In response to injury and inflammation of the CNS, brain cells including microglia and astrocytes secrete tumor necrosis factor-α (TNF). This pro-inflammatory cytokine has been implicated in both neuronal cell death and survival. We now provide evidence that TNF affects the formation of neurites. Neurons cultured on astrocytic glial cells exhibited reduced outgrowth and branching of neurites after addition of recombinant TNF or pretreatment of glial cells to secrete TNF. This effect was absent in neurons of TNF receptor-deficient mice cultured on pretreated glia of wild-type mice and was reverted by blocking TNF with soluble TNF receptor IgG fusion protein. TNF activated in neurons the small GTPase RhoA. By inactivating Rho with C3 transferase, the inhibitory effect of TNF on neurite outgrowth and branching was abolished. These results suggest that glia-derived TNF, as part of an injury or inflammatory process, can inhibit neurite elongation and branching during development and regeneration.

Key words: neurite; morphogenesis; Rho; GTPases; TNF; cytokine; TNF receptor; glia

During development and regeneration, vertebrate neurons undergo profound morphological changes that include substantial cytoskeletal restructuring. These changes are induced by contact with other cells and diffusible extracellular signals (Hu and Reichardt, 1999). Contact-dependent modulation involves membrane-bound molecules such as cadherins, integrins, and cell adhesion molecules of the immunoglobulin superfamily. Diffusible signal molecules include neurotransmitters, neurotrophic factors, and guidance molecules such as semaphorins (Chisholm and Tessier-Lavigne, 1999). In particular, neurotrophins have remarkable effects on neurite outgrowth and arborization (Bibel and Barde, 2000). Recently, a direct link between Rho GTPases and neurotrophin signaling was revealed in experiments demonstrating an interaction between RhoA and the p75 neurotrophin receptor (p75NTR). Members of the Rho family of small GTPases, including Rac, Cdc42, and Rho, have been shown to be crucial intracellular regulators of the cytoskeleton in neurons (Luo et al., 1997; Threadgill et al., 1997; Luo, 2000). In transfected cells, p75NTR was demonstrated to constitutively activate RhoA, whereas neurotrophin binding rapidly reduced the activation of the GTPase (Yamashita et al., 1999). The downregulation of RhoA mediated by p75NTR was proposed to be part of a mechanism accelerating neurotrophin-induced axonal elongation (Yamashita et al., 1999; Tucker et al., 2001). The tumor necrosis factor receptor (TNFR) and the p75NTR receptors are members of the same receptor family. Interestingly, previous experiments have implicated these receptors and their ligand TNF in modulating the cytoskeleton in non-neuronal cells. TNF induced stress fibers and focal adhesion formation mediated by the GTPase Rho in endothelial cells (Wojciak-Stothard et al., 1998), epithelial cells (Koukouritaki et al., 1999), and fibroblasts (Puls et al., 1999).

In the CNS, in contrast to neurotrophins that are mainly produced by neurons, TNF is preferentially produced by activated glial cells, microglia, and astrocytes during brain injury or inflammatory processes (Hopkins and Rothwell, 1995). For instance, TNF protein has been localized in glial cells after experimental autoimmune encephalomyelitis (Renno et al., 1995). TNF protein was also detected in neurons by immunohistochemistry after mechanical (Tchelingerian et al., 1993) or ischemic brain injury (Bruce et al., 1996). Furthermore, TNF gene transcription and protein production have been observed during development in total brain tissue extracts (Munoz-Fernandez and Fresno, 1998; Zhao and Schwartz, 1998). In culture, astrocytes secrete TNF at low level, but cytokine secretion is strongly increased after treatment with inflammatory stimuli such as IFN-γ combined with IL-1β (Chung and Benveniste, 1990). TNF is produced as a propeptide, which integrates into the cell membrane as a stable homotrimer or can be clipped off at the transmembrane domain by the metalloproteinase TNF-α-converting enzyme [TACE, ADAM-17 (Blobel, 1997)]. The biological activity of TNF is mediated via either the TNFR1 (p55 TNF receptor, CD120a) or the TNFRII (p75 TNF receptor, CD120b), which have overlapping signaling capabilities (Rothe et al., 1994; Hsu et al., 1996).

To elucidate the significance of TNF on neuronal morphology, we cocultured hippocampal neurons at low density with glial cells. After treatment with recombinant TNF or stimulation of glial...
cells to elicit TNF, we observed a marked reduction in length and branching of neurites. Furthermore, we found that RhoA becomes activated in cultures of enriched neurites by TNF and provide evidence that this GTPase is causally involved in the inhibitory effect of recombinant or secreted TNF.

MATERIALS AND METHODS

**Mice.** C57BL/6 mice were obtained from the animal house facilities of the Max-Planck Institutes of Neurobiology and Biochemistry ( Martinsried, Germany). C57BL/6 TNFRF-I plus TNFRF-deficient mice were obtained by crossing C57BL/6 TNFRF-deficient mice (backcrossed nine generations with C57BL/6; Klaus Pfeffer, Technical University, Munich). Homozygous mice deficient for both TNF receptors (TNFRF plus TNFRRII) were confirmed by established PCR protocols (K. Pfeffer, Technical University, Munich, Germany/The Jackson Laboratory, Bar Harbor, ME) and then maintained as homozygotes in our animal house facility.

**Marine hippocampal neurons.** Primary hippocampal neuronal cell cultures were prepared as described previously (Neumann et al., 1995). Briefly, hippocampi were isolated from whole brains of embryonic day 16 mice, and the meninges were removed. The trimmed tissue was dissociated by trituration through a fire-polished Pasteur pipette. Cells (5 × 10^5)/ml were plated in astrocyte-enriched medium at 0.5 mg/ml, with fibronectin (Boehringer, Mannheim, Germany) on 0.15 μm boric acid. Cells were cultured in chemically defined medium containing basal medium Eagle (BME; Invitrogen, Gaithersburg, MD) with B27 supplement [2% (v/v), Invitrogen] and glucose [1% (v/v); Sigma; 45%; Sigma]. Cells were treated with TNF (murine recombinant TNF, 10 ng/ml; Roche Products, Hertfordshire, UK), TNF receptor IgG fusion protein (recombinant human TNF receptor p55/IgG1 fusion protein, 10 ng/ml; Perkin-Elmer, Roche Molecular Systems) and 1× PCR buffer (Perkin-Elmer, Roche Molecular Systems) covered with two drops of mineral oil (Sigma). PCR amplification was performed on a programmed thermocycler (MultiCyler, MJ Research Inc.). After denaturing of the cDNA at 95°C for 5 min, denatured primers (100 pmol each) were added at 80°C. PCR was performed for 50 cycles (93°C for 1 min; 60°C for 1 min; 72°C for 1 min) and followed by one final interval at 72°C for 5 min. Ten microliters of the PCR reaction were loaded in parallel with the molecular weight marker (4X 174, HaeIII digested; Amersham Biosciences) on a 1.7% Agarose gel containing ethidium bromide. The membrane was hybridized with digoxigenin-labeled probes, washed with 2 SSC/0.1% SDS at 85°C, and exposed to XAR film.
performed as described previously (Borasio et al., 1989; Jin and Strittmatter, 1997). Briefly, primary hippocampal neurons were suspended in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, with C3 transferase at 0.1 mg/ml directly after isolation from the brain tissue, and passed 50 times through a 200 μm pipette tip. After trituration, neurons were plated on the glial cells as described above.

Flow cytometry for membrane-bound TNF. The astrocyte-enriched glial cells were collected from the culture by short trypsinization (trypsin-EDTA solution; Invitrogen). Cells were washed with PBS, and unspecific binding sites were blocked with 2% bovine serum albumin (Sigma). Then, cells were incubated with polyclonal rabbit anti-TNF antibody (1:100, Genzyme) followed by dichlorotriazinyl amino-fluorescent conjugated goat anti-rabbit antibody (10 μg/ml, Dianova). Polyclonal rabbit IgG (Dianova) followed by dichlorotriazinyl amino-fluorescent conjugated secondary antibody was used as a control. Cells were incubated with 0.5 μg/ml propidium iodide to distinguish living from dead cells and analyzed with a flow cytometer (FACScan, Becton Dickinson).

Measurement of released TNF. To measure TNF secreted by astrocyte-enriched glia, cells were kept in serum-free medium for 6 hr. The supernatant was collected and assayed via ELISA according to the instructions of the manufacturer (OptEIA mouse TNF mono/mono, PharMingen).

RESULTS

TNF receptor expression in primary hippocampal neurons

Primary neurons were cultured from hippocampal tissue derived from embryonic mice. TNF receptor gene transcripts were analyzed in primary neuronal cultures by single-cell RT-PCR of individual neurons. Neurons were first unequivocally identified by whole-cell patch-clamp electrophysiology, and then the cell cytoplasm was collected with the micropipette. Gene transcripts for TNFRI as well as TNFRII were detected in the majority of single neurons (Fig. 1) and confirmed by sequencing. Specifically, 21 of 25 analyzed neurons were positive for TNFRI and 19 of 25 were positive for TNFRII, whereas in 18 of 25 neurons both TNF receptor types were detected.

Protein expression of TNF receptors in primary hippocampal neurons was analyzed by immunohistochemistry. Neurons were immunolabeled with rat monoclonal antibodies directed against mouse TNFRI or TNFRII and subsequently double labeled with antibodies directed against the neuronal cytoskeleton protein β-tubulin III. Background labeling was determined with control rat immunoglobulin. Intracellular granular staining for TNFRI and TNFRII was obtained in the neuronal somata and processes of neurons (Fig. 2). In total, 81.8% (±7.9% SEM) of hippocampal neurons identified by β-tubulin III immunohistochemistry showed TNFRI labeling, and 77.6% (±5.7% SEM) showed TNFRII labeling. Immunolabeling for TNFRI as well as TNFRII gave only background staining in hippocampal neurons derived from TNFRI- plus TNFRII-deficient mice.

Reduced neurite outgrowth and branching of primary hippocampal neurons in response to TNF

Low-density cultures of primary hippocampal neurons were performed by culturing neurons together with glial cells. This low-density culture system allows detailed morphometric analysis of individual neurons. Astrocyte-enriched glial cells were obtained from primary hippocampal brain cells after removal of microglia. Hippocampal neurons derived from E16 embryonic C57BL/6 or TNF receptor-deficient C57BL/6 mice were cultured on these astrocyte-enriched glial cells in chemically defined medium at low density, resulting in individual neurons without neuron–neuron contacts. Within a few hours, the neurons showed outgrowth of neurites up to 100 μm on the glial cell layer.

To analyze the effects of TNF on neurons, recombinant mouse TNF was added a few hours after plating to the neurons on the glial cell layer. After 16 hr, cells were fixed and immunolabeled with β-tubulin III. Neurites were traced and analyzed with an image analysis system. Neurite morphology of TNF-treated cell cultures was completely different from untreated cultures (Fig. 3), whereas neuronal survival was not altered by the TNF treatment (Table 1). Neurons treated with TNF showed reduced branching of their processes, and most neurites were almost equal in length without clear distinction of an axon-like process. The total neurite length per neuron and the axonal length, defined as the length of the longest neurite per neuron, were determined. Treatment with TNF (10 ng/ml, 16 hr) decreased the total neurite length per cell from 453 μm (±18.9 μm SEM) to 230 μm (±27.6 μm SEM) as well as the axonal length from 148 μm (±24 μm SEM) to 70 μm (±15.6 μm SEM) (Fig. 4). No change in the number of primary processes originating from the neuronal somata was detectable after treatment with TNF. However, there was a reduction in the number of branch points per cell in response to TNF. Untreated neurons showed on average 5.5 (±0.46 SEM) branch points per cell, whereas neurons treated with TNF exhibited 1.8 (±0.31 SEM) branch points per cell (Fig. 4). TNF affected neurite length and morphology via neuronal TNF receptors. Hippocampal neurons derived from TNFRI- plus TNFRII-deficient mice were cultured on glial cells derived from C57BL/6 wild-type mice. In this case the neurons did not show alterations in total neurite length, axonal length, and number of branch points after treatment with TNF (Fig. 4). Furthermore, we cultured hippocampal neurons derived from C57BL/6 wild-type mice on glial cells derived from TNFRI- plus TNFRII-deficient mice. TNF again reduced neurite outgrowth and branching of neurons (Fig. 4), indicating that the effect of TNF on neurite morphology was directly mediated via the TNF receptors of the neurons.

TNF production by cytokine-stimulated glial cells

In contrast to normal glial cells, reactive glial cells, in particular activated microglia and astrocytes of the injured brain, have been shown to produce TNF. In culture, gene transcription of TNF in astrocytes can be induced by combined treatment with the pro-inflammatory cytokines IL-1β and IFN-γ. Therefore, we pretreated our astrocyte-enriched glial cells with IFN-γ (100 U/ml) and IL-1β (10 ng/ml) for 24 hr. After pretreatment, gene tran-
scripts for TNF were detected via RT-PCR (Fig. 5). In contrast, no TNF gene transcripts were amplified from untreated glial cell culture. Treatment with IL-1β or IFN-γ alone resulted in low but detectable gene transcription of TNF.

To confirm TNF protein synthesis and secretion by astrocyte-enriched glial cells, we analyzed membrane-bound TNF by flow cytometry. Untreated and cytokine-stimulated glial cells were incubated with antibodies directed against TNF. Membrane-bound TNF was detected in 29% (±5.4% SEM) of glial cells pretreated for 24 hr with IFN-γ plus IL-1β (Fig. 5). Membrane-bound TNF was already seen after treatment with IFN-γ alone (24 ± 9%).

The amount of TNF secreted within 6 hr by glial cells was determined by ELISA. Treatment with IFN-γ plus IL-1β stimulated glial cells to release TNF up to 1000 pg/ml within 6 hr (Fig. 5). Thus, the combined treatment with IFN-γ and IL-1β resulted in significant gene transcription, membrane localization, and secretion of TNF.

Inhibition of outgrowth and branching of neurites by TNF from reactive glia

Primary hippocampal neurons derived from E16 embryonic C57BL/6 or TNF receptor-deficient C57BL/6 mice were cultured on astrocyte-enriched glial cells derived from C57BL/6 wild-type mice now pretreated with IFN-γ and IL-1β to produce sufficient amounts of TNF. After 16 hr, these neurons showed decreased total neurite length, decreased axonal length, and decreased number of branch points (Fig. 6) compared with neurons cultured on unstimulated glial cells (Fig. 4). To analyze whether this reduced outgrowth and branching of neurons on stimulated glia were

### Table 1. Neuronal survival in relation to untreated cultures

<table>
<thead>
<tr>
<th></th>
<th>Untreated cultures</th>
<th>TNF-treated cultures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>1 ± 0</td>
<td>0.84 ± 0.15</td>
</tr>
<tr>
<td>TNFR −/− neurons</td>
<td>1 ± 0</td>
<td>1.07 ± 0.17</td>
</tr>
<tr>
<td>TNFR −/− glia</td>
<td>1 ± 0</td>
<td>1.11 ± 0.14</td>
</tr>
</tbody>
</table>
indeed induced by TNF, we neutralized the endogenously secreted TNF in the culture. The effects of both membrane-bound as well as soluble TNF can be blocked by a TNF receptor IgG fusion protein. Therefore, we treated the cultures with TNF receptor IgG fusion protein and control IgG. Reduced total neurite length, axonal length, and number of branch points were completely antagonized by blockage of TNF activity with TNF receptor IgG fusion protein (Fig. 6). Neurons of cultures treated with TNF receptor IgG fusion protein for 16 hr showed total neurite outgrowth per cell of 502 μm (±18.9 μm SEM) and axonal length of 180 μm (±28.7 μm SEM), whereas neurons of cultures treated with control IgG showed total neurite length per cell of 176 μm (±9.8 μm SEM) and axonal length of 55.6 μm (±4.7 μm SEM) (Fig. 6). No change in neuronal survival was detected after treatment with the TNF receptor IgG fusion protein (Table 2). Hippocampal neurons derived from TNFRI- plus TNFRII-deficient mice did not show substantial reduction in total neurite length, axonal length, and number of branch points after culture on TNF-producing astrocytes (Fig. 6).

In the next step we added the TNF receptor IgG fusion protein to hippocampal neurons cultured on unstimulated glial cells not secreting detectable amounts of TNF (Fig. 7). No change in neurite outgrowth and branching was detected after treatment of unstimulated cultures with the TNF receptor IgG fusion protein, indicating that unstimulated glia and the low-density neurons do not secrete sufficient amounts of TNF to modulate neurite outgrowth and branching.

RhoA activation in hippocampal neurons after TNF treatment
Hippocampal neurons were cultured on poly-L-ornithine-coated dishes without glial cells and were treated with recombinant murine TNF (10 ng/ml). The cells were lysed at different time points after treatment with TNF. The active form of RhoA was precipitated with Sepharose beads conjugated with a GST-tagged binding domain of Rhotekin that specifically binds to GTP–RhoA. The amount of precipitated active RhoA was determined by Western blotting. RhoA was activated by treatment with TNF within 1 hr. The activity of the GTPase gradually increased after treatment with TNF up to 16 hr (Fig. 8).

Reduced neurite outgrowth and branching in response to TNF is mediated by Rho
Next, we analyzed whether the GTPase Rho was involved in the effect of TNF on neurite length and branching of hippocampal neurons cultured on glia. Rho can be inhibited efficiently by
trituration of the cells in the presence of C3 transferase, a procedure allowing cell membrane passage of C3 transferase under special buffer conditions (Borasio et al., 1989; Jin and Strittmatter, 1997). The C3 transferase from C. botulinum specifically ADP ribosylates Rho and thus inactivates the protein. Inhibition of neuronal Rho activity by trituration of neurons together with C3 transferase inhibited the effect of TNF on total neurite length, axonal length, and number of branch points of neurons derived from wild-type, but not TNFR−/− mice. Data are presented as mean ± SEM of three independent experiments.

**Table 2. Neuronal survival in relation to control IgG-treated cultures with wild-type neurons**

<table>
<thead>
<tr>
<th>Glial cells not pretreated</th>
<th>Glial cells pretreated with IFN-γ and IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control IgG</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>TNFR-IgG</td>
<td>0.94 ± 0.09</td>
</tr>
<tr>
<td>Wild-type neurons</td>
<td>0.83 ± 0.11</td>
</tr>
<tr>
<td>TNFR−/− neurons</td>
<td>0.79 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>0.99 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>0.84 ± 0.05</td>
</tr>
</tbody>
</table>

Subsequently, the effects of C3 transferase were analyzed on neurons cultured on astrocyte-enriched glial cells and pretreated with IFN-γ and IL-1β (Fig. 10, Table 3). Again, inhibition of neuronal Rho activity by trituration of neurons together with C3 transferase prevented the effect of TNF-producing astrocytes on neurite length and morphology (Fig. 10).

**DISCUSSION**

This study demonstrates that TNF profoundly modulates neurite branching and extension in hippocampal neurons *in vitro* via a mechanism that requires the small GTPase Rho. Recently, it has become clear that the modulation of the activity of Rho GTPases is an important link between signal transduction by membrane proteins and the actin cytoskeleton (Hall, 1998). Activated Rho family GTPases stimulate specific kinases (p65 PKC or ROCK) to phosphorylate one of the two isoforms of LIM kinase (LIMk1,
NF stimulation. In contrast, TNF activated the GTPases in a highly ordered manner: first Rac, followed by Cdc42, and finally Rho (Puls et al., 1999).

TNF reduced the neurite outgrowth and branching of the hippocampal neurons. This effect was mediated by Rho and abolished after blocking Rho with C3 transferase. Furthermore, two lines of evidence suggest that TNF acts directly on neuronal TNF receptors to modulate neurite morphology and not indirectly via glial cells. First, neurons derived from TNFRI- plus TNFRII-deficient mice and cultured on normal astrocytes showed almost no change in neurite morphogenesis. Second, the inhibitory effect of TNF on neurite outgrowth and branching was still detected in cultures of hippocampal neurons on glia derived from TNFRI- plus TNFRII-deficient mice.

Glia stimulated to secrete TNF reduced neurite outgrowth and branching of cocultured neurons. Although blockade of TNF activity with the receptor fusion protein increased outgrowth and branching of neurons cultured on stimulated glial cells, we did not observe a significant change in neurite morphogenesis by this treatment in nonstimulated glial cells. Thus, nonreactive glial cells and neurons do not produce sufficient amounts of TNF to inhibit neurite outgrowth and branching of neurons. However, we cannot exclude the possibility that our neuronal density was too low to detect such possible effects of neurons.

Gene transcripts and protein expression of both TNF receptors (TNFRI and TNFRII) were detected in cultured hippocampal neurons by single-cell RT-PCR and immunohistochemistry, respectively. In situ, neurons of the hippocampus of adult rats showed no or very low gene transcription of TNFRII under basal conditions, whereas TNFRI was induced and TNFRII upregulated by treatment with inflammatory stimuli or after ischemia (Botchkina et al., 1997; Nadeau and Rivest, 1999). Furthermore, gene transcription and protein expression of TNF and TACE (ADAM-17) have been observed in the CNS during embryonic and different postnatal developmental stages (Munoz-Fernandez and Fresno, 1998; Dziegielew ska et al., 2000; Karkkainen et al., 2000). In particular, TNF was localized by immunohistochemistry in cells with neuronal morphology during early embryonic development of the neocortex (Dziegielew ska et al., 2000). However, it has not been shown that TNF detected in the CNS during development is biologically active and whether the level is comparable to inflammatory processes or injury of the CNS.

No gross anatomical CNS abnormalities of TNFRI- and TNFRII-deficient mice under normal conditions were observed (our unpublished observation). However, a recent report suggested that TNF might be involved in hippocampal synaptic plasticity. The induction of long-term potentiation in region CA1 after stimulation of Schaffer collateral axons was impaired in mice deficient for both TNF receptors (Albensi and Mattson, 2000).

TNF and its receptors show polymorphism in humans, and recent reports indicate that this polymorphism might be associated with a noninflammatory brain disorder. In particular, a TNF gene polymorphism [TNF2(A) allele] has been associated with schizophrenia (Wassink et al., 2000), and patients homozygous for a TNFRII polymorphism (allele 1) showed significantly enlarged ventricles and smaller frontal lobes (Boin et al., 2001).

In our experiments, ligation of TNF receptors by TNF stimu-
related RhoA activity, whereas no effect on survival was observed.
In theory, TNF could induce cell death of pyramidal neurons, as shown with cultured cerebellar neurons by antagonizing insulin-like growth factor I (Venters et al., 1999). In another study, blockade of TNF by soluble TNFR-I significantly reduced focal cerebral ischemic injury in hypertensive rats, indicating a protective potential of TNF in this model (Dawson et al., 1996). TNF can also be “neuroprotective” in pathological situations, because transgenic mice lacking both TNF receptors have increased tissue lesions in response to ischemia (Bruce et al., 1996). Likewise, pretreatment of cultured hippocampal neurons with TNF reduced death of neurons mediated by ischemia (Cheng et al., 1994).

Our finding that TNF affects neurite elongation suggests that TNF might contribute to inhibit outgrowth of CNS axons during inflammation or lesion of the CNS. This is finding of particular importance, because axons do not regenerate after injury of the adult mammalian CNS. Multiple signals converge to regulate neuronal survival and neurite growth and determine the success or failure of axonal regrowth (Goldberg and Barres, 2000). One barrier to regeneration has been shown to be growth inhibition by myelin components such as Nogo (Chen et al., 2000). The synthesis of inhibitory proteins at the glial scar has been revealed as another impediment to axonal growth (Rudge and Silver, 1990; McKeon et al., 1991). Intriguingly, ablation of reactive astrocytes promoted neurite outgrowth after injury in a transgenic model (Bush et al., 1999). In this context, it is interesting to note that TNF is produced by reactive glia after injury (Hopkins and Rothwell, 1995) and deposited in the extracellular matrix where it binds avidly to substrates such as fibronectin and laminin while maintaining full biological activity (Alon et al., 1994; Hershkoziv et al., 1994).

In conclusion, TNF secreted by reactive glia during injury or inflammation might act as an anti-regenerative factor via its ability to activate Rho proteins in neurons.

**REFERENCES**


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