Cellular/Molecular

Differential Regulation of AMPA Receptor and GABA Receptor Trafficking by Tumor Necrosis Factor-α

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The proinflammatory cytokine tumor necrosis factor-α (TNFα) causes a rapid exocytosis of AMPA receptors in hippocampal pyramidal cells and is constitutively required for the maintenance of normal surface expression of AMPA receptors. Here we demonstrate that TNFα acts on neuronal TNFR1 receptors to preferentially exocytose glutamate receptor 2-lacking AMPA receptors through a phosphatidylinositol 3 kinase-dependent process. This increases excitatory synaptic strength while changing the molecular stoichiometry of synaptic AMPA receptors. Conversely, TNFα causes an endocytosis of $GABA_A$ receptors, resulting in fewer surface $GABA_A$ receptors and a decrease in inhibitory synaptic strength. These results suggest that TNFα can regulate neuronal circuit homeostasis in a manner that may exacerbate excitotoxic damage resulting from neuronal insults.

Key words: **GABA; receptor; synaptic; glutamate; trafficking; cytokine**

Introduction

For many decades, the immune system and the CNS were thought to be independent of one another with minimal communication between them because the blood–brain barrier prevented immune cell infiltration into the parenchyma of the brain. It has become clear, however, that many immune molecules may be used constitutively by the CNS as signaling molecules involved in intercellular communication (Pan et al., 1997; Vitkovic et al., 2000; Perry et al., 2002; Golan et al., 2004). Furthermore, immune molecules such as cytokines are also thought to importantly contribute to the brain damage induced by a wide variety of neuronal insults as well as neurodegenerative diseases (New et al., 1998; Lock et al., 1999; Shohami et al., 1999; Nagatsu et al., 2000; Perry et al., 2001; Szelenyi, 2001), while also having some neuroprotective functions in certain circumstances (Morganti-Kossmann et al., 2002; Stoll et al., 2002). Thus, elucidating the detailed mechanisms by which immune molecules affect neuronal function has important implications for understanding both normal brain function and the pathophysiology of a large number of neurological disorders.

One example of immune molecule influence on neuronal function can be seen in the regulation of synaptic transmission by cytokines. It is well established that glutamate is the main excitatory transmitter in the mammalian CNS and signals primarily through AMPA-type ionotropic glutamate receptors (AMPARs). Trafficking of these receptors is thought to underlie, at least in part, both rapid forms of synaptic plasticity and slower homeostatic changes in transmission (Malinow and Malenka, 2002; Turrigiano and Nelson, 2004). The surface expression of AMPARs appears to be tightly regulated in neurons, despite the constant cycling of receptors and rapid increases or decreases in response to stimulation. Recently, the proinflammatory cytokine tumor necrosis factor-α (TNFα) was found to be a novel, glia-released factor that increases the surface expression of AMPARs (Beattie et al., 2002). TNFα appeared to be constitutively released in both primary cell culture and acute hippocampal slices because blockade of TNFα signaling decreased the surface levels of AMPARs. These findings, as well as previous reports (Tancredi et al., 1992; Grassi et al., 1994; Emch et al., 2000), suggest that TNFα can have significant effects on neural circuit function. Furthermore, the TNFα-induced increase in the surface expression of AMPARs may contribute significantly to its putative role in mediating the brain damage resulting from a variety of pathological insults (Gelbard et al., 1993; Chao and Hu, 1994; Le et al., 1997; Hermann et al., 2001).

Here we further investigate the synaptic effects of TNFα focusing on the following: (1) the receptor subtype and intracellular signaling cascade through which TNFα induces exocytosis of AMPARs, (2) TNFα-induced changes in the stoichiometry of AMPARs, and (3) its effects on $GABA_A$ receptor surface expression and inhibitory synaptic transmission. The net effect of TNFα in both dissociated neuronal cultures and hippocampal slices was to change the balance of excitatory and inhibitory synaptic transmission in a manner that should greatly exacerbate the excitotoxicity induced by neuronal insults that increase TNFα levels and/or signaling.

Materials and Methods

Immunostaining. Postnatal hippocampal cultures were prepared as described previously (Beattie et al., 2000). Banker-style cultures were pre-

Received Nov. 1, 2004; revised Jan. 10, 2005; accepted Feb. 17, 2005.

This work was supported by National Institutes of Health Grants MH63394 (R.C.M.) and MH065752 (D.S.). We thank E. Saura and D. Jhung for technical assistance, R. Huganir for kindly providing reagents, and W. Morishita, W. Ju, and X. Yu for helpful discussions.

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DOI:10.1523/JNEUROSCI.4486-04.2005

 0270-6474/05/253219-10$15.00/0

pared as described previously (Goslin et al., 1998). Surface AMPARs were visualized in 15- to 25-d-old cultures after treatment with vehicle or 60 nM TNFα for 15–20 min at 37°C. After treatment, cells were chilled on ice, washed with cold PBS, and fixed with 4% paraformaldehyde. The nonpermeabilized cells were then blocked with 3% bovine serum albumin and 2% normal goat serum in PBS and a rabbit antibody directed at the N terminus of glutamate receptor 1 (GluR1) (gift from R. Huganir, Johns Hopkins University School of Medicine, Baltimore, MD) was applied at a dilution of 1:1000 for 1 h. Cells were sometimes costained with N-terminal antibodies against GluR2 (1:200; Chemicon, Temecula, CA) or the $GABA_A$ β2/3 subunit (1:300, MAB341; Chemicon). An anti-rabbit secondary antibody (Alexa 568; Alexis Biochemicals, San Diego, CA) was applied at a dilution of 1:1000. Anti-mouse Alexa 488 secondary was added in double-labeling experiments. Endocytosed receptors were visualized by adding antibodies to live cells for 30 min and then treating cells for an additional 15 min with TNFα. Cells were then chilled, stripped of remaining surface antibodies with a 3 min treatment of 0.5 M NaCl and 0.2 M acetic acid solution, fixed in paraformaldehyde, permeabilized with 0.1% Triton-X, and labeled with secondary antibodies. Labeled cells were imaged using a 63× objective mounted on a Zeiss (Oberkochen, Germany) Axioskop. Images were obtained using a cooled CCD camera (Hamamatsu, Bridgewater, NJ) and were analyzed using MetaMorph software (Universal Imaging Corporation, West Chester, PA). For individual experiments, images for all conditions were analyzed using identical acquisition parameters, and untreated and treated cells from the same culture preparation were always compared with one another. The images were also collected blind to the experimental condition. The total thresholded area of fluorescently labeled, surface AMPARs was measured automatically by the MetaMorph software and divided by the total cell area, which was determined by setting a lower threshold level to measure background fluorescence produced by the fixed cells. For each experiment, the fluorescence of all cells was normalized by dividing by the average fluorescence of the untreated control cells. Each experimental manipulation was repeated a minimum of three times using different culture preparations. Fixation by paraformaldehyde did not permeabilize the cells, as judged by microtubule-associated protein 2 (MAP2) staining. However, because of variation in the quality of B-27 supplement and possibly other issues of culture health, not all culture preparations were responsive to TNFα. Therefore, all experiments included a positive control of TNFα treatment, and culture preparations that failed to respond to TNFα with a >40% increase in GluR1 surface staining (~20% of total) were excluded from analysis. *n* values in the text represent the number of microscope fields examined. Statistical significance was determined using Student's *t* test. Error bars in figures represent SEM.

Cell surface biotinylation. Determination of the amount of AMPAR subunit surface expression was accomplished using a procedure modified from previous studies (Tsao and von Zastrow, 2000). Briefly, hippocampal cultures were prepared exactly as described for immunofluorescence, with the exception of the final plating procedure; for biochemical analysis, neurons were plated on poly-D-lysine-coated six-well plates and cultured for 3–3.5 weeks before subunit surface expression analysis. TNFα at 60 nM or vehicle was given to neurons for 15 min at 37°C, and neurons were chilled on ice and washed twice with cold PBS. PBS at 1 ml containing 1 mg/ml disulfide N-hydroxysuccininmide ester biotin (catalog #B4531; Pierce, Rockford, IL) was then added to each well. Neurons were incubated at 4°C for 30 min with gentle rocking. The reaction was stopped by washing the neurons three times for 10 min each with chilled Tris-buffered saline or 0.1 M glycine in PBS. Radioimmunoprecipitation assay (RIPA) lysis buffer (1 ml) with a protease inhibitor mixture (catalog #539131; Calbiochem, La Jolla, CA) was used to harvest neurons from three individual wells by cell scraper. This amount of cell material was used for each experimental condition, was subsequently allowed to rotate at 4°C for 30 min, and then was spun out in a tabletop microcentrifuge for 10 min at 10,000 rpm. Lysate (50 μl) was removed to be run on a Bradford protein concentration assay to ensure that samples of the experimental conditions represented the same amount of total protein. The supernatant was immunoprecipitated with streptavidin beads (catalog #20349; Pierce) overnight at 4°C with rotation. Beads were washed with RIPA buffer, eluted in SDS sample buffer, run out using PAGE, and transferred to nitrocellulose; the resulting Western blot was probed for GluR1 or GluR2 with antibodies from Chemicon (GluR1, catalog #AB1504; GluR2, catalog #MAB397). Signal was detected by the use of ECL and BioMax film (Eastman Kodak, Rochester, NY). Band intensities were quantified by ImageQuant Analysis program (Molecular Dynamics, Sunnyvale, CA). *n* values for these experiments represents number of separate culture preparations tested.

Culture electrophysiology. Recordings of miniature EPSCs (mEPSCs) were made from 15- to 24-d-old cultures essentially as described previously (Beattie et al., 2000). Briefly, whole-cell patch-clamp recordings were made with an Axopatch 1D amplifier (Axon Instruments, Union City, CA). Pipette solutions contained the following (in mM): 122 Cs-gluconate, 8 NaCl, 10 glucose, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 0.3 Na_3-GTP, and 2 Mg-ATP, pH 7.2. Cultures were superfused with normal Ringer's solution (in mM: 115 NaCl, 5 KCl, 23 glucose, 26 sucrose, 4.2 HEPES, 2.5 $CaCl_2$, and 1.3 $MgCl_2$, pH 7.2) containing 200 nM tetrodotoxin, 50 μM picrotoxin, and 50 μM D-APV. Data were acquired at 2 kHz with Igor Pro software (WaveMetrics, Lake Oswego, OR) and analyzed with Mini Analysis software (Synaptosoft, Decatur, GA). All mEPSCs above a threshold value set for each cell (5–6 pA) were included in the data analysis, and each mEPSC was verified visually. TNFα (60 nM) was applied to cultures 15–25 min before recording and compared with untreated sister cultures. *N*-(4-hydroxyphenylpropanoyl) (HPP)-spermine (10 μM; Tocris Cookson, Ballwin, MO) was bath applied.

Slice electrophysiology. Transverse hippocampal slices (400 μm) were prepared from 2- to 4-week-old Sprague Dawley rats as described previously (Luscher et al., 1999). Slices were incubated in the external perfusing medium containing the following (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgSO_4$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, 11 glucose, and 0.05 picrotoxin (saturated with 95% O_2/5% CO_2). To examine the effects of TNFα, slices were incubated in external solution containing 600 nM TNFα for a minimum of 2–3 h before experiments commenced. Throughout each experimental day, recordings from control and TNFα-treated slices prepared from the same animal were interleaved. Slices were transferred to a submersion-type recording chamber mounted on an Zeiss Axioskop microscope equipped with infrared-differential interference contrast optics and were continually perfused with perfusing medium at room temperature (2 ml/min, 22–23°C). Whole-cell voltage-clamp or current-clamp recordings were made from CA1 pyramidal cells with an Axopatch 1D amplifier (Axon Instruments). The whole-cell recording solution contained the following (in mM): 120 Cs-gluconate, 10 HEPES, 10 EGTA, 15.5 CsCl, 8 NaCl, 2 MgATP, and 0.3 GTP, pH 7.3. In some experiments, Cs-gluconate was replaced with CsCl (for mIPSCs) or $KMeSO_4$ (for compound postsynaptic potentials). Postsynaptic potentials were evoked by stimulating Schaffer collaterals at a frequency of 0.1 or 0.05 Hz with a glass pipette filled with external solution. Miniature EPSCs and IPSCs were recorded from cells clamped at −70 mV in the presence of 1 μM TTX and 50 μM D-APV plus 2,3-dihydroxy-6-nitro-7-sulfonyl-benzo[*f*]quinoxaline (for mIPSCs) or picrotoxin (PTX) (for mEPSCs). Analysis of mPSCs was performed using Mini Analysis 5 software (Synaptosoft). Each mPSC included in the final data analyses was verified visually. Compound postsynaptic potentials were recorded in current clamp, in the presence of 2 μM of the $GABA_B$ antagonist CGP 55845 and omitting PTX. The amplitude of the EPSP and IPSP were compared from the averaged responses to three to four stimulation intensities per cell. Group results were compared using Student's *t* test, and mPSC cumulative distributions were compared using a Kolmogorov–Smirnov two sample test.

Drug treatments. All drugs were bath applied in culture media at 37°C in the incubator, unless otherwise noted. Cultures were treated for 15–25 min with TNFα (60 nM), interleukin-1β (IL-1β) (50 ng/ml), IL-6 (32 ng/ml), IL-10 (40 ng/ml), or activating antibodies for TNFR1 (2 μg/ml) or TNFR2 (2 μg/ml) and immediately either processed for immunocytochemistry or used for electrophysiology. Inhibitors of protein kinase A (PKA) (PKI, 1 μM), calcium/calmodulin-dependent protein kinase II (CaMKII) (KN-93, 20 μM), cyclooxygenase-2 (COX-2) (aspirin, 10 μM), p38 MAP kinase (SB 203580, 50 μM), p42–44 MAP kinase (PD 98059, 50 μM), and phosphatidylinositol 3 kinase (PI3K) (wortmannin, 100 nM;

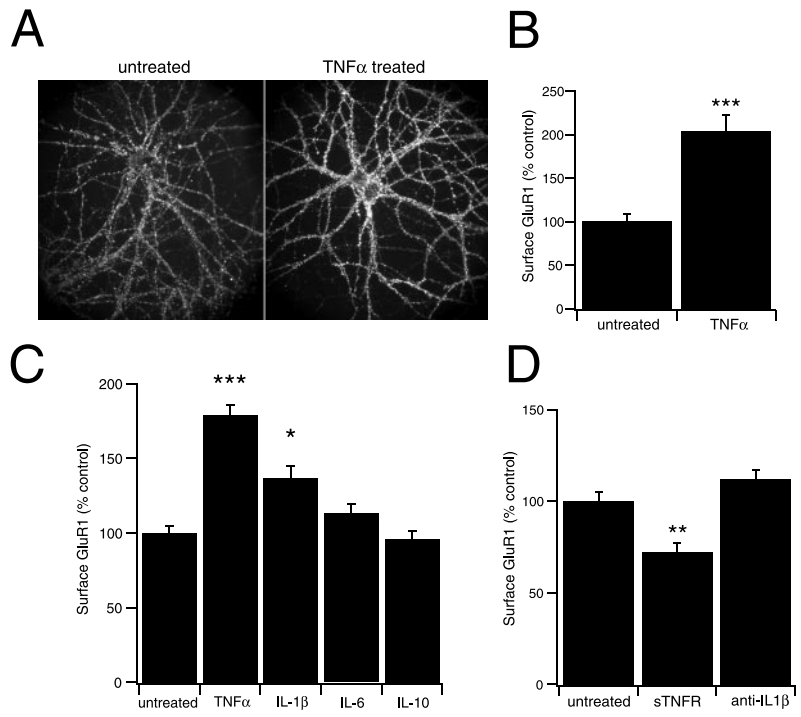

Figure 1. TNFα, unlike other cytokines, acts on neurons to increase surface expression of AMPARs. ***A***, Representative micrographs from sister cultures of nonpermeabilized isolated neurons immunostained for surface expression of the AMPA subunit GluR1. The cells on the right were treated with 60 nM TNFα for 15 min. ***B***, Group data from untreated or TNFα-treated cultures ($n = 70$ for both conditions), showing a substantial increase in surface GluR1 staining. ***C***, Composite data of surface GluR1 from cultures treated with TNFα (60 nM; $n = 214$), IL-1β (50 ng/ml; $n = 140$), IL-6 (32 ng/ml; $n = 159$), or IL-10 (40 ng/ml; $n = 130$). ***D***, Group data of surface GluR1 from cultures treated for 24 h with sTNFR (10 μg/ml; $n = 59$) or an antibody against IL-1β (40 μg/ml; $n = 72$). For this and all subsequent figures, $*p < 0.05$; $**p < 0.001$; $***p < 0.0001$ when compared with untreated cultures.

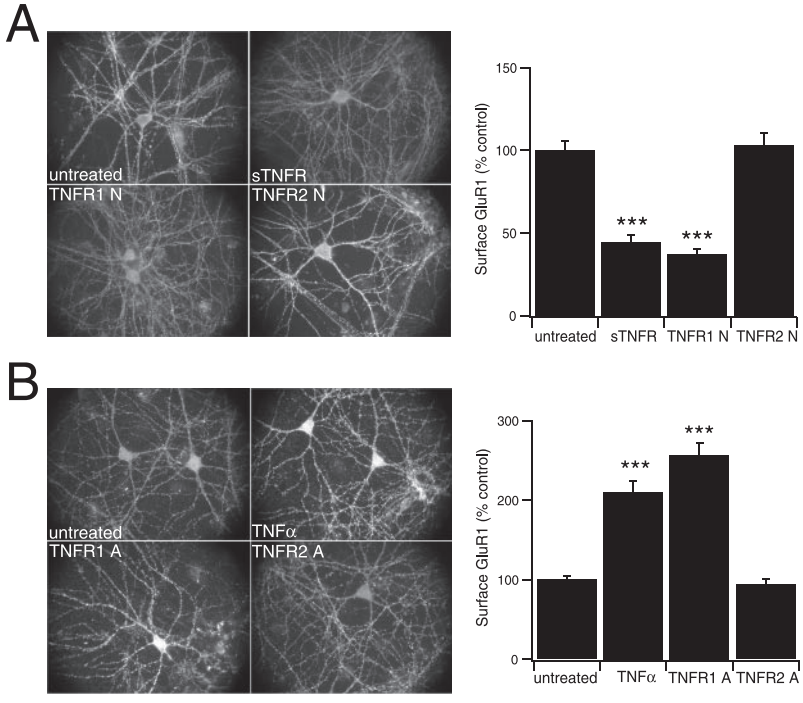

Figure 2. TNFα acts via TNFR1 to increase surface AMPARs. ***A***, Representative micrographs and composite data from cultures treated for 24 h with sTNFR (10 μg/ml; $n = 54$), which decreased the surface expression of GluR1 relative to untreated control cells. Treatment for 24 h with a neutralizing antibody for TNFR1 (TNFR1 N; 5 μg/ml; $n = 66$) also causes a similar decrease in GluR1 surface expression. Neutralizing antibodies for TNFR2 (TNFR2 N; 15 μg/ml; $n = 84$) were ineffective at decreasing GluR1 surface expression. ***B***, Sample micrographs and composite data demonstrating that an activating antibody for TNFR1 (TNFR1 A; 2 μg/ml; $n = 81$) increases the surface expression of GluR1 to a similar degree as sister cultures treated with TNFα ($n = 69$), whereas an activating antibody for TNFR2 (TNFR2 A; 2 μg/ml; $n = 88$) was ineffective at increasing surface GluR1.

and LY 294,002, 50 μM) were applied to cultures for 2 h before TNFα application. Receptor-neutralizing antibodies (TNFR1 N, 5 μg/ml; TNFR2 N, 15 μg/ml), soluble TNF receptors (sTNFRs) (10 μg/ml), or antibodies against IL-1β (40 μg/ml) were applied for 24 h. HPP-spermine (10 μM) was bath applied in culture external solution in the electrophysiology recording chamber. Cytokines and cytokine receptor materials were from R & D Systems (Minneapolis, MN), except the TNFR2 activating antibodies, which were from Abcam (Cambridge, MA). Inhibitors were purchased from Tocris Cookson.

Results

TNFα increases surface AMPARs via activation of neuronal TNFR1

Our previous experiments on TNFα (Beattie et al., 2002) used mixed neuronal–glial cell cultures, and therefore we could not determine whether TNFα was acting directly on the neurons to cause the exocytosis of AMPARs or was inducing glia to release another factor that then acted on the neurons (Bezzi et al., 2001). To address this issue, we removed "Banker cultures" (Goslin et al., 1998) from their glial feeder layers, treated the isolated neurons with TNFα (60 nM, 15 min), and then assayed the surface expression of AMPARs using an N-terminal antibody to GluR1 on nonpermeabilized cells (Fig. 1). TNFα induced a robust increase in the surface expression of AMPARs (204 ± 18% of untreated cultures; $n = 70$) (Fig. 1*B*), an increase indistinguishable from that seen in mixed neuronal–glial cultures (Beattie et al., 2002). This result indicates that the increase in surface expression of AMPARs is attributable to the direct action of TNFα on neurons and not through another cell type.

Several findings suggest that the increase in AMPAR surface expression is relatively specific to TNFα and is not a general effect of cytokines on neurons. A second proinflammatory cytokine, IL-1β (50 ng/ml), induced a smaller although significant increase in AMPAR surface expression (137 ± 8% of untreated cultures; $n = 140$; $p < 0.015$) (Fig. 1*C*). However, IL-1β does not appear to be constitutively released in our cultures, because treating cultures for 24 h with anti-IL-1β antibodies did not alter the surface expression of AMPARs (40 μg/ml; 112 ± 5%; $n = 72$) (Fig. 1*D*). In contrast, consistent with our previous results (Beattie et al., 2002), a recombinant soluble form of the TNFα receptor TNFR1 (sTNFR), which binds endogenous TNFα (Bezzi et al., 2001), decreased surface GluR1 staining (10 μg/ml; 72 ± 5%; $n = 59$) (Fig. 1*D*). A third proinflammatory cytokine, IL-6, induced,

on average, no significant increase in surface AMPAR levels (32 ng/ml; 111 ± 6% of untreated cultures; $n = 159$), whereas the anti-inflammatory cytokine IL-10 had no effect on surface AMPARs (40 ng/ml; 96 ± 5%; $n = 130$) (Fig. 1*C*). These data suggest that, among these cytokines, TNFα alone is used constitutively by the nervous system to regulate the surface expression of AMPARs.

Hippocampal neurons express both forms of TNFα receptor, TNFR1 and TNFR2 (Neumann et al., 2002; Yang et al., 2002). Although the receptors share several of the same downstream signaling cascades, they each also have some unique signaling characteristics, including the presence of a death domain on TNFR1 (Tartaglia et al., 1991; Rath and Aggarwal, 1999; Baud and Karin, 2001). To determine which subtype of TNFα receptor was responsible for the increase in AMPAR surface expression, we examined the effects of neutralizing antibodies for the individual receptors on endogenous TNFα signaling and again compared this with the effects of sequestering TNFα with sTNFR. As expected, incubating these sets of cultures for 24 h with sTNFR significantly reduced the surface expression of AMPARs (45 ± 4% of untreated cells; $n = 54$) (Fig. 2*A*), a finding that demonstrates that endogenous TNFα is present in these cultures and influences AMPAR surface expression. Antibodies that block signaling through TNFR1 caused a similar reduction of surface AMPARs after 24 h (37 ± 3% of untreated cells; $n = 66$), whereas the same treatment with antibodies that block signaling through TNFR2 did not decrease the surface levels of AMPARs (103 ± 7% of untreated cells; $n = 84$). Consistent with a primary role for TNFR1 in mediating the effects of TNFα on AMPARs, stimulation of TNFR1 alone using an activating antibody for 15 min (Diem et al., 2001) caused a clear increase in the surface expression of AMPARs (257 ± 15% of untreated cells; $n = 81$) (Fig. 2*B*), on par with the upregulation seen in sister cultures treated with TNFα (210 ± 15%; $n = 69$). TNFR2 activating antibodies, in contrast, were ineffective at increasing the surface expression of AMPARs (94 ± 7% of untreated cells; $n = 88$) (Fig. 2*B*). These results demonstrate that activation of TNFR1 is necessary for the maintenance of surface expression of AMPARs and is sufficient for their rapid exocytosis.

TNFR1 initiates signaling through an array of downstream pathways, most known for their roles in gene expression and apoptosis (Pan et al., 1997; Baud and Karin, 2001). Because we were studying rapid trafficking events, we concentrated on enzymes and protein kinases that have been reported to be activated by TNFα and also are thought to be involved with various forms of exocytosis. Incubation of cultures with inhibitors of CaMKII, cAMP-dependent PKA, p38 MAP kinase, p42–44 MAP kinase, and COX did not prevent the normal increase in surface expression of AMPARs induced by TNFα treatment (Fig. 3*A*), suggesting that these enzymes are not required for this action of TNFα. However, in contrast, two structurally distinct inhibitors of PI3K, wortmannin and LY 294,002, each completely prevented the TNFα-induced increase in surface levels of AMPARs (Fig. 3*B*,*C*). Specifically, TNFα application to cultures pretreated with wortmannin or LY 294,002 had surface AMPAR levels indistinguishable from pretreated cultures not given TNFα (wortmannin plus TNFα, 111 ± 6% of wortmannin alone, $n = 272$; LY 294,002 plus TNFα, 88 ± 10% of LY 294,002 alone, $n = 60$; $p > 0.27$). Pretreatment with wortmannin or LY 294,002 did not in itself significantly alter the surface expression of GluR1 (data not shown). Thus, similar to the actions of insulin and glycine on cultured neurons (Passafaro et al., 2001; Man et al., 2003), the increase in AMPAR surface expression attributable to TNFα appears to require PI3K activity.

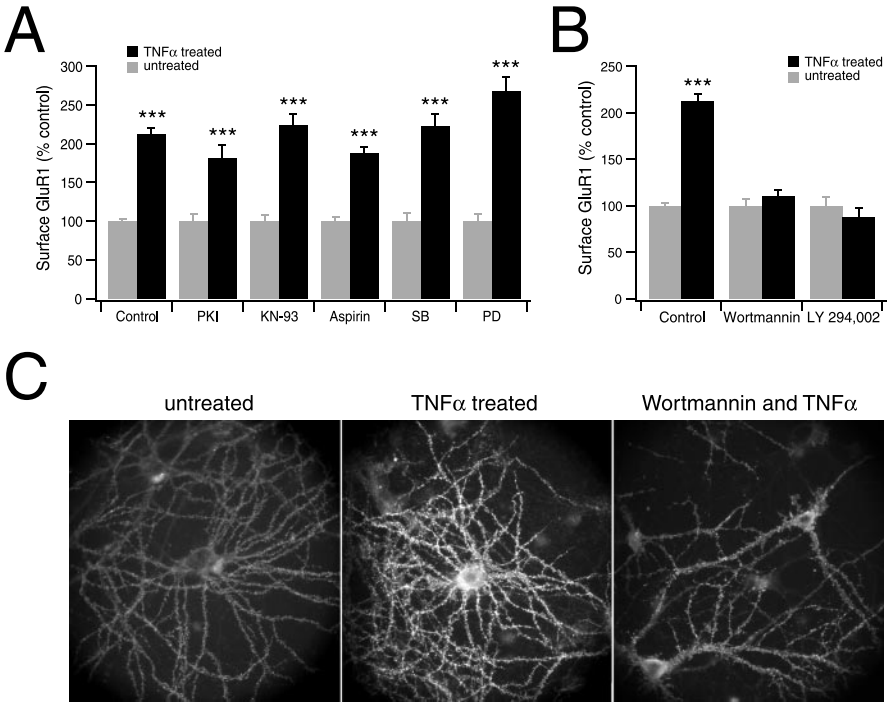

Figure 3. PI3 kinase is required for the increase in surface AMPARs induced by TNFα. ***A***, Pretreatment (2 h) with inhibitors for PKA (PKI, 1 μM), CaMKII (KN-93, 20 μM), COX-2 (aspirin, 10 μM), p38 MAP kinase [SB 203580 (SB), 50 μM], or p42–44 MAP kinase [PD 98059 (PD), 50 μM] all failed to prevent the TNFα-mediated increase in surface GluR1 (black bars; $n = 40–85$ per condition) relative to untreated sister cultures (gray bars). ***B***, Group data indicating that pretreatment with wortmannin (100 nM; $n = 172$) or LY 294,002 (50 μM; $n = 60$) prevented the TNFα-induced increase in surface expression of GluR1 (black bars) compared with untreated sister cultures (gray bars). ***C***, Sample micrographs showing the surface expression of GluR1 from sister cultures: untreated, treated with 60 nM TNFα, or pretreated with wortmannin and then treated with TNFα.

TNFα preferentially increases synaptic expression of GluR2-lacking AMPARs

Endogenous AMPARs in hippocampal pyramidal cells are thought to be heteromultimers, with GluR1 normally forming receptors in conjunction with GluR2 (Wenthold et al., 1996). The presence of edited GluR2 makes the AMPARs relatively impermeable to Ca^{2+} and insensitive to block by polyamines such as spermine (Washburn et al., 1997; Mainen et al., 1998; Dingledine et al., 1999). Our measurements of surface AMPARs thus far depend solely on tracking the GluR1 subunit of endogenous AMPARs. Because recent results suggest that increases in synaptic strength as a result of homeostatic synaptic scaling (Turrigiano and Nelson, 2004) involve increased synaptic expression of GluR2-lacking AMPARs (Thiagarajan et al., 2003; Ju et al., 2004), it was of interest to examine whether TNFα also increased the surface expression of GluR2. Surprisingly, although TNFα induced a robust twofold increase in GluR1 surface expression, in

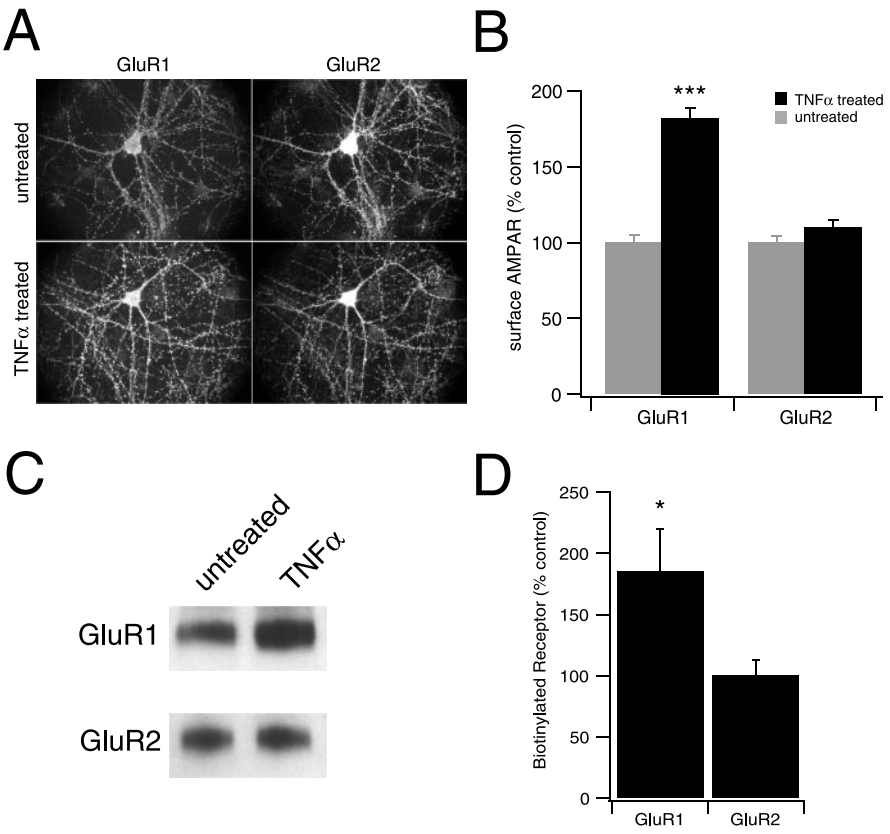

Figure 4. TNFα does not increase the surface expression of GluR2. ***A***, Representative micrographs from cultured neurons double labeled for surface GluR1 and GluR2, after treatment with TNFα. Despite a clear increase in GluR1 surface expression, GluR2 surface levels are not increased relative to untreated cultures. ***B***, Quantification of all doubled-labeled cells, showing a significant increase in GluR1 surface expression after TNFα treatment (black bars; $n = 177$) compared with untreated cells (gray bars; $n = 156$), whereas the same cells had no significant increase in GluR2 surface expression relative to untreated controls. ***C***, Representative blots from surface biotinylation experiments from sister cultures, probed for GluR1 or GluR2, after treatment with TNFα. ***D***, Quantification of all experiments for the levels of biotinylated receptors ($n = 7$ cultures for GluR1, 6 for GluR2).

the same cells, it caused no significant increase in GluR2 surface expression (110 ± 5%; $n = 177$) (Fig. 4*A*,*B*). It is possible that the antibodies to GluR1 might somehow sterically inhibit antibody staining of surface GluR2, and, therefore, we used an alternative technique, biotinylation of surface AMPARs (Ehlers, 2000; Kim and von Zastrow, 2003), to reexamine whether GluR2 surface expression was increased by TNFα. Consistent with the immunostaining results, TNFα caused a clear increase in surface biotinylated GluR1 (185 ± 35% of untreated cultures; $n = 9$) but no increase of surface GluR2 (100 ± 13%) (Fig. 4*C*,*D*).

These results suggest that many, if not most, of the new surface AMPARs lack the GluR2 subunit, resulting in receptors that are calcium permeable, inwardly rectifying, and inhibited by polyamines such as HPP-spermine (Washburn et al., 1997; Mainen et al., 1998; Dingledine et al., 1999). To directly test whether, after TNFα treatment, the properties of synaptic AMPARs had changed in a manner consistent with the previous results, we recorded mEPSCs and examined the effects of HPP-spermine. Consistent with previous results (Ju et al., 2004), cells from control cultures showed no sensitivity to HPP-spermine, with the mEPSC amplitude remaining unchanged after bath application of HPP-spermine (10 μM; 100 ± 3% of initial mEPSC amplitude; $n = 10$) (Fig. 5*A–C*). In contrast, HPP-spermine caused a reduction in mEPSC amplitude in cells treated for 20 min with TNFα (Fig. 5*A–C*). This was evident in both the measurements of mean mEPSC amplitude (82.7 ± 2.4% of initial mEPSC amplitude; $n = 12$) (Fig. 5*B*) and a leftward shift of the cumulative amplitude distribution (Fig. 5*C*). Furthermore, whereas the mean mEPSC amplitude (untreated, 14.9 ± 1.5 nA, 13 cells; TNFα, 20.2 ± 2.1 nA, 15 cells; $p < 0.05$) (Fig. 5*D*) and cumulative mEPSC amplitude distribution (Fig. 5*E*) before HPP-spermine application was significantly different between TNFα-treated and untreated cells ($p < 0.0012$), the post-HPP-spermine mean amplitude and cumulative distributions were not significantly different ($p > 0.5$). These results suggest that TNFα caused a significant increase in the proportion of GluR2-lacking AMPARs at synapses.

TNFα decreases $GABA_A$ receptor surface expression and inhibitory synaptic transmission

Our previous work demonstrated that TNFα does not cause a change in the synaptic localization of NMDA receptors (Beattie et al., 2002), suggesting that TNFα does not, nonspecifically, affect the trafficking of all glutamate receptors. To further examine the specificity of TNFα for synaptic receptors, we assayed its effects on the surface expression of $GABA_A$ receptors: the receptors that are primarily responsible for mediating fast inhibitory synaptic transmission. The vast majority of hippocampal $GABA_A$ receptors are believed to contain a β2 or β3 subunit, because the β subunit is thought to be a required subunit for receptor function, and β1 has negligible expression in the hippocampus (Persohn et al., 1992; Sperk et al., 1997). Using an antibody to the $GABA_A$ receptor β2/3 subunit (Richards et al., 1987), there was no increase in the level of surface expression of $GABA_A$ receptors after 15 min of TNFα treatment compared with untreated cultures, despite a nearly twofold increase in the surface AMPARs that were measured in the same cells (Fig. 6*A*). In fact, TNFα caused a small but significant reduction in $GABA_A$ receptor surface expression (88 ± 4%; $n = 237$; $p < 0.01$).

A reduction in surface levels of $GABA_A$ receptors is most likely attributable to an increase in endocytosis of the receptors. We tested this prediction by labeling surface $GABA_A$ receptors in live cells, treating the cells with TNFα, allowing endocytosis to occur, stripping the antibody off the remaining surface receptors, and then immunostaining the internalized receptors (for details, see Materials and Methods). Treatment with TNFα nearly doubled the amount of internalized $GABA_A$ receptors compared with untreated control cells (196 ± 17%; $n = 55$) (Fig. 6*B*) but had no effect on the amount of endocytosed AMPARs. The amount of constitutive endocytosis of $GABA_A$ receptors is much lower than for AMPARs (data not shown), so that a large proportional increase in the amount of endocytosed $GABA_A$ receptors would still only be equivalent to a modest proportion of the total number of surface receptors. Together, these data suggest that TNFα decreases the levels of surface $GABA_A$ receptors while simultaneously increasing surface AMPAR levels.

To test whether the effects of TNFα on AMPAR and $GABA_A$

receptor trafficking in cultured neurons occurred in more intact preparations and had the predicted effects on excitatory and inhibitory synaptic transmission, we recorded mEPSCs and mIPSCs from acute hippocampal slices pretreated with TNFα. Incubation of slices in TNFα caused an increase in the mean amplitude of mEPSCs (125 ± 7% of control; $n = 10, 12$) (Fig. 7*A*) and a rightward shift in the cumulative distribution of mEPSC amplitudes. In contrast, the mean amplitude of mIPSCs was decreased in TNFα-treated slices (86 ± 5% of control; $n = 7, 8$) (Fig. 7*B*) when compared with control slices and, there was a significant leftward shift in the cumulative amplitude distribution (Fig. 7*B*). (Changes in the frequency of mEPSCs and mIPSCs were in the expected directions but did not reach statistical significance.)

Modest increases in mEPSC amplitude combined with small decreases in mIPSC amplitude could combine to result in much larger changes in the ratio of excitatory to inhibitory synaptic transmission and thereby significantly affect network properties. To test this prediction, we evoked compound postsynaptic potentials consisting of an initial EPSP followed by a $GABA_A$ receptor-mediated IPSP (recordings were made in the presence of a $GABA_B$ receptor antagonist to isolate the $GABA_A$ component of the IPSP). The ratio of EPSP to IPSP amplitude from acute slices incubated in TNFα was increased more than twofold when compared with untreated slices from the same animal (EPSP/IPSP ratio, TNFα treated cells, 7.4 ± 1.4, $n = 11$; untreated cells, 3.1 ± 0.8, $n = 10$) (Fig. 7*C*). Together, these data indicate that treatment with TNFα can substantially influence the relative contribution of excitatory and inhibitory synaptic transmission and presumably have a significant effect on neural circuit behavior.

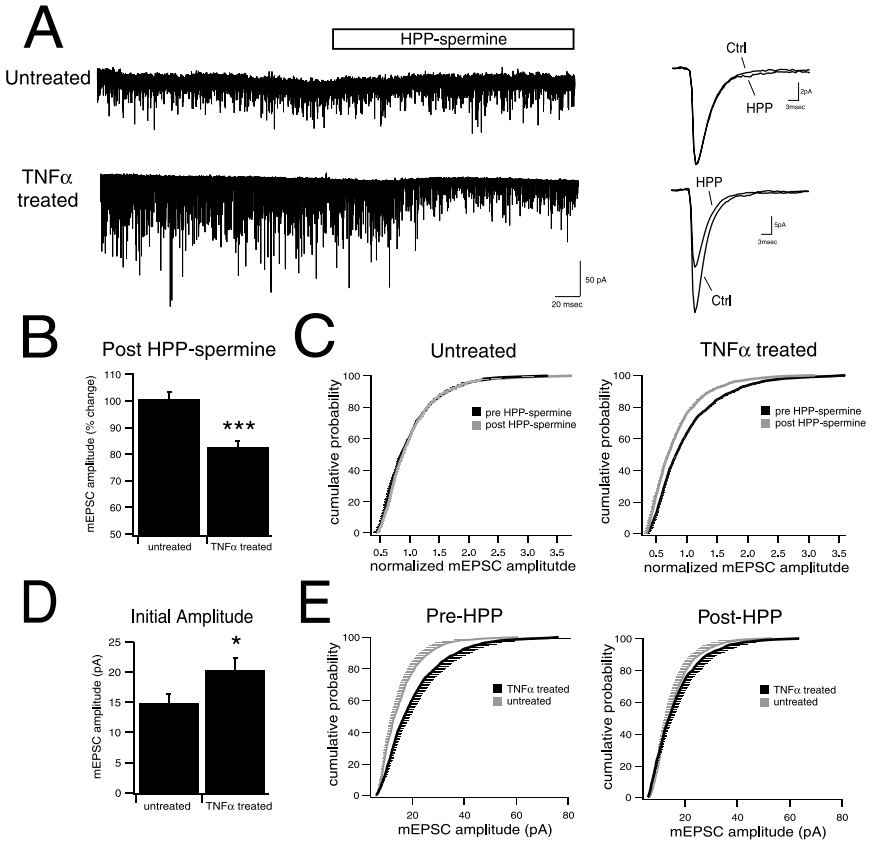

Figure 5. TNFα treatment induces surface expression of GluR2-lacking AMPARs at synapses. ***A***, Voltage-clamp recording of mEPSCs from untreated and TNFα-treated cultured hippocampal neurons, before and during application of HPP-spermine (10 μM). Traces at right show the average mEPSC before and after HPP-spermine application. Ctrl, Control. ***B***, Group data showing the normalized mEPSC amplitude after HPP-spermine for control ($n = 10$) and TNFα-treated cells ($n = 12$). ***C***, Cumulative probability graphs from control cells (left) and TNFα-treated cells (right) of normalized mEPSC amplitudes before (black) and after (gray) HPP-spermine application. ***D***, Group data of average mEPSC amplitude of untreated ($n = 13$) and TNFα-treated ($n = 15$) cells, showing a significant increase in average mEPSC amplitude. ***E***, The cumulative distribution of mEPSC amplitudes (left graph) demonstrates a significant rightward shift of TNFα-treated mEPSC amplitudes (black) compared with untreated cells (gray). The right graph shows the cumulative distribution of mEPSC amplitudes after the application of HPP-spermine, in which there is no significant difference between the untreated (gray) and TNFα-treated (black; $p > 0.5$) cells.

Discussion

TNFα is a proinflammatory cytokine that has been implicated in playing an important role in the neuronal damage caused by a variety of brain insults, such as stroke and head trauma, as well as that which occurs during neurodegenerative disorders (New et al., 1998; Lock et al., 1999; Shohami et al., 1999; Nagatsu et al., 2000; Perry et al., 2001; Szelenyi, 2001). In addition, there is evidence that it also plays an important role in normal neural circuit development and function (Pan et al., 1997; Vitkovic et al., 2000; Beattie et al., 2002; Neumann et al., 2002; Perry et al., 2002; Golan et al., 2004). Here we focused on the synaptic effects of TNFα, specifically on its effects on AMPAR and $GABA_A$ receptor trafficking and the consequences for excitatory and inhibitory synaptic transmission. We show that TNFα, acting on neuronal TNFR1 receptors, increases surface AMPARs through a PI3 kinase-dependent pathway. Many of the newly exocytosed AMPARs lacked or had lower stoichiometric amounts of GluR2, as evidenced by their sensitivity to HPP-spermine (Washburn et al., 1997; Dingledine et al., 1999). This change in the stoichiometry of synaptic AMPARs presumably will make at least some proportion of the receptors Ca^{2+} permeable. Surprisingly, we found that TNFα also simultaneously decreased surface $GABA_A$ receptors, resulting in a decrease in inhibitory synaptic transmission. Thus, the net effect of TNFα was to alter the balance of excitation and inhibition in a manner, to our knowledge, unlike previously studied neuromodulators.

We note that we observed amplitude changes in mEPSCs in both culture and slices after TNFα treatment, whereas in our previous paper (Beattie et al., 2002), we primarily observed a change in mEPSC frequency. This apparent discrepancy probably arises from the different recording techniques used in the two papers. The previous technique (recording from single cells before and after direct application of TNFα) would allow detection of frequency changes much more readily than the technique used in this work (cross cell comparisons of TNFα-treated and untreated coverslips) because of the very high variability of mEPSC frequency across cells. We also frequently observed a small rundown of mEPSC amplitudes over time during recordings from control cells, and this did not occur in cells treated with TNFα (Beattie et al., 2002, their supplementary material). This may have obscured detecting increases in mEPSC amplitude in the

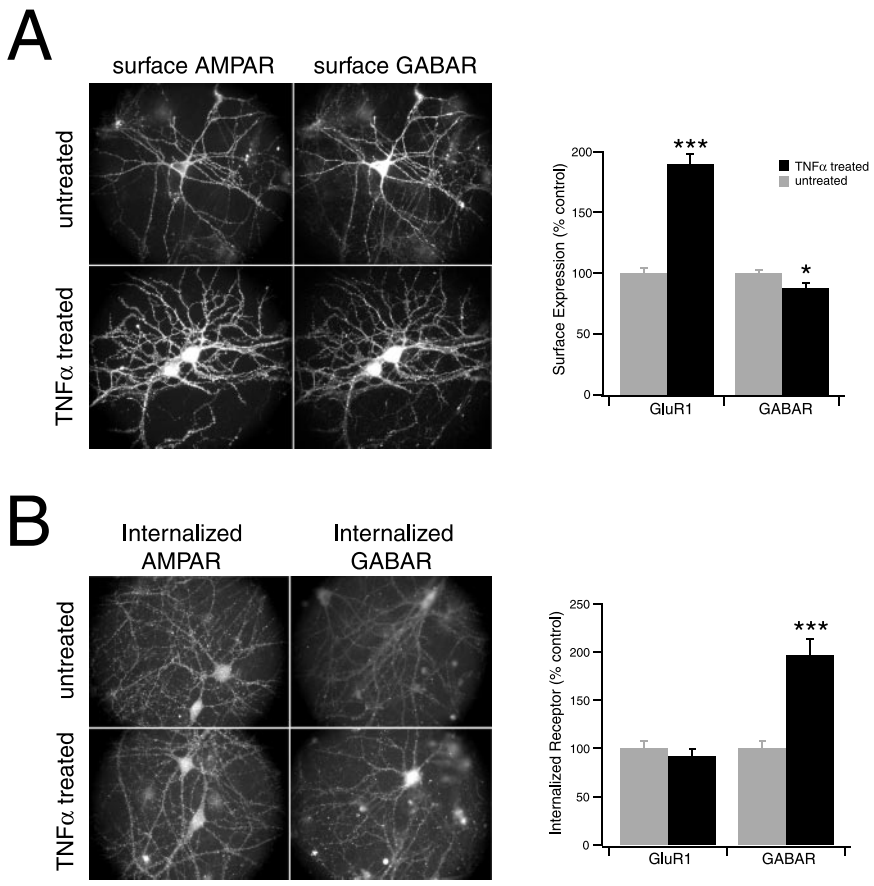

Figure 6. TNFα decreases the surface expression of $GABA_A$ receptors. ***A***, Representative micrographs and group data from cells double labeled for surface GluR1 and the β2/3 subunit of the $GABA_A$ receptors (GABAR). In cells showing a robust increase in GluR1 after TNFα treatment, there was a small but significant decrease in surface expression of GABAR compared with cells from untreated sister cultures. ***B***, Sample images and composite data from cells labeled for endocytosed GluR1 or GABAR. TNFα treatment (black bars) increased the endocytosis of GABAR but not GluR1 relative to cells from untreated cultures (gray bars).

previous experiments. In addition, the current method looked at a slightly later time point (20–40 min after TNFα) than previously (10–15 min), and mEPSC amplitude changes may occur at a slower rate. Finally, we used bath application of TNFα (identical to what was used for immunocytochemistry), which may simply be a more effective method of application, resulting in greater changes in amplitude.

We addressed the question of whether the effects of TNFα on receptor trafficking and synaptic transmission represent a general feature of most or all cytokines by examining the effects of a number of different proinflammatory and non-inflammatory cytokines on AMPAR surface expression. Our results suggest that TNFα has a unique role in the neuronal regulation of neurotransmitter receptors. IL-1β was less efficacious than TNFα at increasing surface AMPARs, and we found no evidence for the constitutive regulation of AMPARs in culture by IL-1β, unlike TNFα. Furthermore, other proinflammatory (IL-6) and anti-inflammatory (IL-10) cytokines were ineffective at altering the surface expression of AMPARs. These results suggest that TNFα is a member of the subset of cytokines that are used endogenously by the nervous system for the regulation of neurotransmission.

TNFα can activate two distinct receptors, TNFR1 and TNFR2, which couple to distinct but overlapping intracellular signaling pathways (for review, see Rath and Aggarwal, 1999; Baud and Karin, 2001; MacEwan, 2002). Our results suggest that activation of TNFR1 is both necessary and sufficient for the TNFα-induced increase in surface expression of AMPARs. However, soluble TNFα, which was used for many of our experiments, binds TNFR1 with much higher affinity than TNFR2 (MacEwan, 2002). It is therefore possible that TNFR2 could be capable of initiating the same or a complementary signaling cascade, resulting in an increase in the surface expression of AMPARs. We could not formally rule out this possibility because we do not know whether the TNFR2 agonist used in these experiments achieved maximal activation of TNFR2. However, using a TNFR2 neutralizing antibody, we determined that TNFR2 activation is not necessary for the maintenance of surface AMPARs. Considered together with our other results, this strongly suggests that endogenous TNFα acts solely on TNFR1 to regulate AMPAR surface expression.

Experiments using inhibitors of a variety of protein kinases, as well as COX, suggest that, downstream of the TNFR1 receptor, PI3K activity is required for the increase in AMPAR surface expression. This is perhaps not surprising given that the similar increases in AMPAR surface expression elicited by insulin and glycine treatments also require PI3K activity (Passafaro et al., 2001; Man et al., 2003). However, glycine treatment caused an increase in surface expression of both GluR1 and GluR2 (Lu et al., 2001; Man et al., 2003), although we observed a large increase in surface GluR1 with TNFα but no significant change in surface GluR2. Importantly, electrophysiological experiments indicated that this change in the subunit composition of AMPARs also occurred at synapses because HPP-spermine decreased mEPSC amplitude soon after TNFα treatment. These new synaptic AMPARs are likely replaced over time by GluR2-containing receptors, because HPP-spermine had no effect on mEPSCs recorded from control neurons despite the finding that constitutive TNFα release is necessary to maintain normal levels of surface AMPARs. However, even the short-term appearance of a significant population of GluR2-lacking, Ca^{2+}-permeable AMPARs could have important functional implications for synaptic plasticity as well as excitotoxicity (see below). Furthermore, these results suggest that a reserve pool of non-GluR2-containing AMPARs exists near, but not on, the membrane and can be readily trafficked to the surface by TNFα signaling. The rapid, constitutive cycling of AMPARs may then replace GluR2-lacking receptors with GluR2-containing ones, returning the GluR2-lacking receptors to the reserve pool. TNFα may also increase the size of the pool of non-GluR2-containing AMPARs, because 24 h treatment with TNFα has been reported to increase the expression of GluR1, but not other AMPAR subunits, in a neuronal cell line (Yu et al., 2002).

A surprising finding was that TNFα causes the endocytosis of $GABA_A$ receptors and a decrease in inhibitory synaptic strength. The increase in excitatory synaptic transmission combined with the decrease in inhibitory transmission is, to our knowledge, a

unique feature of TNFα action unlike that caused by other more extensively studied neuromodulators. This change in circuit behavior is, however, similar to the homeostatic mechanisms that appear to come into play when prolonged changes in activity level occur (Turrigiano and Nelson, 2004). Specifically, when circuit activity is pharmacologically reduced, excitatory synapses are strengthened and inhibitory synapses weakened (Turrigiano and Nelson, 2004), at least in part through changes in the synaptic surface expression of AMPARs and $GABA_A$ receptors (Kilman et al., 2002; Turrigiano and Nelson, 2004). The mediators of this process are unknown. Our data suggest one possible mechanism that may contribute to this homeostatic plasticity. Glia, sensing circuit activity levels possibly through glutamate spillover or extracellular ionic concentrations, might release TNFα in an inverse relationship with activity levels. As activity falls, TNFα release would be increased, strengthening excitatory synapses but weakening inhibitory ones. In the context of this hypothesis, it is interesting to note that the new AMPARs that appear during homeostatic "synaptic scaling" induced by activity blockade also lack GluR2 (Thiagarajan et al., 2003; Ju et al., 2004).

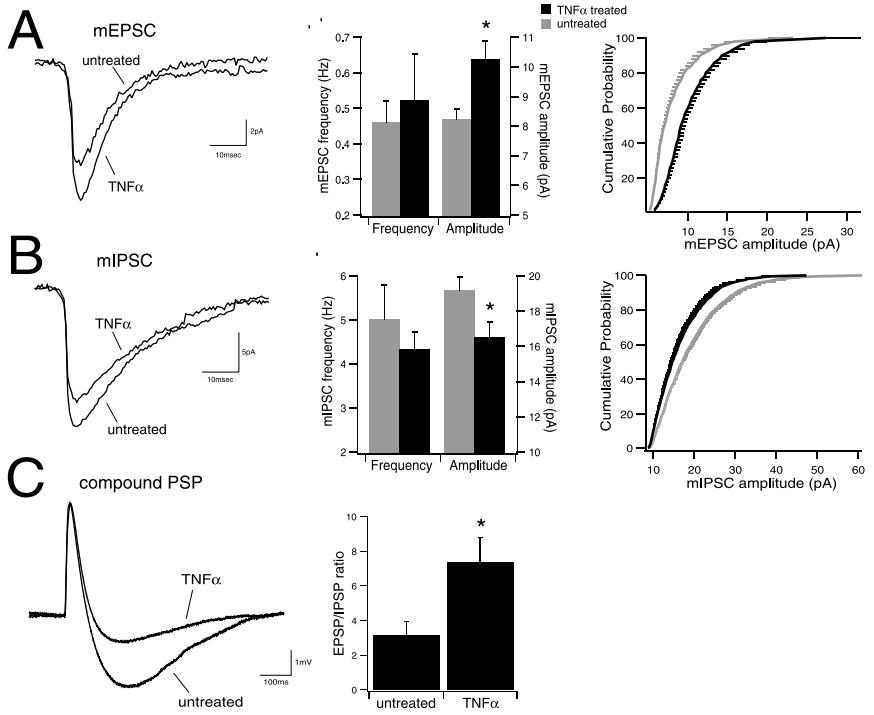

Figure 7. TNFα increases the ratio of excitatory to inhibitory synaptic transmission in acute hippocampal slices. ***A***, TNFα increases mEPSC amplitude. Left, Representative average mEPSCs from cells treated with TNFα and untreated slices. Middle, Composite data of average mEPSC frequency and amplitude of cells from untreated (gray; $n = 10$) and TNFα-treated (black; $n = 12$) slices. Right, Cumulative probability of mEPSC amplitude from the same cells, showing a significant rightward shift in TNFα-treated cells. ***B***, Representative mIPSCs (left), composite average mIPSC frequency and amplitude (middle), and cumulative probability of mIPSC amplitudes (right) of cells from TNFα-treated (black; $n = 12$) and untreated (gray; $n = 13$) hippocampal slices. ***C***, Representative traces of compound postsynaptic potentials of cells from TNFα-treated slices and untreated slices from the same animal. TNFα-treated cells ($n = 11$) had a significantly higher ratio of the EPSP to IPSP amplitude than untreated cells ($n = 10$).

Independent of whether or not the actions of TNFα reported here play a role in adaptive neural circuit function, the effects of TNFα on AMPAR and $GABA_A$ receptor trafficking have important implications for the role of TNFα in neuropathology. The release of TNFα is upregulated by a variety of neuronal insults and disease states (New et al., 1998; Lock et al., 1999; Nagatsu et al., 2000; Perry et al., 2001), and preventing its signaling *in vivo* can, for example, prevent or reduce neuron loss after cerebral ischemia (Dawson et al., 1996; Barone et al., 1997; Meistrell et al., 1997) and head injury (for review, see Barone et al., 1997; Shohami et al., 1999). The pathological contributions of TNFα to neural injury are likely, at least in part, attributable to its ability to potentiate glutamate excitotoxicity (Gelbard et al., 1993; Chao and Hu, 1994; New et al., 1998; Epstein and Gelbard, 1999; Hermann et al., 2001), which importantly contributes to much of the secondary damage after brain insults (Choi, 1994; Wrathall et al., 1994). The findings presented here, specifically an increase in surface AMPARs, a change in their stoichiometry to make them Ca^{2+} permeable, and a decrease in inhibition, all will enhance the excitotoxicity that accompanies neuronal insults and some autoimmune and neurodegenerative diseases that may involve TNFα (Epstein and Gelbard, 1999; Weiss and Sensi, 2000). Indeed, a number of neuronal insults, including hypoxia, ischemia, epileptiform activity, and spinal cord contusions, show an increase in non-GluR2-containing AMPARs (Ying et al., 1997; Grossman et al., 1999; Grooms et al., 2000; Sanchez et al., 2001), and increasing the proportion of calcium-permeable AMPARs greatly increases neuronal vulnerability (Feldmeyer et al., 1999; Oguro et al., 1999). Together, these data suggest that dysregulation of the trafficking of a reserve pool of non-GluR2-containing AMPARs could underlie aspects of several neurological disorders. Similarly, the effects of TNFα could contribute to the modification of circuit behavior that underlies the hyperalgesia after peripheral nerve damage, a neuropathological state that involves glia-released cytokines, including TNFα (Watkins et al., 2001; Milligan et al., 2003). Additional work will be necessary to determine whether pharmacological manipulations can be developed that will interfere with the pathological consequences of increased TNFα signaling while not significantly inhibiting its potential role in adaptive neural circuit reorganization.

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