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Tenascin-R Plays a Role in Neuroprotection via Its Distinct Domains That Coordinate to Modulate the Microglia Function*

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Microglia are one of the main cell types activated by brain injury. In the present study, we have investigated how domains of the extracellular matrix molecule tenascin-R (TN-R) modulate microglia function. We found that epidermal growth factor-like repeats inhibited adhesion and migration of microglia via a protein kinase A-dependent mechanism. In contrast, fibronectin 6–8 repeats promoted adhesion and migration of the primary microglia via a protein kinase C-dependent mechanism. Both domains of TN-R induced an up-regulation in the secretion of cytokines, such as chemokine-induced cytokine 3 and tumor neurosis factor α. Interestingly, epidermal growth factor-like repeats and fibronectin 6–8 induced a dramatic up-regulation in the secretion of brain-derived neurotrophic factor/transforming growth factor-β and nerve growth factor/transforming growth factor-β, respectively, and conditioned medium from activated microglia was able to promote neurite outgrowth of N1E-115 cells and primary cortical neurons. These results suggest that TN-R plays a role in neuroprotection through distinct domains coordinating to modulate microglia function.

Microglia are recognized as one of main players in the response to brain and spinal cord injury because these cells are rapidly activated in response to even minor pathological changes in the central nervous system (1, 2). This action leads to a secondary injury in which neurons and glial cells die in the hours, days, or even weeks following the initial injury. After central nervous system injury, the first therapeutic goal is to preserve as many cells as possible because functional recovery cannot be established if the nerve cells are going to die. Thus, any approach to neural regeneration that takes into account the actions of microglia is essential, *e.g.* suppressing or eliminating the migration of the activated microglia to the injury site. It had been found that TN-R[1] was anti-adhesive for activated microglia *in vitro* and down-regulated in the lesioned nucleus after peripheral nerve axotomy (3). However, the molecular mechanism of TN-R involved in microglia migration remains unclear.

TN-R is expressed predominantly by oligodendrocytes during the onset and early phases of myelin formation and remains expressed by oligodendrocytes in the adult as well as some interneurons in the spinal cord, retina, cerebellum, and hippocampus (4–7). TN-R comprise a cysteine-rich amino-terminal part that is involved in multimerization, a region consisting of epidermal growth factor-like repeats (EGFL), a region consisting of fibronectin type-like (FN) homologous repeats, and a fibrinogen-like domain (FG) at the carboxyl-terminus. Two major TN-R molecules of apparent molecular masses of 160 and 180 kDa have been isolated. Interestingly, TN-R co-localizes with other glial-derived molecules, such as myelin-associated glycoprotein and a phosphacan-related molecule, at high density in central nervous system myelinated axons (8). TN-R is a multifunctional molecule that promotes neurite outgrowth when presented as a uniform substrate, inhibits growth cone advance when offered as a sharp substrate boundary, induces axonal defasciculation *in vitro*, resulting from interaction with its neuronal receptor, F3/contactin, and interacts with sodium channels at nodes of Ranvier, modulating the function of sodium channels (9–15). Because TN-R is a glia-derived molecule with multiple domains in the central nervous system, it is intriguing to explore its direct effects in modulating the activated microglia, which indirectly influences neurons. In the present study, taking the advantages of the subcloned domains of TN-R (13), we have shown that distinct TN-R domains may work together to modulate the microglia function during neuroprotection.

EXPERIMENTAL PROCEDURES

Primary Microglia Culture—Mixed cell culture was prepared following the described method (16) with some modifications. Briefly, cerebral cortex from embryonic Wista rat (embryonic day 18–19) was stripped of meninges, chopped into small chunks, and dissociated in trypsin-EDTA (0.25%) before being seeded into T75 tissue culture flasks in Dulbecco's modified Eagle's medium/nutrient mixture F-12 supplement with 10% fetal calf serum. Subsequent medium change was carried out every 3 days. After 10–14 days in culture, floating cells and weakly attached cells on the mixed primary culture cell layer were isolated by gently shaking of the flask for 1 h. The resulting cell suspension was transferred to a Petri dish and allowed to adhere at 37 °C for 1 h. Unattached cells were discarded, and microglia was isolated as strongly adhering cells. About 95% of these attached cells were positive for Mac-1, a marker for microglia cell types.

Adhesion Assays—4-well plates were coated with methanol-solubilized nitrocellulose as described previously (17, 13) and air-dried under a sterile hood. For adhesion assays, 2 μl spots of different glutathione *S*-transferase (GST) fusion proteins, each at a concentration of 50 μg/ml, were applied in duplicate to the nitrocellulose-coated surfaces of the plate and incubated overnight at 4 °C. Subsequently, the spots were washed three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution. The plates were flooded with Ca^{2+}-and Mg^{2+}-free Hanks'

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[1] The abbreviations used are: TN-R, tenascin-R; EGFL, epidermal growth factor-like repeats; FN, fibronectin type-like repeats; FG, fibrinogen-like domain; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; TGF-β, transforming growth factor-β.

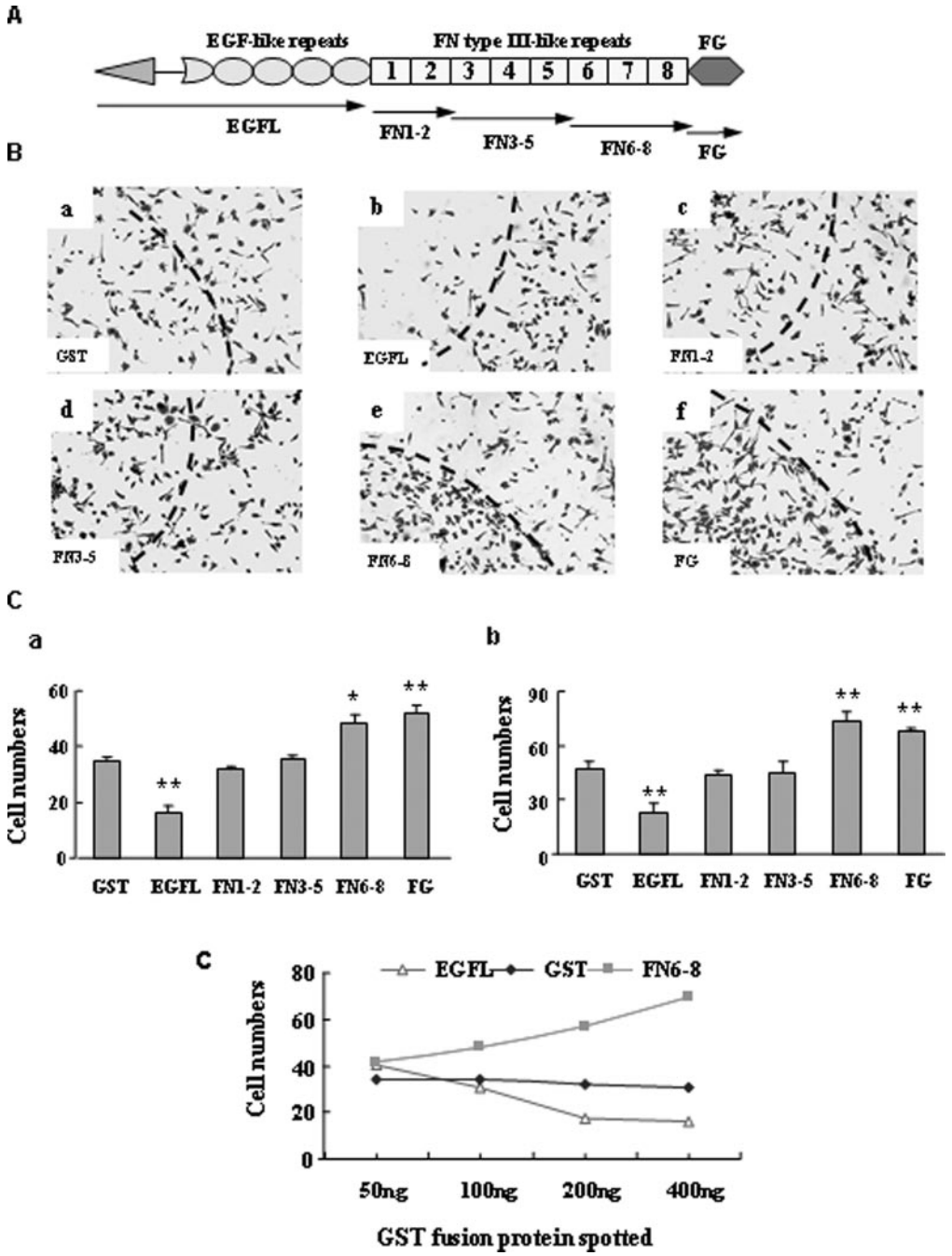


FIG. 1. **The effect of different tenascin-R domains on microglia adhesion.** *A*, TN-R polypeptide chain comprises a series of structural motifs: EGF-like repeats (*ovals* and *triangle*), FN type III-like repeats (*squares*), and an FG knob (*hexagon*). *Arrows* represent the domains of TN-R included in the GST fusion proteins used in our experiments. *B* and *C*, microglia were plated onto different TN-R domain coated substrates for the short term (1 h, *C*, *a*) and long term (24 h, *B* and *C*, *b* and *c*) adhesion assays. The *dotted lines* indicate the coated protein in each panel (*B*, *a*, GST; *b*, EGFL; *c*, FN1–2; *d*, FN3–5; *e*, FN6–8; *f*, FG). Either EGFL induced anti-adhesion or FN6–8 induced adhesion occurred in a dose-dependent manner (*C*, *c*). All data points are expressed as cell numbers per visual field and represent the mean ± S.E. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$ compared with the GST group.

balanced salt solution containing 2% heat-inactivated fatty acid-free bovine serum albumin (Sigma) and incubated for at least 0.5 h in a 37 °C incubator to block residual nonspecific protein binding sites. Then the plates were washed three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, and freshly dissociated microglia were seeded in 300 μl of medium for adhesion assay. After 1 h (short term) or 24 h (long term), the plate was washed three times with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution, and cells were fixed with 2.5% glutaraldehyde and stained with Coomassie blue. The numbers of cells adhering to different GST fusion protein spots were counted under phase microscopy. All experiments were performed at least three times.

Assays for Testing Signaling Pathways Involved—To investigate which signaling pathways are involved in the adhesion assays, microglia were incubated with various kinase and phosphatase inhibitors and activators, respectively, for 30 min prior to plating onto EGFL or FN6–8 coated substrates. The concentration used was as follows: 100 μM erbstatin, 100 μM H7, 100 nM calphostin C, 1 nM okadaic acid, 0.1, 0.5, and 1.0 mM dbcAMP (protein kinase A activating molecule), 0.1, 1.0, and 5.0 nM phorbol 12-myristate 13-acetate.

Transwell Migration Assay—Costar Transwell polycarbonate filters (5-μm pore size) were used in a migration assay to examine the ability of microglia migration (18). The undersurfaces of the 6.5-mm transwell membranes were coated with different TN-R domains and GST (50 μg/ml) in PBS overnight at 4 °C. The undersurfaces were blocked with 2% heat-inactivated fatty acid-free bovine serum albumin, and then 2.5×10^5 cells/ml were plated in serum-free culture medium into the upper chamber and allowed to migrate through the pores onto the coated undersurfaces at 37 °C in a CO_2 incubator. After 24 h, cells from

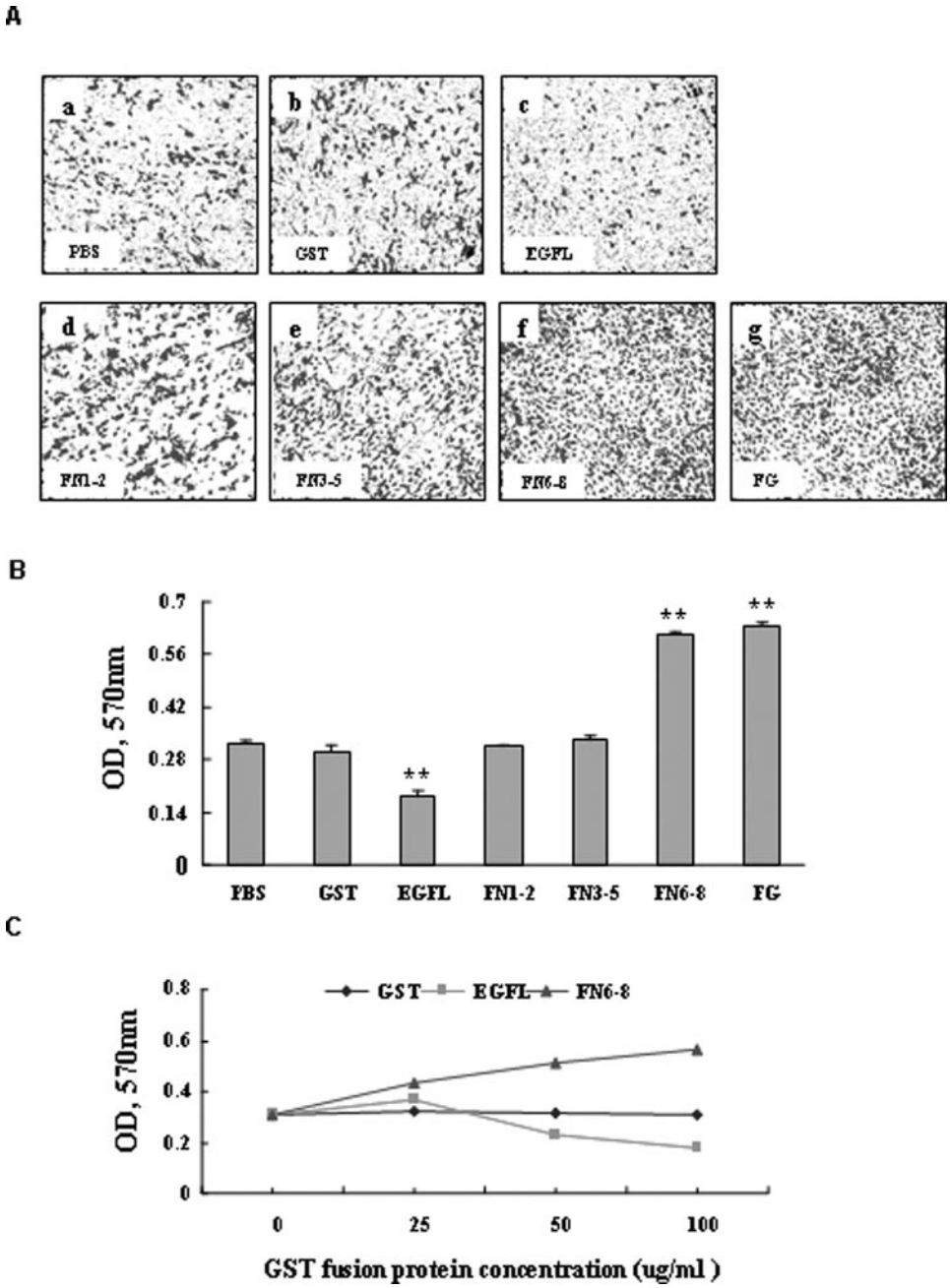


FIG. 2. **The effect of different TN-R domains on microglia migration.** The transwell membrane undersurface was coated with different TN-R domains. In *A*, *a*, PBS; *b*, GST; *c*, EGFL; *d*, FN1–2; *e*, FN3–5; *f*, FN6–8; *g*, FG. Relative numbers of cells transmigrating through membrane were determined by staining cells on the undersurface of the transwell membrane with crystal violet followed by cell lysis and measurement of absorbance value at 570 nm. Dye levels were directly proportional to numbers of microglia (*B* and *C*). Data were presented as mean ± S.E. **, $p < 0.01$ compared with control group.

the inner surface of the insert were gently wiped out with cotton-tipped swabs, and the inserts were fixed with 2.5% glutaraldehyde and stained with crystal violet solution (0.2%). After a final PBS washing, the cells were examined under a microscope to confirm proper morphology, and then dye was extracted with 10% acetic acid. The absorbance was measured at 570 nm using a microplate reader. Dye levels are directly proportional to the numbers of microglia. Data are presented as mean ± S.E.

Cytokine Array Assay—Purified microglia were cultured in serum-free Dulbecco's modified Eagle's medium/nutrient mixture F-12 medium. Microglia were treated for 8 h in the presence or absence of GST-fusion protein (100 μg/ml GST, EGFL, FN6–8, respectively) in a 37 °C incubator, and then culture medium was changed with fresh serum-free Dulbecco's modified Eagle's medium/nutrient mixture F-12 medium. After 16 h, supernatants were collected as conditioned medium for cytokine assay. Cytokine array assay was carried out with RayBio™ rat cytokine array kit (RayBiotech, Inc.) according to the manufacturer's protocol. Each cytokine array membrane was scanned and the intensities of signals were quantitated by densitometry (Bio-Rad).

Primary Cortical Neuron Culture—Primary cortical neuron culture was prepared as described previously (19) with some modifications. Briefly, cerebral cortexes were isolated from embryonic day 18 Wistar rat, and meninges were gently removed from cortex. All cortexes were chopped finely and cortical neurons were dissociated by repeated trituration. Dissociated cells were filtered through a 40-μm cell strainer (BD Falcon™) to remove any remaining clumps of cells. The cells were cultured at a concentration of 1×10^5/ml in 48-well plates precoated with poly-L-lysine (0.1 mg/ml) in neurobasal medium containing B27 supplement and penicillin/streptomycin (Invitrogen). Cells were cultured in a humidified incubator in 5% CO_2 at 37 °C for 1 day then used in a neurite outgrowth experiment. Under these conditions, about 95% of cells were positive for MAP2, a marker for neuron cell types.

Neurite Outgrowth Assay—Microglia conditioned media (obtained from the above method) were collected for the assay of neurite out-

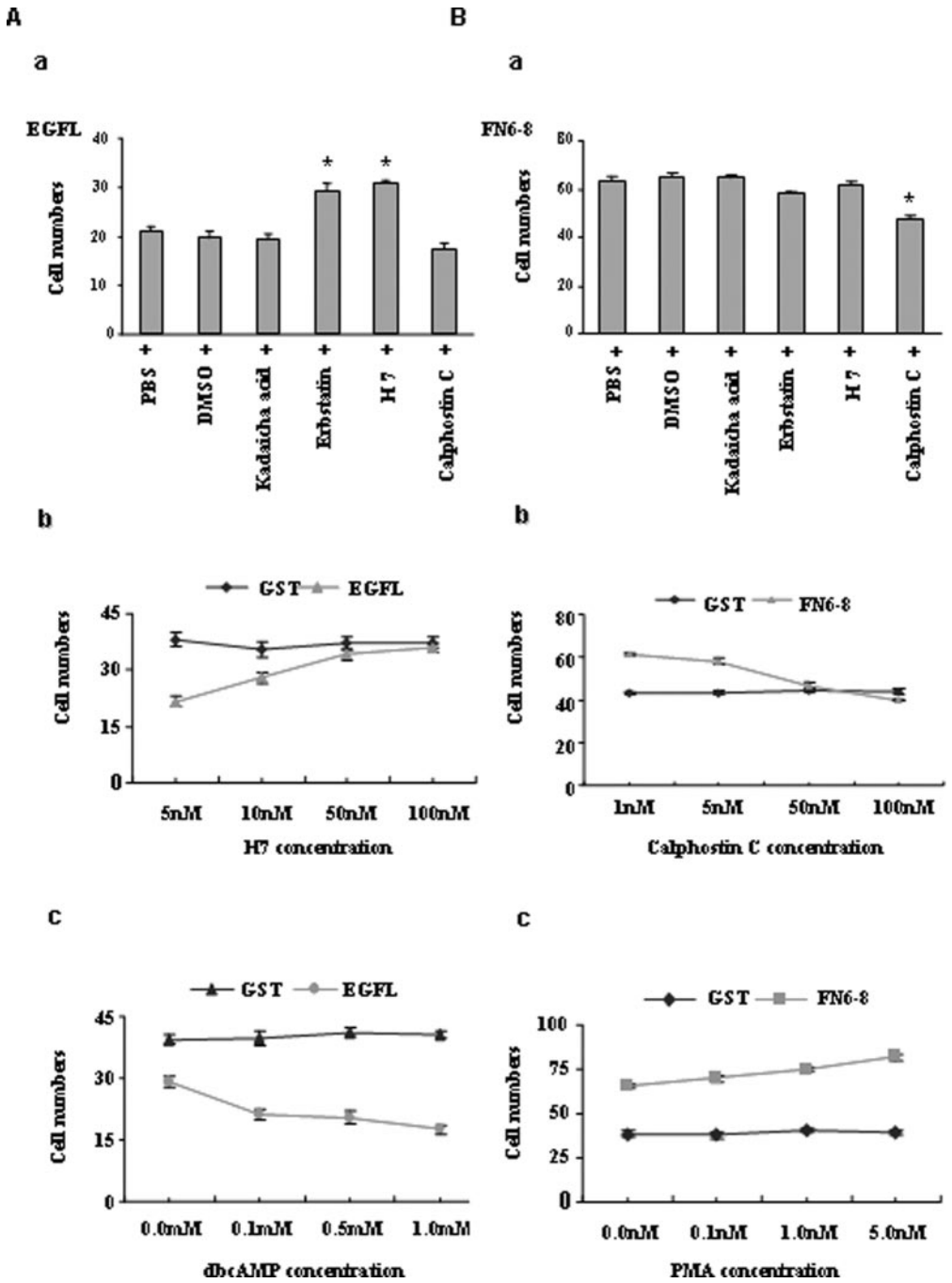
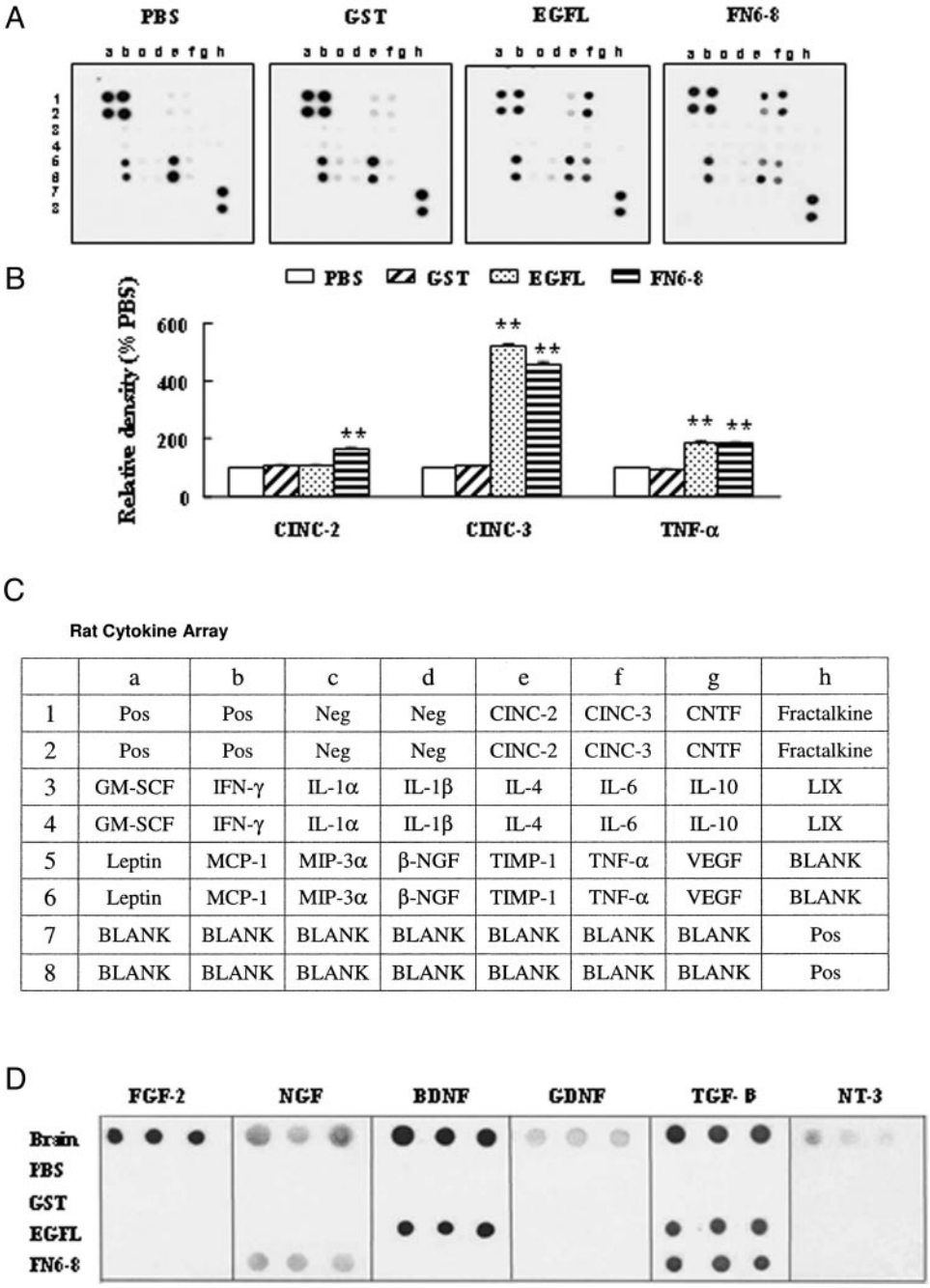


FIG. 3. **Signaling pathways involved in modulating microglia functions induced by TN-R domains.** *A*, protein kinase A involved in the EGFL induced microglia anti-adhesion. Microglia adhesion assay was performed in the presence of various kinase inhibitors (100 μM erbstatin, 100 μM H7, 100 nM calphostin C) and phosphatase inhibitor (1 nM okadaic acid) on the coated EGFL domain (*a*). The influence of different concentrations of H7 (*b*) and dbcAMP (*c*) on EGFL induced microglia anti-adhesion is shown. *B*, protein kinase C involved in the FN6–8 induced microglia adhesion. Microglia adhesion assay was performed in the presence of various kinase inhibitors (100 μM erbstatin, 100 μM H7, 100 nM calphostin C) and phosphatase inhibitor (1 nM okadaic acid) on the coated FG6–8 domain (*a*). The influence of different concentrations of calphostin C (*b*) and phorbol 12-myristate 13-acetate (*c*) on FN6–8 induced microglia adhesion. Me_2SO and GST were used as controls. All data points were expressed as cell numbers per visual field and represent the mean ± S.E. of three experiments. *, $p < 0.05$ compared with the GST group.

growth in neuroblastoma cell line (N1E-115) and primary cortical neurons. NIE-115 was cultured in Dulbecco's modified Eagle's medium supplement with 10% fetal calf serum. NIE-115 and cortical neurons were cultured in a 48-well plate; medium was replaced with microglia conditioned medium (300 μl). After 24 h, NIE-115 and cortical neurons were fixed with 2.5% glutaraldehyde, and NIE-115 was stained with Coomassie blue. The wells were viewed under a microscope (Carl Zeiss). Only cells that did not have contact with other cells were evaluated. Neurites were defined as those processes with a length equivalent to at least one cell body diameter. The number of cells with neurite was calculated from about 90 cells from three independent experiments.

Dot Blot—After TN-R domain stimulated microglia for 8 h, microglia conditioned media were collected and dotted on the nitrocellulose membrane (2 μl/do and then membranes were incubated with growth factor antibody (pαNGF purchased from Sigma; pαFGF-2, pαBDNF, pαGDNF, pαTGF-$\beta$and pαNT-3, obtained from Santa Cruz Biotechnology, 1:500) for 12h at 4 °C after blocked with 5% skim milk for 1 h at room temperature. Blots were reacted with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:5000) for 3 h at room temperature and developed using ECM™ chemiluminescent detection reagents for visualization.

Data Analysis—All data were expressed as mean ± S.E. Statistical evaluations were achieved by one-way analysis of variance. Differences were considered to be significant when $p < 0.05$.



	a	b	c	d	e	f	g	h
1	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fractalkine
2	Pos	Pos	Neg	Neg	CINC-2	CINC-3	CNTF	Fractalkine
3	GM-SCF	IFN-γ	IL-1α	IL-1β	IL-4	IL-6	IL-10	LIX
4	GM-SCF	IFN-γ	IL-1α	IL-1β	IL-4	IL-6	IL-10	LIX
5	Leptin	MCP-1	MIP-3α	β-NGF	TIMP-1	TNF-α	VEGF	BLANK
6	Leptin	MCP-1	MIP-3α	β-NGF	TIMP-1	TNF-α	VEGF	BLANK
7	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	BLANK	Pos
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FIG. 4. **Detection of cytokine and growth factor expression from microglia conditioned medium induced by EGFL and FN6–8 domains.** *A*, cytokine array patterns of microglia conditioned medium induced by PBS, GST, EGFL, and FN6–8. *B*, cytokine arrays were scanned, and the intensities of signals were quantitated by densitometry. Data are presented as mean ± S.E. **, $p < 0.01$ compared with the control group. *C*, rat cytokine array layout. *D*, the *dot blots* show the profile of growth factor expression by microglia triggered by either EGFL or FN6–8 domains.

RESULTS

The Effects of Different TN-R Domains on Microglia Adhesion—Given that TN-R is an extracellular matrix molecule with multiple domains, we first investigated the effects of different TN-R fragments on activated microglia using cell adhesion assay. Expression of different TN-R domains fused to GST has been described (13). The position of the TN-R-derived domain in GST-fusion protein relative to the TN-R primary structure is indicated in Fig. 1*A*. Cell adhesion assay was performed in which different fragments were applied as spots to the cell culture plate coated with translucent nitrocellulose. After the plate was blocked with bovine serum albumin, freshly dissociated rat microglia were plated. After adhering for either 1 h (Fig. 1*C*, *a*) or 24 h (Fig. 1*C*, *b*), the number of microglia markedly increased in adherence to FN6–8 and FG domains and significantly reduced to EGFL domain, and there was no significant change to FN1–2 and FN3–5 domains compared with GST (Fig. 1*B*, *a–f*, and *C*). Moreover, different concentrations of EGFL and FN6–8 domains were applied to confirm their distinct roles in microglia adhesion. As shown in Fig. 1*C*, *c*, either the adhesive effect induced by the FN6–8 domain or the anti-adhesive effect induced by the EGFL domain occurred in a dose-dependent manner.

The Effects of Different TN-R Domains on Microglia Migration—Given that the distinct TN-R domains have different effects on microglia, next we investigated whether they could

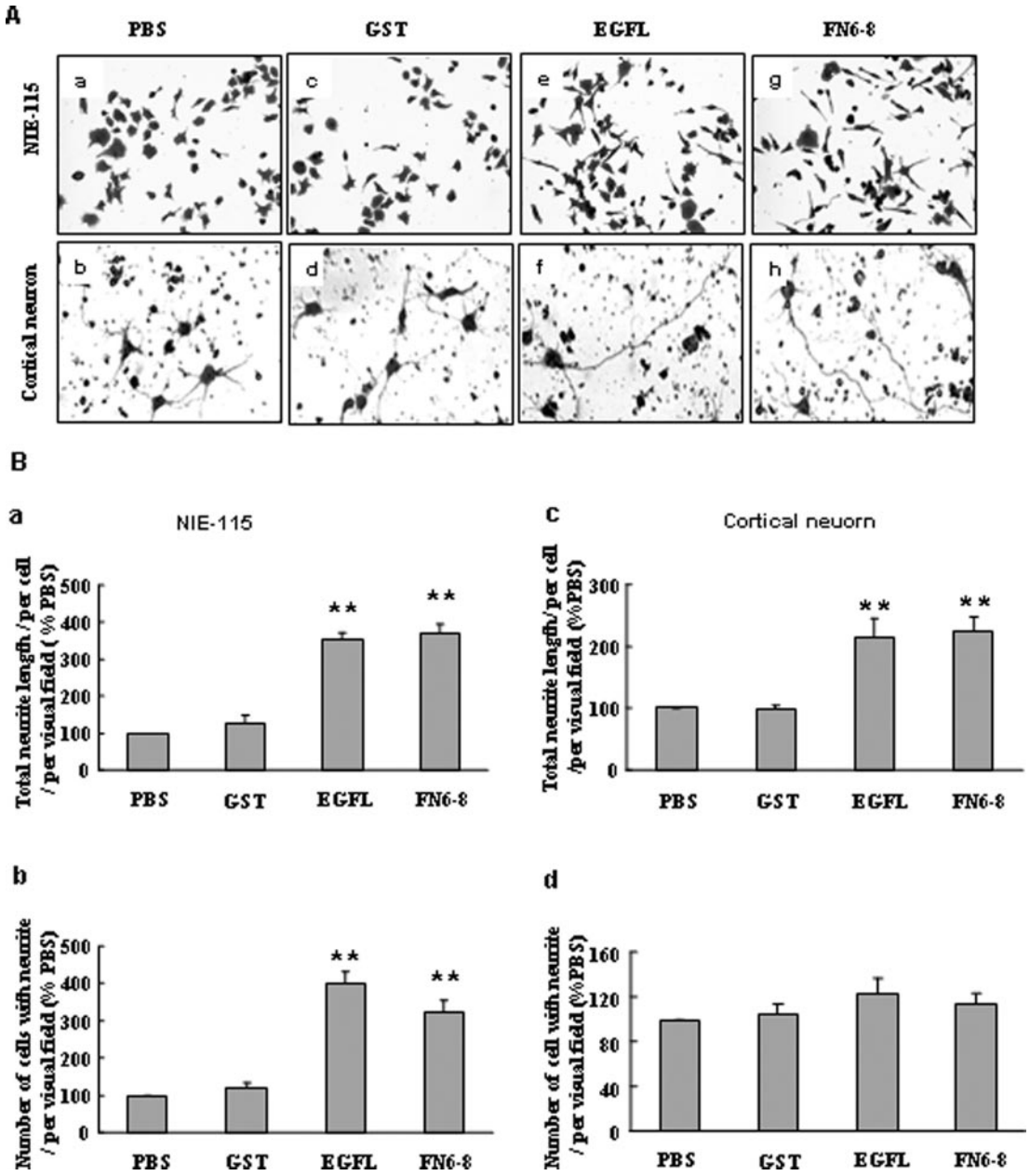

FIG. 5. **Effects of the microglia activated by EGFL and FN6–8 domains on neurite outgrowth.** The microglia conditioned medium induced by EGFL and FN6–8 domains affect the neurite outgrowth of N1E-115 cells and primary cortical neuron. *A*, photomicrograph of cultured N1E-115 (*a*, *c*, *e*, and *g*) and primary cortical neuron (*b*, *d*, *f*, and *h*) treated with microglia conditioned medium induced by PBS, GST (100 μg/ml), EGFL (100 μg/ml), and FN6–8 (100 μg/ml) domains, respectively. *B*, statistical assessment of the number of N1E-115 cells (*B*, *b*) and primary cortical neurons (*B*, *d*) with neurite. Total neurite length of N1E-115 cells (*B*, *a*) and primary cortical neurons (*B*, *c*) were assayed quantitatively. Data are presented as mean ± S.E. **, $p < 0.01$ compared with control group.

also play distinct roles in microglia migration. Transwell assay was conducted to examine the ability of microglia migrating onto undersurfaces precoated with TN-R different domains as well as GST as control. Under same condition, the number of migrating microglia was significantly increased by both FN6–8 and FG domains and inhibited pronouncedly by the EGFL domain, and there was no changes by the FN1–2 and FN3–5 domains compared with GST (Fig. 2*A*, *a–g*, and *B*). Notably, the effect exerted by either the FN6–8 or the EGFL domain occurred in a dose-dependent manner (Fig. 2*C*). Consistent with the results from adhesion assay, these observations further indicate that distinct TN-R domains play different roles in the microglia migration.

Protein Kinase A Is Involved in the EGFL Domain Induced Microglia Anti-adhesion—To explore the signaling pathway involved in the EGFL related microglia anti-adhesion, various phosphatase inhibitors and kinase inhibitors were applied in the microglia adhesion assay. As shown in Fig. 3*A*, the number of microglia on the coated EGFL spots was increased significantly by either erbstatin, a tyrosine kinase inhibitor, or H7, a protein kinase A inhibitor, but not by calphostin C, a protein kinase C inhibitor, and okadaic acid, a phosphatase inhibitor (Fig. 3*A*, *a*), implicating that protein kinase A may be involved in the EGFL domain induced microglia anti-adhesion. This notion was further supported by the observations that the number of microglia on the EGFL coated spots was increased by H7 (Fig. 3*A*, *b*) and decreased by dbcAMP (Fig. 3*A*, *c*), a protein kinase A activator, in a dose-dependent manner.

Protein Kinase C Is Involved in the FN6–8 Domain Induced Microglia Adhesion—Next we investigated the signaling path-

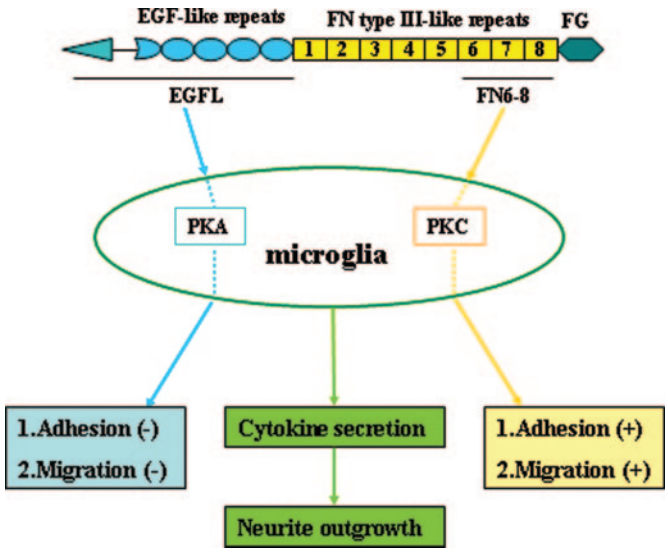

FIG. 6. **Schematic diagram of TN-R domains modulating the microglia function.** Shown is the EGFL domain inhibited microglia adhesion and migration via the protein kinase A signaling pathway. However, the FN6–8 domain promoted microglia adhesion and migration via the protein kinase C signaling pathway. Both EGFL and FN6–8 domains triggered microglia and displayed the same functions in inducing the cytokine secretion of microglia and promoting neurite outgrowth.

way involved in the FN6–8 induced microglia adhesion. As shown in Fig. 3*B*, *a*, the number of the microglia on the coated FN6–8 domain was significantly decreased by calphostin C but not by okadaic acid, erbstatin, and H7. Further, the FN6–8 domain induced microglia adhesion was either decreased by calphostin C (Fig. 3*B*, *b*) or enhanced by phorbol 12-myristate 13-acetate (Fig. 3*B*, *c*), a protein kinase C activator in a dose-dependent manner. These observations imply that protein kinase C may involve in the FN6–8 domain induced microglia adhesion.

Both EGFL and FN6–8 Domains Stimulated Microglia to Display Distinct Cytokine Expression Patterns—In light that different domains of TN-R have different functions on microglia adhesion and migration, especially EGFL and FN6–8 domain, next we examined whether EGFL and FN6–8 have a different effect on microglia in respect to cytokines secreted by microglia. Surprisingly, the cytokines secreted by microglia treated by EGFL and FN6–8 displayed a distinct expression pattern in which chemokine-induced cytokine 3 and tumor neurosis factor-α were significantly up-regulated after both EGFL and FN6–8 triggered microglia, but chemokine-induced cytokine-2 was obviously up-regulated in respect to FN6–8 stimulation compared with PBS as control (Fig. 4, *A* and *B*). These observations imply that TN-R may play one unique role in neuroprotection via microglia although its distinct domains can trigger different pathways in microglia.

Both EGFL and FN6–8 Domains Triggered Microglia to Express Distinct Growth Factors—To determinate which growth factors released from microglia were stimulated by TN-R, we performed dot blots to detect microglia conditioned medium using antibodies against basic fibroblast growth factor, nerve growth factor, brain-derived neurotrophic factor, glial cell line-derived neurotrophic factor, TGF-β, and neurotrophin-3. As shown in Fig. 4*D*, TGF-β was secreted by microglia stimulated by either EGFL or FN6–8 domain. Interestingly, nerve growth factor secretion was triggered by FN6–8 domain, and brain-derived neurotrophic factor secretion was induced by EGFL domain. The function of secretion of the nerve growth factor/TGF-β and brain-derived neurotrophic factor/TGF-β pairs may be to synergize in neuroprotection. Because the effects of the EGFL domains and FN6–8 domains on growth factor secretion was similar in contrast to their effects on adhesion/migration, the results suggest that TN-R plays a unique role in neuroprotection.

Both EGFL and FN6–8 Domain-activated Microglia Promote Neurite Outgrowth—To support the notion that TN-R-activated microglia play one unique role in neuroprotection, we further investigated whether microglia conditioned medium triggered by both EGFL and FN6–8 have a similar effect on neurite outgrowth. Microglia-conditioned media induced by PBS, GST, EGFL, and FN6–8 were added into the culture media of both NIE-115 cells and primary cortical neuron. Interestingly, both EGFL and FN6–8-treated microglia conditioned media significantly increased the total neurite length of either N1E-115 cells (Fig. 5, *A* and *B*, *a*) or primary cortical neurons (Fig. 5, *A* and *B*, *c*) compared with control (PBS). These two conditioned media also significantly increased the number of cells with neurites of the NIE-115 cells (Fig. 5, *A* and *B*, *b*) but not of primary cortical neurons (Fig. 5, *A* and *B*, *d*). These results further incriminate that TN-R may plays a role in neuroprotection via its distinct domains coordinately modulating the microglia function.

DISCUSSION

Proliferation of microglia cells is one of the hallmarks of central nervous system responses to neural injury. These responses are likely to play important roles in neuronal survival and functional recovery after the central nervous system injury. Here, we have shown that TN-R plays a role in neuroprotection via the coordination of its distinct domains in modulating the microglia (Fig. 6).

Role of TN-R Domains in Modulating Microglia Migration and Adhesion—Given that TN-R is a glia-derived molecule with multiple domains in the central nervous system, we have identified distinct TN-R domains that confer different effects on neuronal cell functions such as neuronal cell adhesion, neurite outgrowth, and modulation of sodium channels (13, 15). In addition, it had been found that TN-R also plays a role in neuroprotection via microglia (3). In the present study, we have shown that the EGFL domain inhibits cell adhesion in a protein kinase A-dependent manner. In contrast, the FN6–8 domain stimulates cell adhesion in a protein kinase C-dependent manner. In addition EGFL inhibits whereas FN6–8 stimulates microglia migration. These observations suggest that the distinct TN-R domains may coordinate effects on adhesion/migration via interacting with different receptors on the microglia cell surface.

Role of TN-R Domains in Neuroprotection—Microglia cells have been generally considered as aggressive cells, capable of inducing neuronal and oligodendroglial damage through their secretory products, such as nitrogen and oxygen radicals, inflammatory cytokines, glutamate, and other excitotoxins. However, activated microglia are bifunctional (beneficial or harmful) in response to neuropathological conditions (20, 21). It is unclear how the ECM molecules, such as TN-R, modulate microglia in the central nervous system environment. In the present study, we have found both EGFL and the FN6–8 domain activated microglia display a similar cytokine and growth factor secretion pattern. TN-R domains EGFL and FN6–8 induced microglia to release brain-derived neurotrophic factor/TGF-β and nerve growth factor/TGF-β, respectively. Furthermore, conditioned medium derived from EGFL and FN 6–8 activated microglia promotes neurite outgrowth in both N1E-115 neuroblastoma cells and primary rat cortical neurons. Altogether, these observations support the notion that TN-R may play a unique role in neuroprotection via modulating microglia (Fig. 6) in addition to its direct effects on neuronal

cells (13). These data may help to develop novel strategies to regulate the regenerative state and remodeling of the brain by targeting beneficial microglia responses.

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