domains cannot be explained by varying concentrations of protein on the culture substrate, as evidenced by systematic analysis of coating efficiencies using radiolabeled tracers (Table II). In particular, proteins of similar mass adsorbed to comparable extents to nitrocellulose or plastic carriers, as expected, yet exerted different effects on cell binding and growth, e.g., TNfnB,D as compared to TNfn4,5 and TNfn7,8 (Table II; Fig. 5). Therefore, the behavior of these neurons on the different culture substrates might reflect distinct cell interactions with recognition sites, presumably via selective receptors. This notion was reinforced by systematic perturbation studies with specific mono- and polyclonal antibodies. Thus, the mAbs J1/tn1 and J1/tn2 specifically interfered with the attachment of cerebellar neurons to the proteins containing their epitopes, which are TNfnA1,2,4 and TNfnA1,2,4,B,D, and TNfnB,D and TNfnA1,2,4,B,D, respectively, at concentrations as low as 10 μg/ml (Table III). The polyclonal antibody pTN (batch KAF9(2); Faissner and Kruse, 1990) reduced cell binding to the recombinant proteins it recognizes according to ELISA and Western blot in a concentration-dependent manner (Table III, and not shown) and polyclonal antibodies generated against TNfnA1,2,4 were most efficient in suppressing attachment to their antigen (Table III). These results support the view that the recombinant proteins are correctly folded and expose specific recognition sites for cerebellar neurons. Summarizing at this point, TN-C contains cell-binding sites for both hippocampal and cerebellar neurons. On the other hand, the

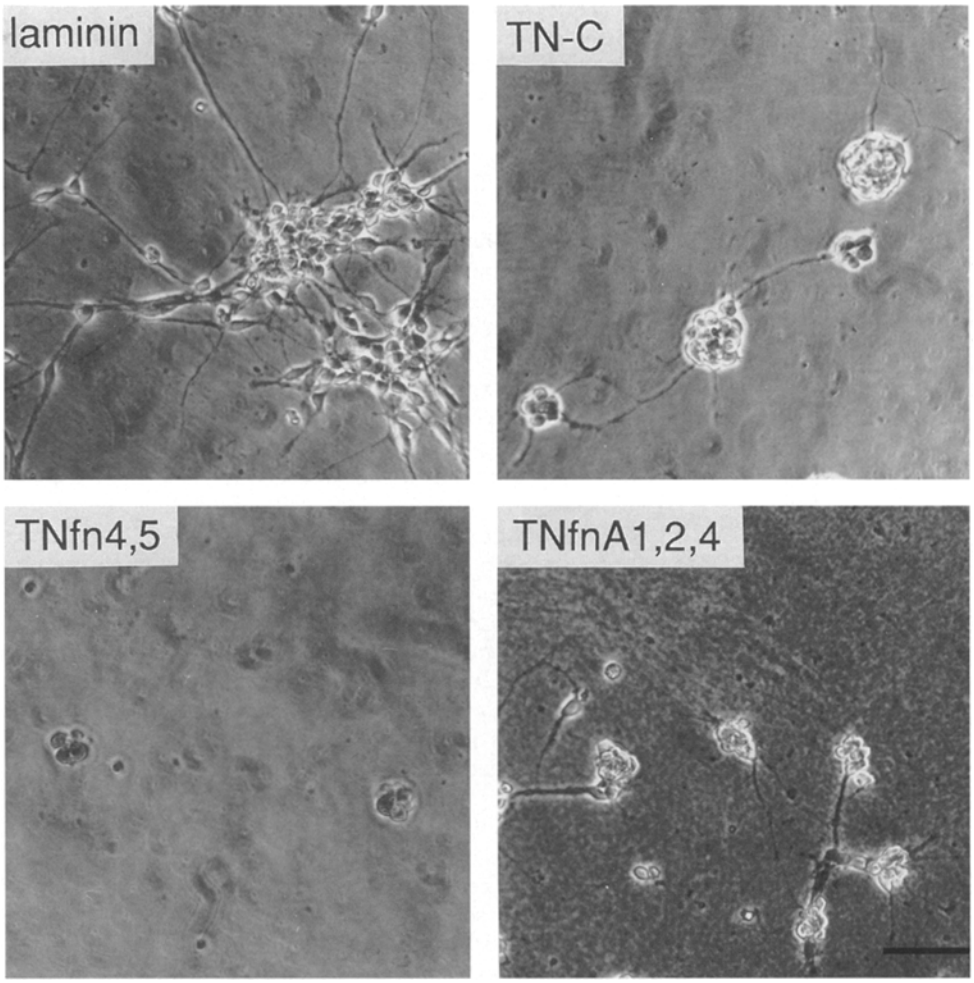


Figure 6. Growth of E18 hippocampal neurons on TN-C domains. Phase contrast micrographs of hippocampal neurons maintained for 24 h on nitrocellulose coated with laminin-1, TN-C, TNfn4,5, and TNfnA1,2,4. Hippocampal neurons differentiate on laminin-1 and form interconnected aggregates on TN-C (0.94 pmol/cm^2, Table II) and TNfnA1,2,4 (5.8 pmol/cm^2). Note that no process outgrowth is observed on TNfn4,5 (4.4 pmol/cm^2). For comparison with other proteins see Table II. Bar, 50 μm.

intact glycoprotein proves a bad culture substrate for these cell types. This apparent contradiction probably results from the presence of anti-adhesive sites which override the conducive properties, as described below.

Identification of Domains Repulsive for Neuronal Cells

Although TN-C contains cell-binding sites, the glycoprotein exerts anti-spreading, repulsive effects on most cell types (Chiquet-Ehrismann, 1991). With the aim to uncover domains which might underlie these anti-adhesive effects, the recombinant domains were applied in a patterned fashion to poly-ornithine (PORN)–conditioned coverslips. The composition of the substrate as compared to the position of cell bodies was visualized by indirect immunofluorescence using a mAb against the T7 tag of the fusion proteins. Cerebellar neurons were plated at high density and their distribution monitored after various culture periods. Under these conditions, P6 cerebellar neurons avoided areas which contained TN-C, TNegf, TNfnA1, 2,4,B,D, or TNfnA1,2,4 and arranged in stripes on the adjacent PORN-conditioned, protein-free parts of the coverslips (Fig. 8). When neurons grew on surfaces coated with these recombinant proteins, they formed aggregates interconnected by fasciculating neurites. These effects could already be observed after 12–24 h, and did not change over the following culture period, as described earlier for intact TN-C (Faissner and Kruse, 1990). In contrast, patterned substrates consisting of PORN/fibronectin, -/BSA, -/TNfn1-3, -/TNfn4,5, -/TNfnB,D, -/TNfn6, -/TNfn7,8, and -/TNfbg did not significantly influence culture morphology. Interestingly, analogous observations were collected when E18 hippocampal instead of P6 cerebellar neurons were used. Differing from the results obtained with cerebellar neurons, however, TNfn4,5 also exhibited weak repulsive effects towards hippocampal neurons (Table IV). These experiments suggest that TNegf and the upstream half of the alternatively spliced region of TN-C, e.g., TNfnA1,2,4, encode repulsive sites for central nervous system neurons. To assess whether this holds also true for growing neurites, cerebellar explants instead of single cell suspensions were used. These permit us to monitor the development of an impressive neuritic halo and of both astrocytic and neuronal emigration on supportive substrates (Künemund et al., 1988). On patterned substrates, fibers were specifically deflected by TN-C, TNegf, and TNfnA1,2,4, whereas the other recombinant proteins were not effective (Fig. 8). Neurites which crossed the boundaries showed a more fasciculated morphology than neurites growing on PORN alone. Deflection of neurites at step gradients between PORN and TN-C or the repulsive recombinant domains was apparent as soon as neurites grew to sufficient lengths

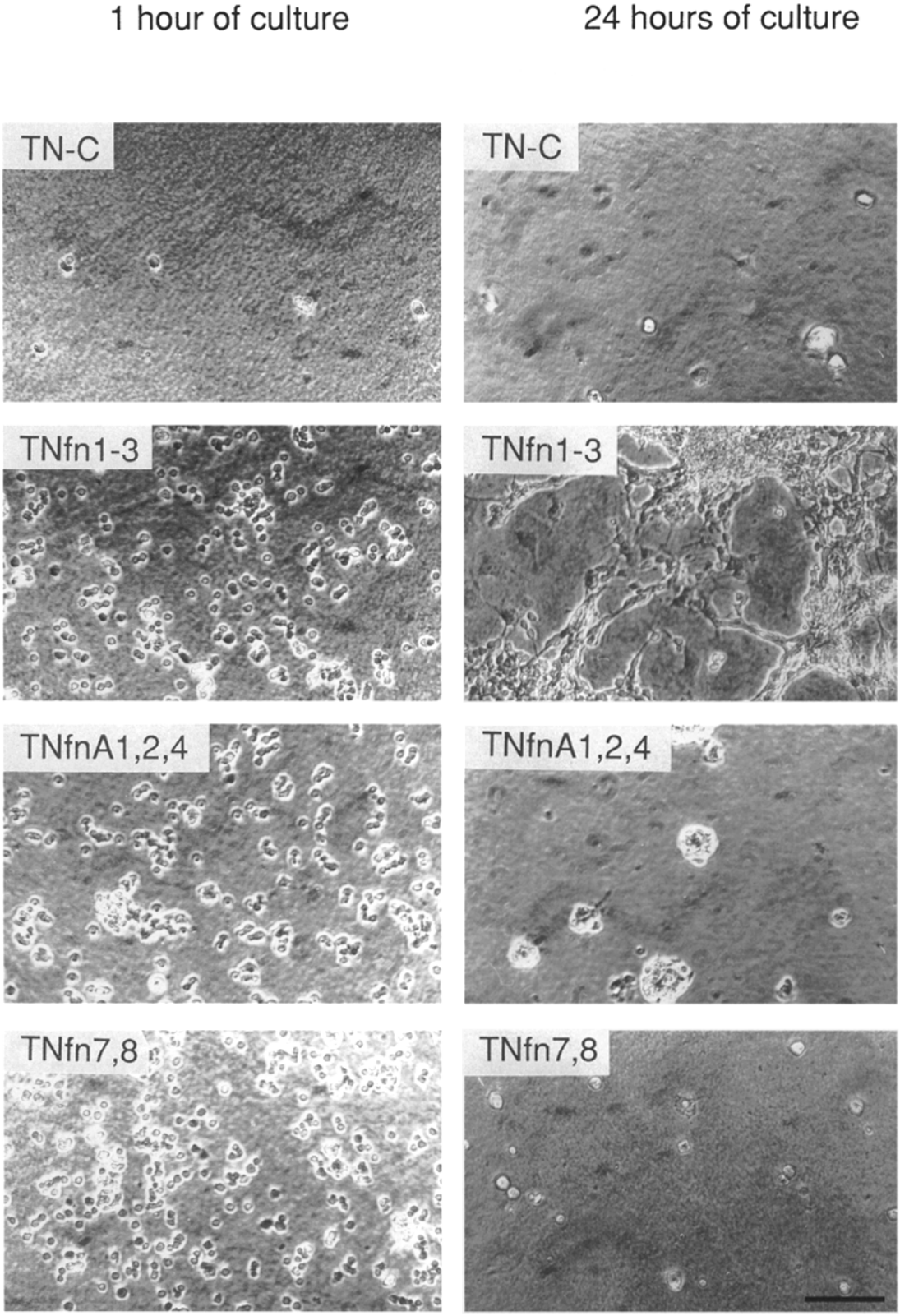


Figure 7. Attachment of P6 cerebellar neurons on TN-C-domains. Phase contrast micrographs of cerebellar neurons maintained for 1 h or 24 h on nitrocellulose coated with TN-C, TNfn1-3, TNfnA1,2,4, and TNfn7,8 (50 μg/ml). Neurons were plated as detailed in legend to Fig. 5. Note that cerebellar neurons do not adhere to TN-C (0.94 pmol/cm^2, Table II), form a monolayer on TNfn1-3 (1.76 pmol/cm^2), elaborate clusters on TNfnA1,2,4 (5.80 pmol/cm^2), and totally detach from TNfn7,8 (2.03 pmol/cm^2) within 24 h. Concentration differences do not account for the distinct properties of adsorbed proteins. In particular, TNfn1-3 supports medium term culture, although its molar concentration on the substrate is lower than the one for TNfnA1,2,4 and TNfn7,8. For comparison with other proteins see Table II. Bar, 50 μm.

Table II. Coating Efficiency of Recombinant Proteins and TN-C on Cell Culture Substrates

Carrier	Nitrocellulose			Tissue culture plastic (Nunc TC)					
Protein concentration	50 μg/ml			50 μg/ml			20 μg/ml		
Coating efficiency		μg/cm²	pmol/cm²		μg/cm²	pmol/cm²		μg/cm²	pmol/cm²
		%			%			%	
Protein									
TN-C	7.51 ± 3.59	0.22 ± 0.11	0.94 ± 0.45	3.03 ± 1.23	0.24 ± 0.10	0.99 ± 0.40	4.11 ± 0.91	0.13 ± 0.03	0.53 ± 0.12
TNegf	2.99 ± 1.97	0.09 ± 0.06	1.24 ± 0.82	3.12 ± 0.58	0.24 ± 0.05	3.52 ± 0.65	5.50 ± 1.13	0.17 ± 0.04	2.48 ± 0.51
TNfn1-3	1.86 ± 0.92	0.06 ± 0.03	1.76 ± 0.87	1.83 ± 0.44	0.14 ± 0.03	4.68 ± 1.13	3.04 ± 0.90	0.09 ± 0.03	3.10 ± 0.92
TNfn4,5	3.56 ± 0.67	0.11 ± 0.02	4.40 ± 0.83	0.69 ± 0.14	0.05 ± 0.01	2.50 ± 0.50	1.64 ± 0.77	0.05 ± 0.02	2.38 ± 1.12
TNfnA1,2,4	6.07 ± 0.61	0.18 ± 0.02	5.80 ± 0.58	3.34 ± 0.31	0.26 ± 0.02	8.76 ± 0.82	6.24 ± 1.15	0.20 ± 0.04	6.54 ± 1.21
TNfnB,D	2.78 ± 0.43	0.04 ± 0.01	2.00 ± 0.31	1.46 ± 0.25	0.11 ± 0.02	5.64 ± 0.96	6.04 ± 6.48	0.19 ± 0.20	9.34 ± 10.02
TNfnA1,2,4,B,D	5.12 ± 1.83	0.08 ± 0.03	1.60 ± 0.57	2.51 ± 1.80	0.20 ± 0.14	4.21 ± 3.02	5.89 ± 1.05	0.18 ± 0.03	3.96 ± 0.71
TNfnD,6	3.68 ± 2.10	0.11 ± 0.06	4.40 ± 2.50	1.08 ± 0.27	0.08 ± 0.02	3.92 ± 0.99	1.65 ± 0.49	0.05 ± 0.02	2.40 ± 0.71
TNfn6	4.50 ± 1.46	0.13 ± 0.04	8.44 ± 2.74	1.96 ± 0.32	0.15 ± 0.02	12.36 ± 2.00	3.91 ± 1.13	0.12 ± 0.04	9.86 ± 2.85
TNfn7,8	1.74 ± 0.87	0.05 ± 0.02	2.03 ± 1.00	0.40 ± 0.27	0.03 ± 0.02	1.47 ± 0.99	0.99 ± 0.87	0.03 ± 0.03	1.47 ± 1.29
TNfbg	10.93 ± 4.84	0.33 ± 0.15	11.10 ± 4.92	1.99 ± 1.41	0.16 ± 0.11	5.91 ± 4.20	4.35 ± 2.31	0.14 ± 0.07	5.17 ± 2.74

TN-C and recombinant domains were coated as indicated with ^{125}I-labeled tracer protein. The percent-fraction of bound protein was determined (1st column) and consequent substrate concentrations were calculated in μg/cm² (2nd column) and pmol/cm² (3rd column). Molarities were calculated for TN-C monomers assuming an apparent molecular mass of 240 kD. At least three singular measurements were performed per protein and values obtained in three independent experiments were pooled and used for the determination of standard deviations. Note that the percent-fractions of protein bound to the substrate appear lower on plastic than on nitrocellulose, although comparable substrate concentrations were reached. This reflects the fact that larger volumes, and hence absolute amounts of protein, were incubated in the wells of microtiter plates as compared to the nitrocellulose surface. No differences were found when the cell culture plastic was pretreated with poly-DL-ornithine (1.5 μg/ml) before the application of protein solutions. The substrate concentrations obtained with the present design compare well with earlier reports for laminin-1 and fibronectin. Thus, fibronectin coated at 50 μg/ml resulted in 740 ng/cm² on polystyrene (Calof and Lander, 1991), and laminin-1 yielded 79 and 295 ng/cm² on polystyrene when coated at 5 and 50 μg/ml, respectively (Calof and Lander, 1991), and 1,000 ng/cm² on tissue culture plastic when applied at 10 μg/ml (Buettner and Pittman, 1991). Saturation of cell-binding effects was observed in these studies with coating concentrations of 15 μg/ml, comparable to results reported for TN-C (Aukhil et al., 1991).

to be confronted with the choice situation, which is after 24 h. The emerging outgrowth pattern was not modified even when the culture period was extended over more than 72 h, which suggests that substrate conditioning by components released from cerebellar explants is not a critical factor in this assay. Repulsion of both neuronal cell bodies and their neurites was dependent on the coating concentration, with good results obtained at 50 μg/ml. The quantitation of coating efficiencies shows that repulsiveness is not merely a reflection of molar densities of proteins on the culture substrate. For example, TNegf is repulsive for cell bodies and growth cones, in contrast to TNfn6, although the latter is exposed at a much higher molarity (Table IV). Yet, despite their repulsive qualities TN-C or derived recombinant domains did not inhibit fiber formation when P6 cerebellar microexplants were maintained on homogeneous substrates composed of PORN coated with these proteins. Rather, neurite growth was similar on all the proteins tested. This is consistent with earlier observations that homogeneous TN-C substrates enhance neurite outgrowth by most CNS neurons, although the glycoprotein displays repulsive effects on outgrowing neurites in choice situations (summarized by Faissner et al., 1994*a*).

Neurite Outgrowth Promoting Domains in TN-C

It had been concluded from perturbation studies with mAbs that neurite outgrowth promoting properties of TN-C exposed as homogeneous substrate are encoded by domain(s) different from the one(s) involved in neurite deflection in choice situations (Faissner and Kruse, 1990; Lochter et al., 1991). To examine this hypothesis, neurite outgrowth assays were performed with TN-C and the library of derived recombinant domains. E18 hippocampal neurons were grown on substrates containing recombinant proteins or TN-C coated onto poly-DL-ornithine in chamber slides (Lochter et al., 1991). Under these conditions, TNfnA1,2,4,B,D, TNfnB,D, and TNfn6 increased the fraction of process bearing cells within a 24-h assay period (Fig. 9). This would locate a neurite outgrowth promoting domain around the distal splice site of TN-C, confirming predictions derived from in vitro perturbation assays with mAb J1/tn2 (Lochter et al., 1991). Consistent with this conclusion, TNfnB,D and TNfn6 stimulated total neurite lengths of hippocampal neurons (Fig. 10, Table V). In view of former reports describing neurite outgrowth promoting properties of TN-C for peripheral neurons, P0/P1 mouse dorsal root ganglia (DRG) were explanted onto TN-C or the library of recombinant domains adsorbed to tissue culture plastic. TN-C was as efficient as laminin-1 in supporting fiber growth from DRG-explants and several domains, e.g., TNfnB,D, TNfnD,6, and TNfn6 supported the establishment of a vigorous halo of neurites (Fig. 11). The formation of a neuritic halo was observed in 10 of 11 explants on laminin-1 and in 6 of 9 samples on TNfnD,6 in this case. The neurite outgrowth promoting effect of TNfnD,6 was reduced by addition of mAb J1/tn2 to the culture system, with only 2 of 11 explants showing neurite outgrowth, whereas mAb J1/tn1 could essentially not block the effects seen with TNfnD,6 (9 of 11). The same outcome was obtained in three independent experiments. We conclude from these observations that the FNIII domains bordering the distal splice site of TN-C encode a neurite outgrowth promoting site for central and peripheral neurons.

Discussion

Generation of a Library of Recombinant Mouse TN-C Domains

TN-C has been implicated in several key events of neuro-

Table III. Inhibition (%) of Short-Term Cerebellar Neuron Binding by Antibodies to TN-C

Concentration of mAb J1/tn1 (μg/ml)	10	50	100
Protein			
LN-1	5.13 ± 0.24 (ns)	7.69 ± 0.14 (ns)	4.96 ± 0.12 (ns)
TNfnA1,2,4	−36.80 ± 13.83 (ns)	−47.40 ± 6.92 (*)	−35.40 ± 5.26 (*)
TNfnB,D	−16.81 ± 3.20 (ns)	−16.95 ± 3.27 (ns)	−11.30 ± 2.35 (ns)
TNfnA1,2,4B,D	−42.73 ± 8.16 (*)	−51.14 ± 14.20 (*)	−63.40 ± 16.03 (*)
Concentration of mAb J1/tn2 (μg/ml)	10	50	100
Protein			
LN-1	−1.85 ± 0.12 (ns)	−5.31 ± 0.47 (ns)	0.92 ± 0.05 (ns)
TNfnA1,2,4	8.49 ± 2.74 (ns)	14.39 ± 2.56 (ns)	0.71 ± 0.17 (ns)
TNfnB,D	−70.20 ± 22.12 (*)	−83.05 ± 21.56 (*)	−83.47 ± 17.64 (*)
TNfnA1,2,4B,D	−32.82 ± 3.05 (*)	−40.12 ± 4.49 (*)	−54.70 ± 15.17 (*)
Concentration of pTN-C (μg/ml)	50	250	500
Protein			
LN-1	16.17 ± 2.85 (ns)	15.00 ± 1.20 (ns)	13.10 ± 4.20 (ns)
TNfn1-3	26.13 ± 7.35 (ns)	−83.93 ± 42.83 (*)	−90.33 ± 51.32 (*)
TNfnA1,2,4	−15.13 ± 4.24 (ns)	−47.98 ± 6.24 (*)	−47.10 ± 9.12 (*)
TNfnB,D	−14.00 ± 13.20 (ns)	38.00 ± 13.99 (ns)	37.33 ± 13.94 (ns)
TNfnA1,2,4,B,D	3.51 ± 1.32 (ns)	5.70 ± 0.45 (ns)	−26.32 ± 3.67 (*)
TNfn6	−45.52 ± 5.87 (ns)	−73.88 ± 33.90 (*)	−96.08 ± 31.30 (*)
TNfn7,8	38.63 ± 12.92 (ns)	−27.34 ± 15.32 (ns)	−91.46 ± 57.70 (*)
Concentration of pTNfnA1,2,4 (μg/ml)	50	250	500
Protein			
LN-1	7.05 ± 0.45 (ns)	2.61 ± 0.47 (ns)	11.23 ± 1.35 (ns)
TNfn1-3	−32.45 ± 8.36 (*)	−25.33 ± 1.44 (*)	−49.33 ± 6.11 (*)
TNfnA1,2,4	−92.82 ± 4.64 (*)	−97.41 ± 60.70 (*)	−93.90 ± 5.52 (*)
TNfnA1,2,4,B,D	−69.09 ± 33.54 (*)	−93.30 ± 30.50 (*)	−93.54 ± 14.08 (*)

Short-term (60 min)–binding assays of P6 cerebellar neurons were performed as described in legend to Fig. 5 with proteins exhibiting nonambiguous cell-binding sites. Mono- and polyclonal antibodies were preincubated with the protein spots for 60 min and added to the culture medium during the assay at the concentrations indicated. Numbers of neurons attached to the spots were determined in triplicate and the percent inhibition (I) of neuron binding in the presence (N_{test}) and absence ($N_{control}$) of antibodies was determined as $I = (N_{test} - N_{control})/N_{control} \times 100$. Negative values indicate the degree of inhibition of cell attachment in percent as compared to the control. Note that mAbs J1/tn1 and J1/tn2 inhibit cell binding to the proteins which contain their respective epitopes, but not to the neighboring domains. Thus, J1/tn2 supresses attachment to TNfnB,D, but not to TNfnA1,2,4, and only partially reduces the binding to the alternatively spliced segment which contains both components. pTN-C interferes with binding of cerebellar neurons to all recombinant proteins tested, except for TNfnB,D, which is also not recognized in Western blot and ELISA (not shown). Three independent experiments were carried out and statistically evaluated with the Mann-Whitney U-test. The molarities of proteins adsorbed to the nitrocellulose carrier are detailed in Table II. (*) $0.01 < P < 0.05$; *ns*, nonsignificant.

histogenesis such as neuron migration and neurite outgrowth, and also anti-adhesive properties for neuronal cell bodies and growth cones have been attributed to the glycoprotein. These diverse functions may be mediated by separate domains (for reviews see Faissner and Steindler, 1995; Faissner et al., 1995). To probe the proposed structure-function relationships of TN-C for CNS and PNS neurons, a library of recombinant mouse TN-C domains was generated and used in several in vitro bioassays. We show here that cell binding, anti-adhesive, and neurite outgrowth promoting sites for neurons are located in distinct regions of TN-C. Several of the recombinant domains could be purified in physiological salt buffers, while others required additional urea for solubilization. The resulting proteins were further characterized by electron microscopy of rotary-shadowed preparations, size exclusion chromatography, SDS-PAGE, Western blot, and ELISA using defined mono- and polyclonal antibodies and exhibited the expected properties (Aukhil et al., 1993). As compared to the FNIII domains, TNegf was produced at lower levels and showed partial degradation. Others have described fusion of these EGF-type repeats to glutathione-*S*-transferase (GST; Prieto et al., 1992), and electron microscopy pictures of EGF-type repeats linked to β-galactosidase displayed bulky globules and emanating thin rods comparable in length and thickness to those in intact TN-C (Spring et al., 1989). Renaturation of the eukaryotic EGF-type repeats was, however, effective to some extent because mAb J1/tn3, which reacts with this motive (Husmann et al., 1992), bound to the recombinant protein with the same efficiency as intact TN-C.

Identification of Cell-binding Sites for Neurons on TN-C

Polyclonal antibodies to TN-C had been found to reduce the binding of neurons to astrocyte surfaces in short-term adhesion assays (Kruse et al., 1985; Grumet et al., 1985; Faissner et al., 1988). Yet, subsequent studies did not reveal TN-C as efficient substrate for neuron culture (for reviews see Faissner et al., 1994*a*; Tucker, 1994; Faissner and Steindler, 1995). In this regard, neurons resemble various nonneural cell types which exhibit initial attachment to intact TN-C and fail to spread thereafter. To investigate cell recognition sites for neurons in TN-C, short-term assays on recombinant fragments derived from mouse TN-C were carried out. Substantial binding of P6 cerebellar neurons could be detected on all recombinant fragments, except for TNegf, TNfn4,5, and TNfbg. Adhesion appears to be specific since not all recombinant domains yielded the

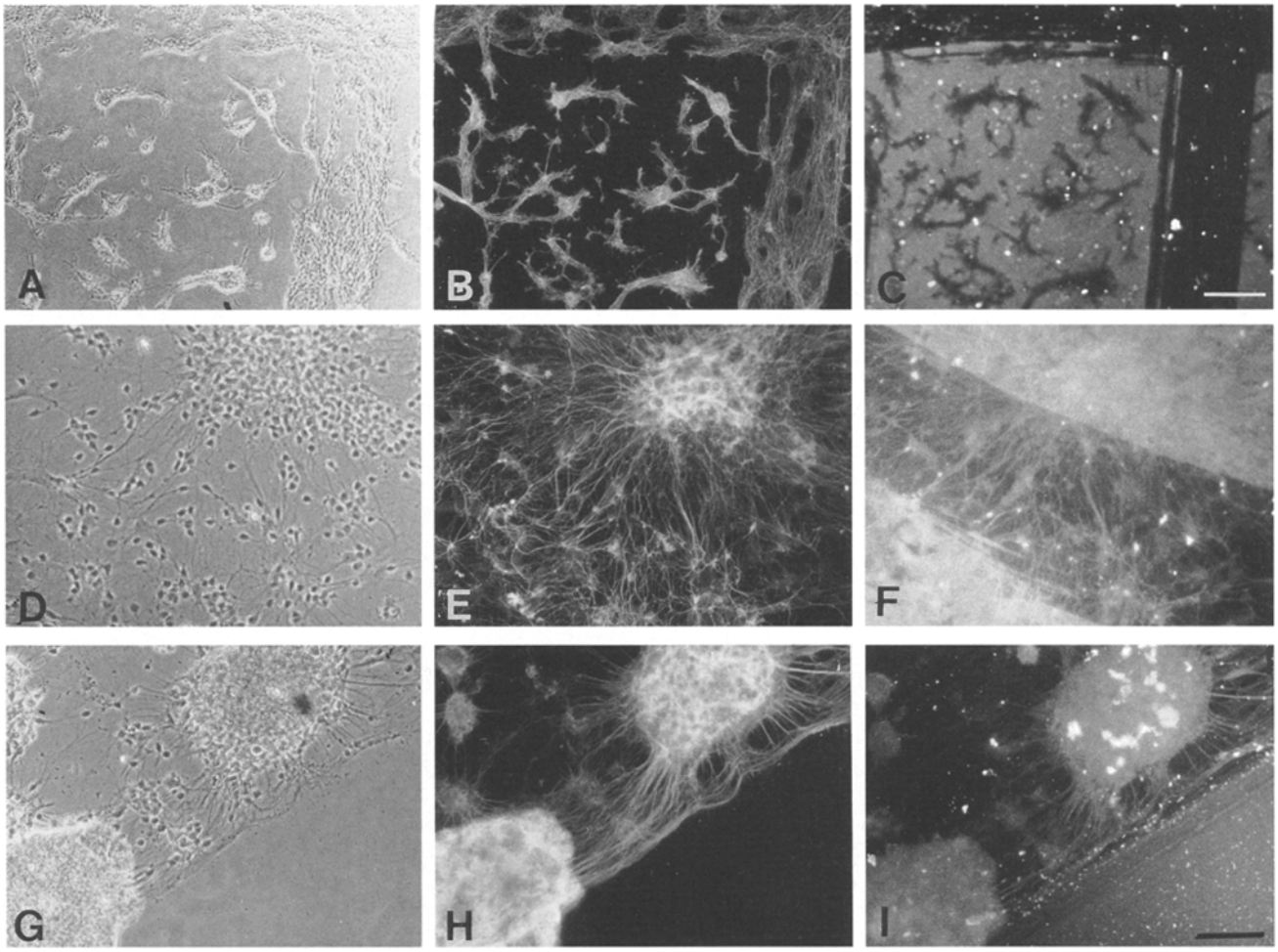


Figure 8. Repulsion of cerebellar neurons and their processes by TN-C domains. P6 cerebellar neurons (*A–C*) or microexplants (*D–I*) were cultivated on patterned substrates consisting of poly-DL-ornithine and TNfnA1,2,4 (*A–C*), TNfn1-3 (*D–F*), and TNegf (*G–I*). Cultures were fixed after 72 h and stained with polyclonal antibodies to the cell adhesion molecule L1 and anti-rabbit FITC (*B*, *E*, and *H*) or stained with monoclonal antibody to the NH_2 terminus of the fusion proteins and anti-mouse TRITC (*C*, *F*, and *I*). Note that dissociated cerebellar neurons avoid TNfnA1,2,4 to some extent and form clusters. The explants attach to TNfn1-3 and avoid areas coated with TNegf. Emigration of granule cells occurs on TNfn1-3 and outgrowing neurites cross areas coated with TNfn1-3, but are deflected by TNegf. The amounts of protein coated to the culture substrates are detailed in Table IV. Bars: (*A–C*) 140 μm; (*D–I*) 70 μm.

same results and because the interactions could be blocked by specific antibodies. E18 hippocampal neurons showed a similar behavior, although binding to the substrate appeared overall weaker. Within 24 h small neuronal aggregates interconnected by fasciculating fibers formed which could be detached from the culture by mild shearing forces. In contrast, cerebellar neurons grew and differentiated on TNfn1-3 within 24 h, yet not as pronounced as on the control substrate laminin-1. The cell-binding site in TNfn1-3 was even more attractive for the neu-7 glioma cell line. In this context, it is interesting that a cell-binding site which is recognized by the integrin αvβ3 has been reported in TNfn3 for U251MG glioma cells (Prieto et al., 1993), fetal bovine endothelial cells, and cell lines derived therefrom (Joshi et al., 1993). In human and chicken TN-C, this cell-binding site is dependent on the RGD sequence, a motif which has not been identified in the mouse glycoprotein (Weller et al., 1991). Yet, a mutation of the RGD sequence to a RAD or RVD, the motifs expressed in mouse TNfn3, did not abolish the adhesive sites for glioma cells in TNfn3 (Prieto et al., 1993). Whether integrins are involved in the binding of cerebellar granule cells or hippocampal neurons to TNfn1-3 is currently not known. Interestingly, P6 cerebellar neurons detached from the domains TNfnA1,2,4, TNfnA1,2,4,B,D, TNfnB,D, TNfn6, and TNfn7,8 within 1 d of culture. Several reasons are conceivable for this process. Thus, the hypothesized receptors for these domains could be downregulated or degraded during the culture period, the domains could be modified, e.g., by proteolysis or, finally, anti-adhesive events could be launched in the responsive neuron which would lead to cell rounding and release from the substrate. The latter possibility might hold true for TNfnA1,2,4,B,D and TNfnA1,2,4, as detailed below. No significant attachment of cerebellar neurons to TNfbg could be found. The carboxy-terminal globular domain is believed to harbor a cell-binding site for fibroblasts, primary glia, U251MG glioma, and endothelial cells (Friedlander et al., 1988; Prieto et al., 1992; Aukhil et al.,

Table IV. Repulsion of Neuronal Cell Bodies and Processes by TN-C Domains

Substrate	E18 hippocampal Neurons	P6 cerebellar Neurons	P6 cerebellar Explants	Coating efficiency		
				%	μg/cm²	pmol/cm²
BSA	−	−	−	ND		
TN-C	+++	+++	+++	28 ± 18	0.74 ± 0.48	3.1 ± 1.99
TNegf	+++	+++	+++	12 ± 8	0.32 ± 0.21	4.4 ± 2.93
TNfn1-3	−	−	−	12 ± 2	0.32 ± 0.05	9.4 ± 1.57
TNfn4,5	(+)	−	−	17 ± 3	0.45 ± 0.08	18.0 ± 3.18
TNfnA1,2,4	+++	+++	+++	23 ± 12	0.61 ± 0.32	19.7 ± 10.28
TNfnB,D	−	−	−	18 ± 11	0.47 ± 0.29	23.3 ± 14.24
TNfnA1,2,4,B,D	+++	+++	+++	18 ± 15	0.47 ± 0.39	9.4 ± 7.83
TNfn6	−	−	−	18 ± 8	0.47 ± 0.21	29.4 ± 13.07
TNfn7,8	−	−	−	12 ± 5	0.32 ± 0.13	13.8 ± 5.75
TNfbg	−	−	−	18 ± 13	0.47 ± 0.34	15.8 ± 11.41

Repulsion of hippocampal and cerebellar neurons and outgrowing processes by TN-C domains. Suspensions of E18 hippocampal and P6 cerebellar neurons or microexplants were cultured on patterned substrates consisting of poly-DL-ornithine–conditioned glass (20 μg/ml) alternating with areas additionally containing TN-C or recombinant domains (Faissner and Kruse, 1990). The protein coating concentration for each protein was 50 μg/ml. Cultures were fixed after 48 or 72 h and monitored for repulsive effects. Representative examples are shown in Fig. 8 and analogous results were obtained in at least three independent experiments for each distinct protein and culture type. The coating concentrations were determined by adding ^{125}I-labeled TN-C or derived recombinant proteins as tracers to the coating solutions. The percent-fraction of adsorbed protein (1st column) was used to calculate the substrate concentrations in μg/cm² (2nd column) or pmol/cm² (3rd column). At least three coverslips were measured per protein and three independent experiments were carried out. Molarities were calculated for TN-C monomers assuming an apparent molecular mass of 240 kD. Note that the repulsive properties do not correlate with the molar concentrations on the substrate, e.g., TN-C is repulsive at 3.1 pmol/cm², whereas TNfn1-3 or TNfn6 do not exert repulsive effects at 3- and 10-fold higher molar densities, respectively.

1993; Joshi et al., 1993). Comparable to these studies, neu-7 cells and hippocampal neurons adhered to some extent on TNfbg, but kept a round morphology and did not differentiate further.

A Search for Anti-Adhesive Domains

Although TN-C contains cell-binding sites, the glycoprotein displays anti-adhesive properties for many cell types including neurons and it had been proposed that additional, independent sites are responsible for antagonistic effects (Chiquet-Ehrismann, 1991; Tucker, 1994; Faissner and Steindler, 1995). In support of this assumption, already the first study characterizing bacterially expressed segments concluded that anti-adhesive properties for L929 cells can be allocated to TNegf, while a cell-binding site was attributed to the carboxy-terminal end of TN-C (Spring et al., 1989). Subsequent investigations reinforced this notion for several cell types including N2A neuroblastoma cells (Prieto et al., 1992). Our data are consistent with these findings in that TNegf showed clear repulsive effects for P6 cerebellar and E18 hippocampal neurons on patterned substrates where the recombinant protein as coated on poly-DL-ornithine alternates with the bare polycation (Faissner and Kruse, 1990). Interestingly, anti-adhesive properties of EGF-type repeats were detected regardless of the expression vector system, e.g., β-galactosidase– (Spring et al., 1989), GST- (Prieto et al., 1992), or, in the present study, poly-histidine–containing fusion proteins. Another anti-adhesive area for NIH-3T3 fibroblasts had been reported in TNfn7-8 (Prieto et al., 1992). Yet, neurons proved indifferent to this protein, which could indicate lineage-dependent differences. In contrast, the alternatively spliced insert TNfnA1,2,4,B,D showed pronounced anti-adhesive qualities for both cerebellar and hippocampal neurons in choice situations and fostered aggregation of these cells when used as plain substrate. Likewise, this recombinant protein and TNegf deflected fibers extending from P6 cerebellar explants on patterned substrates, suggesting that repulsion also affects growth cones. The repulsive properties of the alternatively spliced segment could be further circumscribed to TNfnA1,2,4. Thus, the initial attachment to TNfnA1,2,4,B,D and the anti-adhesive properties of these FNIII repeats apparent after longer periods could reflect a sequential interaction with cell binding, and, subsequently, repulsive domains which would result in inhibition of spreading through cellular responses which are controlled by unknown second messengers (Faissner, 1993; Faissner et al., 1994*a*, 1995). In this perspective, the insertion of supplementary anti-adhesive FNIII repeats could serve the fine tuning of the repulsive properties of TN-C. One functional implication might be the creation of an environment favorable to cellular movement by promoting both attachment and detachment from neighboring cells and the surrounding matrix. This hypothesis is supported by our finding that mAb J1/tn1 which retards cerebellar granule cell migration (Husmann et al., 1992) maps to the repulsive domains TNfnA1,2,4. Along these lines, the expression of high molecular weight TN-C isoforms peaks during phases of cell and growth cone motility; for example, in the mouse cerebellum where the ratio of high to low M_r TN-C shifts from 5:1 to 1:3 during postnatal development (Bartsch et al., 1992); or in the developing cornea, which is invaded by migrating neural crest cells (Kaplony et al., 1991). This interpretation is consistent with earlier studies which reported that the alternatively spliced FNIII-motives of human TN-C induce anti-spreading in bovine aortic endothelial cells, presumably by inducing downregulation of focal contact sites (Murphy-Ullrich et al., 1991). This recombinant TN-C fragment also prevents the adhesion of uterine epithelial cells to Matrigel (Julian et al., 1994). In addition, indirect evidence supports the concept that the alternatively spliced FNIII sequence in TN-C harbors anti-adhesive site(s). Thus, maximal attachment of N2A neuroblastoma cells was achieved at lower amounts of TNfn1-6 including the

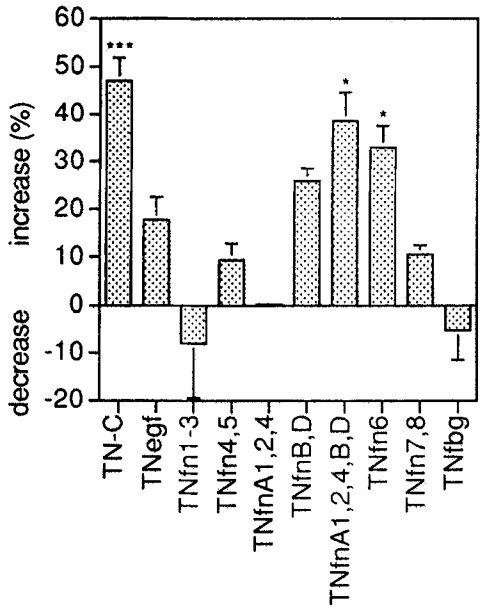
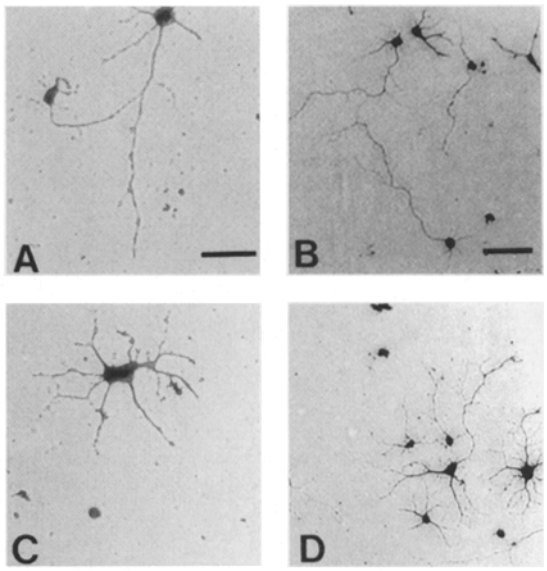


Figure 9. Neurite outgrowth stimulation of hippocampal neurons on TN-C domains. E18 hippocampal neurons were seeded at low density in tissue culture plastic chamber slides on PORN substrates coated with different TN-C domains (50 μg/ml). The proportion of cells with neurites was determined as a fraction of at least 200 cells per well selected at random and given in percent. At least 1,200 neurons in total were analyzed in three independent experiments for each substrate. Percentage of increase P of the fraction of process bearing cells on test proteins (F_{test}) as compared to the poly-ornithine control (F_{ctrl}) was determined as $P = (F_{test} - F_{ctrl})/F_{ctrl} \times 100$. Mean values ± SD are shown. The alternatively spliced FNIII repeats TNfnB,D and TNfnA1,2,4,B,D, and TNfn6 increase the fraction of process bearing neurons. Note that TNfn1-3 does not enhance this parameter although its substrate concentration (4.68 pmol/cm^2) is comparable to the one of TNfnB,D (5.64 pmol/cm^2) or TNfnA1,2,4,B,D (4.21 pmol/cm^2). For concentrations of proteins adsorbed to the culture substrates see Table II. Results were statistically analyzed with the Mann-Whitney U-test and the symbols mean: * $0.05 > P > 0.01$ and *** $0.005 > P > 0.001$. The values obtained for TNfn1-3 and TNfn6 confirm earlier observations (Faissner et al., 1995).

alternatively spliced segment (FN2) as compared to TNfn1-6 without that part of TN-C (FN1), but fibroblasts growing on a mixed substrate of fibronectin plus FN2 were significantly more aggregated than on fibronectin combined with any other fragment (Prieto et al., 1992).

Neurite Outgrowth Promoting Domains in TN-C

Promotion of neurite outgrowth by homogeneous TN-C–containing substrates had been described for chicken E3 spinal cord and E7-8 DRG explants (Wehrle and Chiquet, 1990; Wehrle-Haller and Chiquet, 1993). Similarly, TN-C promotes outgrowth from dissociated E18 hippocampal and P6 cerebellar neurons (Lochter et al., 1991; Husmann et al., 1992). Based on in vitro perturbation assays with mAb J1/tn2, a neurite outgrowth promoting domain was assigned to the distal splice site of TN-C, which is TNfnD or TNfn6 (Lochter et al., 1991). Our studies with recombinant domains clearly support this conclusion. Thus, the fraction of process bearing E18 hippocampal neurons and the lengths of their longest process were increased on TNfnB,D,



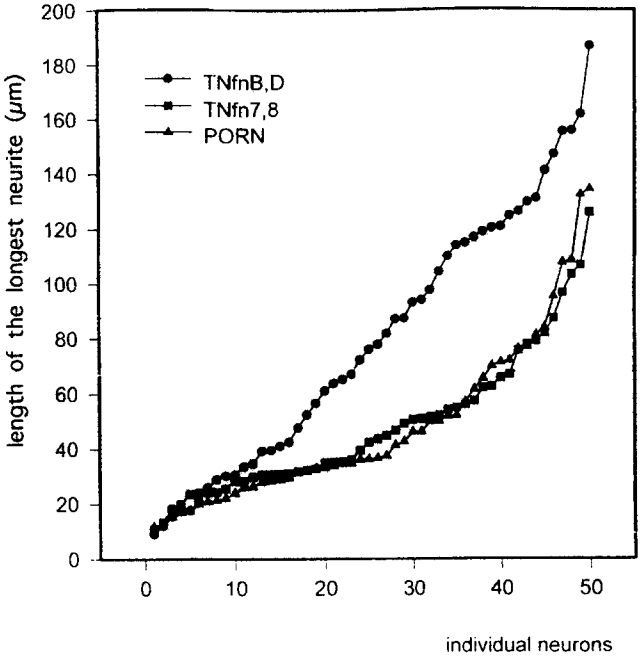


Figure 10. Length distribution of hippocampal neurites on TN-C domains. E18 hippocampal neurons were grown in tissue culture plastic chamber slides on PORN coated at 50 μg/ml with TNfnB,D (*A* and *B*) and PORN plus TNfn7,8 (*C* and *D*) for 24 h, fixed, and stained for tubulin. The lengths of the longest process of 50 hippocampal neurons were plotted for different substrates, as indicated. According to the Mann-Whitney U-test, the length distributions of the longest processes on PORN and on PORN/TNfn7,8 (1.47 pmol/cm^2, Table II) did not significantly differ from one another, while the length distribution on PORN/TNfnB,D (5.64 pmol/cm^2) exhibited a clear bias towards higher values ($P < 0.001$). As further control, TNfnA1,2,4 (8.76 pmol/cm^2) did not result in increased neurite lengths (not shown). Bars in *A*, 40 μm; in *B*, 80 μm.

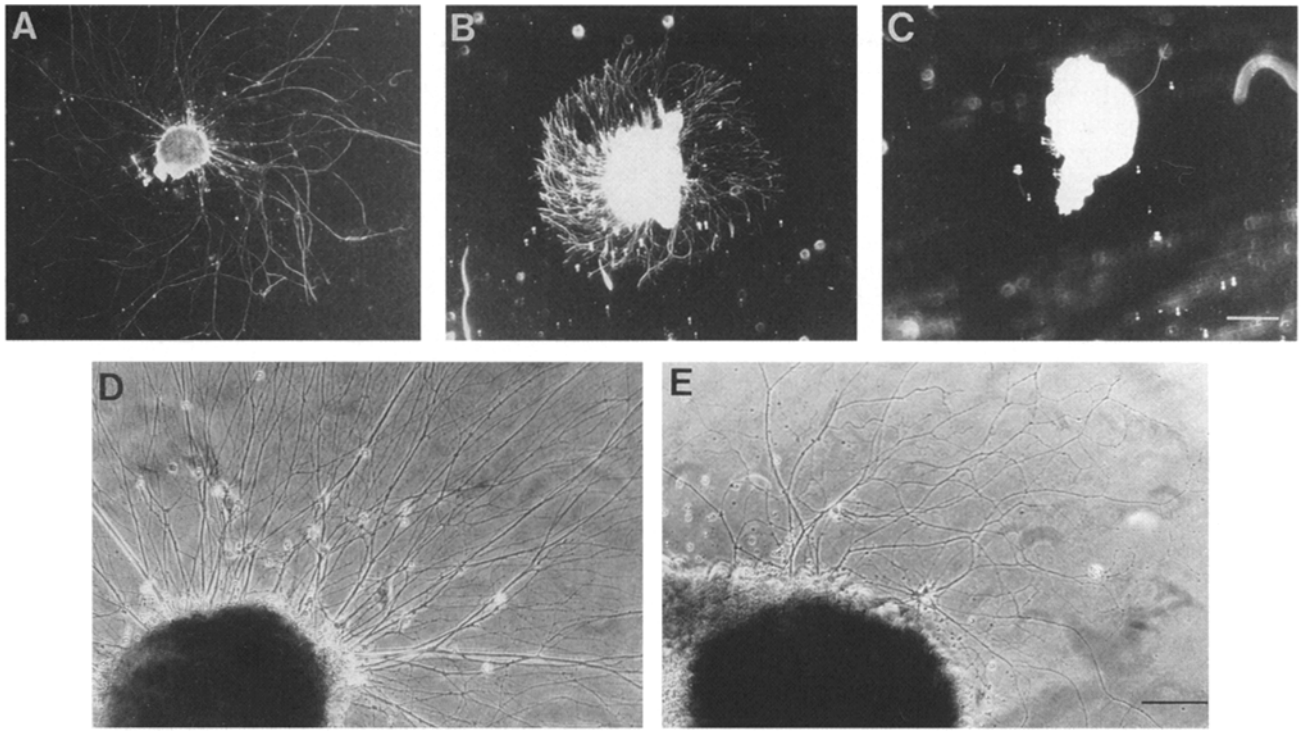


Figure 11. Dorsal root ganglia explants on homogenous TN-C domain substrates. DRG explants from P0/1 mice were cultured for 48 h on tissue culture plastic dishes coated with TN-C (*A*), TNfnD,6 (*B* and *C*), laminin-1 (*D*), and TNfnB,D (*E*, all at 20 μg/ml). Neurite outgrowth of DRG explants is supported on TN-C (*A*, 0.53 pmol/cm^2, Table II). Note that the neurite outgrowth promoting activity of TNfnD,6 (2.4 pmol/cm^2) is not affected by mAb J1/tn1 (*B*), but is totally blocked by mAb J1/tn2 (60 μg/ml in the culture medium, *C*). *A–C* are dark-field micrographs. Laminin-1 fosters the formation of a vigorous halo of neurites from DRG-explants (*D*, phase contrast). TNfnB,D (9.34 pmol/cm^2) is also a supportive substrate for DRG neurites (*E*, phase contrast). At least 10 explants were seeded per well and protein substrate, and three independent experiments were carried out with analogous results. Bars (*A–C*) 300 μm; (*D* and *E*) 100 μm.

TNfnD,6, and TNfn6 after 24 h. The fact that hippocampal neurons did not bind to TNfn6, and only moderately to TNfnB,D in the short-term cell-binding assay, might reflect that the hypothesized receptor(s) for this domain is not or only weakly expressed on the cell body shortly after plating and begins to operate once the growth cone forms and moves over the culture substrate. Interestingly, mAb J1/tn2 which blocks neurite outgrowth promotion properties of TN-C maps to TNfnD. Because TNfn6 promotes neurite outgrowth by itself, the blocking activity of J1/tn2 on intact TN-C probably occurs in part through steric hindrance of domains neighboring its binding site. In addition to the FNIII domains, TNegf enhanced neurite lengths to some extent, an effect which might be linked to its anti-adhesive properties. It has been proposed that release of cell-substrate adhesion forces favors axogenesis and that anti-adhesive properties of TN-C might underlie the increase of neuronal polarity induced by this glycoprotein (Chamak and Prochiantz, 1989; Rousselet et al., 1990; Lochter and Schachner, 1993). However, TNfnB,D, TNfnD,6, and TNfn6 did not display anti-adhesive properties in repulsion assays or choice situations for neuronal cell bodies and growth cones. Therefore, neurite outgrowth promotion effected by these proteins probably occurs through another route than the one implicated in the activity of the EGF-type repeats. The neuronal Ig-superfamily member F3/F11/contactin has been found to bind to TNfn5,6 (Zisch et al., 1992; Vaughan et al., 1994). It is presently not known whether this adhesion molecule is involved in the promotion of neurite outgrowth by TN-C. This seems unlikely because F11 does not react with TN-C isoforms which contain alternatively spliced FNIII domains and thus would not interact with TNfnB,D or TNfnD,6, domains which

Table V. Quantitative Analysis of the Longest Neurites

Substrate	Longest neurites of 50 cells	SD	SE	Percent increase (as compared to control)	
	μm	μm	μm		
PORN (control)	2431	254	147	–	
TNegf	4046	92	53	67.5 ± 15.2	(**)
TNfnB,D	4047	306	176	66.8 ± 4.9	(**)
TNfnD,6	3581	252	145	47.6 ± 5.6	(**)
TNfn6	3250	287	166	35.3 ± 24.8	(*)
TNfn7,8	2846	333	192	17.7 ± 16.5	(ns)
TN-C	4100	359	208	70.1 ± 26.9	(**)
LN-1	3352	244	172	45.9 ± 1.3	(*)

Hippocampal neurons were cultured under distinct conditions as detailed in legend to Fig. 10 and morphometrically analyzed as specified in Materials and Methods. 50 process-bearing neurons per individual well were collected at random and the length of the longest neurite was determined for each single cell. Since neurite lengths were not normally distributed the values for individual neurites of a well were summed. 300 neurons from three independent experiments were sampled. The average values and corresponding standard deviations of the single sums were calculated. The significance of the differences between the mean values of sums of the longest neurite was estimated with the *t*-test and is indicated next to the percent increase with the symbols meaning (**): $P < 0.005$, (*): $0.01 < P < 0.05$, and (*ns*): nonsignificant. Percentages were calculated as percent increase = $(L_{test} - L_{control})/L_{control} \times 100$. For substrate concentrations of the different proteins, see Table II. Abbreviations are *PORN*, polyornithine; *LN*, laminin; *SD*, standard deviation; *SE*, standard error of the mean.

clearly stimulate neurite outgrowth in our assays. Whether the integrin α8β1, which has recently been implicated in promotion of neurite outgrowth of chicken motoneurons by TN-C (Varnum-Finney et al., 1995), binds to these proteins remains to be seen. It has to be kept in mind, though, that TN-C does not promote neurite outgrowth of every neuron. Lineage dependence is indicated by the fact that retinal neurons do not grow any neurite on a homogeneous TN-C–containing surface (Perez and Halfter, 1993; Taylor et al., 1993). Furthermore, as discussed above, most neurites tested so far are deflected at sharp boundaries of TN-C alternating with a conducive substrate (Faissner and Kruse, 1990; Crossin et al., 1990; Taylor et al., 1993; Krull et al., 1994*a*,*b*).

Conclusions

The present study has substantiated and confirmed the structure-function model of TN-C for CNS neurons proposed earlier (Faissner, 1993; Faissner et al., 1994*a*, 1995). Short-term assays have revealed several interaction sites which are recognized with low affinity by CNS neurons. One of these, TNfn1-3, supports growth and differentiation of cerebellar neurons in medium term culture. With regard to anti-adhesive effects, two independent areas could be circumscribed, TNegf and the alternatively spliced FNIII domains TNfnA1,2,4, which could synergize to produce overall repulsive effects of TN-C on CNS neurons. Finally, neurite outgrowth-promoting properties could be assigned to the region covered by TNfnD,6. Notably, the downstream alternatively spliced FNIII repeats TNfnB,D by themselves promoted neurite outgrowth. Thus, our observations emphasize that the developmental regulation of alternative splicing is functionally significant, consistent with the preferential expression of the high M_r TN-C isoforms during phases of increased cell migration and neurite growth (Crossin et al., 1989; Steindler et al., 1989*a*,*b*; Husmann et al., 1992; Bartsch et al., 1992). Several receptors have been described for TN-C some of which, e.g., F3/F11/contactin, the integrin α8β1, neurocan and phosphacan, are expressed in neural tissues (Zisch et al., 1992; Maurel et al., 1994; Barnea et al., 1994; Grumet et al., 1994; Varnum-Finney et al., 1995). Interestingly, phosphacan is a member of the tyrosine-phosphate-phosphatase (PTP) family of membrane components. This opens an avenue to study whether TN-C affects second messenger cascades.

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