Inflammatory events have long been implicated in initiating and/or propagating the pathophysiology associated with a number of neurological diseases. In addition, defects in Ca\(^{2+}\)-handling processes, which shape membrane potential, influence gene transcription, and affect neuronal spiking patterns, have also been implicated in disease progression and cognitive decline. The mechanisms underlying the purported interplay that exists between neuroinflammation and Ca\(^{2+}\) homeostasis have yet to be defined. Herein, we describe a novel neuron-intrinsic pathway in which the expression of the type-1 inositol 1,4,5-trisphosphate receptor is regulated by the potent pro-inflammatory cytokine tumor necrosis factor-\(\alpha\). Exposure of primary murine neurons to tumor necrosis factor-\(\alpha\) resulted in significant enhancement of Ca\(^{2+}\) signals downstream of muscarinic and purinergic stimulation. An increase in type-1 inositol 1,4,5-trisphosphate receptor mRNA and protein steady-state levels following cytokine exposure positively correlated with this alteration in Ca\(^{2+}\) homeostasis. Modulation of Ca\(^{2+}\) responses arising from this receptor subtype and its downstream effectors may exact significant consequences on neuronal function and could underlie the compromise in neuronal activity observed in the setting of chronic neuroinflammation, such as that associated with Parkinson disease and Alzheimer disease.

Although the brain was once believed to be an immunoprivileged site, it is presently evident that immune-related activities, including inflammation, underlie normative and pathological brain function. The physiological events that govern the transition of inflammatory reactions associated with self-limited events to chronic activation of inflammatory cascades found in the setting of neurodegeneration are of considerable interest. Various neurological disorders, including Parkinson disease, Alzheimer disease, and traumatic brain injury, display inflammation-related hallmarks, including cytokine and chemokine expression, the activation of central nervous system-residing macrophage-related cells (microglia), and in some cases, immune cell infiltration from peripheral sites via the brain microvasculature (reviewed in Ref. 1). The roles these processes play in disease pathogenesis are, however, less clearly defined.

Tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) is a prototypical pro-inflammatory cytokine, has been studied primarily in the context of immune cell/tumor cell biology and induction of innate immunity and apoptosis (2–4). In the central nervous system, TNF-\(\alpha\) is produced by brain-resident astrocytes, microglia, and neurons in response to numerous intrinsic and extrinsic stimuli (5). Radioactive binding assays have also indicated the presence of TNF receptors in several regions in the brain, including the cortex (6). However, its role within the central nervous system has been controversial. TNF-\(\alpha\) has been shown in some studies to lead to positive outcomes, as in the case of remyelination (7), and its ability to prevent neurons from apoptosis when exposed to amyloid-\(\beta\), a possible causative agent of Alzheimer disease (8). Alternatively, TNF-\(\alpha\) induces neuronal apoptosis though an excitotoxic mechanism mediated through alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (9) and activates microglia, which may lead to the propagation of various neurological diseases (5).

Few studies have detailed the effects of TNF-\(\alpha\) on ionic signaling cascades within neuronal cells, and most of these have focused on the modulation of plasma membrane ion channels. For example, it was shown that TNF-\(\alpha\) can cause the insertion of Ca\(^{2+}\)-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors in the plasma membrane of hippocampal pyramidal neurons (10) and that TNF-\(\alpha\) can increase N-methyl-\(d\)-aspartic acid receptor activity in mouse cortical neurons (11). These events both result in an increase in neuronal stress and possibly death. Our present study examined the effects of TNF-\(\alpha\) on intracellular ion channels, specifically the inositol 1,4,5-trisphosphate (IP\(_3\), R) and ryanodin (RyR) receptors. We posited that changes in channel expression and/or activity contribute to alterations in neuronal...
Enhanced IP₃R Levels by TNF-α

functioning observed during neuroinflammation. Modulation of responses arising from these receptors may have significant consequences for neuronal function and survival, ranging from membrane hyperpolarization (12) to the alteration of global protein expression (13). To this end, we report a novel neuro-intrinsic pathway through which TNF-α regulates type-1 IP₃R expression at least partially through c-Jun N-terminal protein kinase (JNK) activation. Perturbation of Ca²⁺ signaling through activation of this IP₃R subtype and its downstream effectors may significantly impact neuronal function and could ultimately compromise neuronal activity in the setting of chronic neuroinflammation.

EXPERIMENTAL PROCEDURES

Primary Cell Cultures—Baby hamster kidney (BHK) cells were maintained as previously described (14). Neuronal cultures were obtained from E₁₄.₅ cortical homogenates by micro-dissection and trypsin digestion. Neurons were then plated on polyethyleneimine-coated coverslips at a density of 3 × 10⁵ cells/ml in B-27-supplemented Neurobasal Media (with 0.5 mM glutamine plus glutamic acid). One-half of the supplemented neurobasal media was changed every 4 days. Transwell co-cultures were established by culturing glia obtained from P₀ C57BL/6 mice on plastic inserts (Corning). P₀ cortices were microdissected, mechanically homogenized, and grown in minimal essential medium with 10% fetal bovine serum for 12 days post-dissection (Invitrogen). Microglial cultures were obtained by gentle agitation of the T-75 flask, removal of the media, centrifugation, and plating at a density of 5 × 10⁴ cells/insert in 5% fetal bovine serum. After 1 week of growth, supplemented minimal essential medium was removed from the inserts, and co-cultures were established for 4 days before murine recombinant TNF-α addition (11 neuronal days in vitro).

Ca²⁺ Imaging—Days 12, 13, and 14 in vitro (24, 48, and 72 h, respectively) neuronal cultures were removed from their culturing media and loaded with Fura2-AM (Tefabs, 1 μM) in HEPES-buffered physiological saline solution (in mM): 5.5 glucose, 0.56 MgCl₂, 4.7 KCl, 1 Na₂HPO₄·7H₂O, 10 HEPES, 1.2 CaCl₂, pH 7.4. Ryanodine (100 μM) or Ru360 (10 μM) was loaded in conjunction with Fura2-AM for the RyR and mitochondrial blocking experiments, respectively. Coverslips were mounted in an imaging chamber and transferred to an inverted Nikon microscope with a 40× oil immersion objective lens (numerical aperture, 1.3). During experiments, HEPES-buffered solution, or an La³⁺–Ca²⁺ reduced solution (300 μM La³⁺ with 0.5 mM Ca²⁺), and agonists were perfused using a gravity-fed system (Warner Instruments), and excitation at 340 nm/380 nm was provided by a monochromator illumination system under the control of TILL Vision software. A SENSICAM-QE (Cooke) was used to detect 510 nm emission from 20 ms exposures at a sampling rate of 1 Hz. Reciprocal increases in fluorescence from 340 nm excitation and decreases in 380 nm excitation fluorescence corresponded to an increase in intracellular Ca²⁺. During agonist addition, the average deviation from baseline was calculated for each neuron, and these data were then pooled for each coverslip (n). A change of two standard deviations from baseline was considered a viable response. Mathematical and statistical analyses of the Ca²⁺ signals were performed using Origin 6.1 software.

Quantitative Real-time RT-PCR—RNA was extracted from neuronal cultures by the TRIzol (Invitrogen) phenol-chloroform method. A 2-μg aliquot of RNA was converted to cDNA using the High-Capacity cDNA Archive Kit (Applied Biosystems). Quantitative real-time PCR was performed using an Assay-on-Demand primer probe set (Applied Biosystems) specific to the assayed gene and quantified by comparison to a serially diluted plasmid containing the gene of interest. Samples were analyzed on a Real Time 7300 PCR machine (Applied Biosystems) and 18S rRNA and/or SERCA mRNA was utilized as a normalizing internal control for each sample.

Western Blotting—For IP₃R blots, primary neurons (6 × 10⁴) were mechanically isolated from culture plates and homogenized through a 21.5-gauge needle in Solution A (5 mM NaN₃, 0.1 mM EGTA, 1 mM 2-β-mercaptoethanol, 20 mM HEPES-NaOH, pH 7.4). A 1,000 × g, 4 °C centrifugation step was then utilized to remove insoluble debris, and the remaining supernatants were transferred to 2-ml (Beckman) ultracentrifuge tubes. The homogenates were then centrifuged at 100,000 × g for 1 h at 4 °C in a TLA-100.3 rotor (Beckman). Pellets were resuspended in appropriate volumes of Solution B (110 mM KCl, 10 mM NaCl, 5 mM KH₂PO₄, 1 mM 2-β-mercaptoethanol, 50 mM HEPES-KOH, pH 7.2). For cell surface receptor blots, primary neurons (3 × 10⁴) were mechanically isolated with a rubber policeman in Modified radioluminescence precipitation assay buffer and homogenized through a 21.5-gauge needle. All fractionation solutions were spiked with 1× Protease Inhibitor Mixture (Sigma). Fractions were then quantified by an Amido Black assay (15), and 25 μg of each sample was then subjected to SDS-PAGE on a 5% gel for the IP₃Rs or a 12% gel for the surface receptors. Proteins were transferred to polyvinylidene difluoride membranes and incubated overnight with a type-1 IP₃R antibody (Chemicon, 1:1,000), type-2 IP₃R antibody, pan-SERCA antibody (AbCam and Sigma, 1:1,000), a pan-PMCA antibody (AbCam 1:1,000), or a TNF-R antibody (AbCam, 1:1,000). Affinity-purified anti-rabbit/mouse horseradish peroxidase-conjugated IgG antibodies (Sigma) were then used in combination with Western Lightning reagent (PerkinElmer) for protein visualization.

TNF-α Protein and Activity Assessment—Cell culture media was removed from TNF-α-treated neuronal cultures at 24, 48, and 72 h post-addition and assayed for the levels of murine TNF-α by enzyme-linked immunosorbent assay (R&D Systems) in accordance with manufacturers’ directions. Media samples were further assayed for TNF-α biological activity similarly to a previously described method (16). A reporter plasmid that contained multimerized NFkB binding elements and minimal promoter upstream of the recombinant firefly luciferase reporter gene was transiently transfected into BHK cells with Lipofectamine 2000 (Invitrogen) in Opti-MEM. 6 h (Tₐ) after transfection, the transfection mixture was removed, and neuronal culture media described above was added. At T₁₈, the BHK monolayers were lysed using Passive Lysis Buffer (Promega) and examined for luciferase activity using the Luciferase Assay system (Promega) and a Lumicount luminescent plate reader (Packard). Transfected, PBS-treated background luminescence was subtracted from the data set.
Apoptosis Assay—Neuronal cultures were exposed to murine recombinant TNF-α for 24, 48, and 72 h and then fixed with 4% paraformaldehyde. Hoechst 33342 dye (Invitrogen) suspended in a Tris-buffered saline solution was added to the coverslips. Coverslips were then imaged on an IX70 microscope (Olympus) with 350 nm excitation from a mercury lamp (Chiu) and captured on a Progressive 3CCD (Sony) camera. Pyknotic and diffuse nuclei were counted offline using ImageJ (National Institutes of Health) software.

Statistical Analysis—Statistical analysis was preformed using Microsoft Excel and Origin 6.1 software. A two-tailed Student’s t-test or one-way analysis of variance was performed on resultant data sets as indicated. Statistical differences with p values of \(p \leq 0.05\) were considered significant.

RESULTS

Carbachol-mediated Ca\(^{2+}\) Signals Are Enhanced in Primary Neuronal Cultures Treated with TNF-α—To examine the impact of the pro-inflammatory cytokine, TNF-α, on endoplasmic reticulum (ER)-derived Ca\(^{2+}\) signals, we established neuronal cultures from C57BL/6 mouse embryos on glass coverslips. The cell composition of these cultures was determined through immunocytochemistry. NeuN, a neuronal nuclear protein, immunoreactivity overwhelmingly demonstrated the presence of primary neurons, whereas the absence of glial fibrillary acidic protein reactivity suggested minimal glial contamination (data not shown). Under low power magnification, it was evident that the primary neurons progressively elaborated processes during the course of the culturing period, a finding similar to that previously reported (17).

Cytosolic Ca\(^{2+}\) levels in neurons were then examined by calcium imaging following 24-, 48-, and 72-h treatment with 100 ng/ml TNF-α. The concentration of 100 ng/ml (\(\approx 5\) nM) murine recombinant TNF-α was chosen based on previously published literature (8), as well as our own analysis of neuronal viability in the presence of the cytokine (supplemental Fig. S1). Perfusion of the neurons with the muscarinic agonist, carbachol (CCh) was performed to determine if exposure to the pro-inflammatory cytokine leads to modulation of IP\(_3\)-releasable Ca\(^{2+}\) pools, whereas perfusion with caffeine was used to determine if RyR release was altered with cytokine exposure. TNF-α treatment led to a significant up-regulation in peak height (2-fold, \(p < 0.05\) by Student’s t test) of CCh-mediated Ca\(^{2+}\) signals at 24- and 48-h post exposure with a return to control peak levels at 72 h after initial cytokine application (Fig. 1, A and B). This result depicting the CCh response (brackets represent the change in 340/380 ratio) in single neurons after 24-h treatment with PBS or TNF-α. B, peak CCh responses were averaged for each coverslip and are shown as a diamond in the box plots. C, the percentage of neurons with a response greater than two standard deviations over background during the addition of 50 µM CCh are shown for each treatment group. D and E, 25 mM caffeine was perfused over neuronal monolayers to elicit a RyR-mediated Ca\(^{2+}\) response. D, average responses from each coverslip are again plotted in box plot form, and the percentage of cells that responded to 25 mM caffeine (E) is also shown for 24, 48, and 72 h of PBS or TNF-α treatment. Box plot parameters are as follows: diamonds represent the mean peak response from 8 neurons per coverslip, small boxes are the mean of the coverslip averages, large boxes indicate one standard error of the mean, and the whiskers indicate one standard deviation of the mean. p values were obtained from two-tailed Student’s t testing and analysis of variance analysis was performed in relation to the TNF-α variable.
Enhanced IP₃R Levels by TNF-α

was further analyzed by an analysis of variance for which the variable of TNF-α was significant (p = 0.006) in altering the peak CCh responses. The numbers of neurons per field that responded to 50 μM CCh were also analyzed, and no significant change was observed in the percentage of responding cells (Fig. 1C). In addition, basal [Ca²⁺]ᵢ levels of control and cytokine-treated cultures were analyzed by averaging the initial 20 s of the 340/380 ratio before CCh addition and were observed to not change with TNF-α addition (data not shown).

A direct measurement of RyR release was also obtained by the addition of 25 mM caffeine. In contrast to the observed alterations in the CCh-induced Ca²⁺ signals, no change in RyR-mediated Ca²⁺ signals was observed after caffeine exposure either as a function of time or with TNF-α exposure (Fig. 1D). Moreover, there was no significant difference in the percentage of cells that responded to 25 mM caffeine (Fig. 1E), a concentration chosen based on previously published reports (12, 18).

Transwell co-cultures, containing both microglia and neurons, were also tested for the ability of TNF-α to enhance muscarinic signals (see supplemental Fig. S2). These cultures displayed a similar trend, suggesting at least in this paradigm that microglia were not playing a role in buffering the inflammatory signal. Collectively, these data indicate exogenously applied TNF-α modulates neuronal muscarinic Ca²⁺ signals but does not affect signals emanating from the RyR.

TNF-α Potentiates Purinergic Receptor Ca²⁺ Signaling—The ability of TNF-α to increase neuronal Ca²⁺ signals was further analyzed to determine the ability of the cytokine to alter other Gₛ-coupled Ca²⁺ signals. Because neurons also express purinergic receptors (19), ATP was utilized to further assess inositol-induced Ca²⁺ mobilization. Ca²⁺ release was isolated by reducing the Ca²⁺ concentration in the perfusion buffer (1.2 to 0.5 mM), and through the addition of 300 μM La³⁺, which has been shown to block Ca²⁺ entry (20–22). Fifty micromolar ATP induced an increase in [Ca²⁺]ᵢ, that was significantly larger with 24-h TNF-α pretreatment (Fig. 2, A and B). Similar to the muscarinic data, there was no alteration in the percentage of neurons that responded to the ATP perfusion (Fig. 2C). The alteration of both CCh and ATP signals, even in the presence of microglia, suggested that TNF-α can alter the IP₃R-mediated Ca²⁺ signaling pathway, either at the level of Ca²⁺ release, clearance, or store-operated entry.

Enhanced Signals Arise from Increased IP₃R-mediated Ca²⁺ Release—The TNF-α-enhanced signals could arise from a multitude of factors besides a simple enhancement of IP₃R-mediated Ca²⁺ release. Increased Ca²⁺ entry, or Ca²⁺-induced Ca²⁺ release, as well as decreased Ca²⁺ clearance, could all play a role. Therefore, we sought to determine if any of these latter mechanisms were responsible. 24-h TNF-α-treated and PBS-treated control coverslips were assayed by Ca²⁺ imaging in La³⁺-blocking solution to eliminate store-operated Ca²⁺ entry (Fig. 3, A and B). The blocking solution successfully eliminated all Ca²⁺ influx as observed by the absence of a Ca²⁺ signal with the addition of 25 mM KCl (Fig. 3A). However, the enhanced CCh signals observed with cytokine treatment remained present (Fig. 3B). Removal of the La³⁺ block after agonist addition also allowed for the direct measurement of store-operated Ca²⁺ entry (Fig. 3, C and D). No difference in the peak signal

![Figure 2. Effect of TNF-α on purinergic Ca²⁺ signaling.](image-url) Primary neurons were treated with 100 ng/ml TNF-α for 24 h and subsequently loaded with the calcium imaging dye, Fura2-AM. Emitted fluorescence from alternating 340/380 nM excitation was monitored after exposure to 50 μM ATP in the presence of a La³⁺ block solution to eliminate Ca²⁺ influx. A, representative traces depicting the ATP response are shown for both a control and cytokine treated neuron. B, average peak responses from six coverslips (n) for each condition were analyzed and are shown in box plot format (for parameters see Fig. 1). C, the percentage of neurons that responded (change in 340/380 ratio of ≥2 S.D. from the baseline) in response to perfusion with ATP addition is plotted.
elucidate the components of the muscarinic cascade affected by cytokine values for Ca\textsuperscript{2+} on the CCh-mediated signals but completely blocked KCl-induced A

C57BL/6 primary neurons in reduced Ca\textsuperscript{2+}-containing external perfusion solution, and CCh signals were again obtained from Ca\textsuperscript{2+} entry (Fig. 3D) or in the kinetics (time constant (\(\tau\); Con = 14.7 \(\pm\) 3.3 s, TNF-\(\alpha\) = 16.1 \(\pm\) 4.9 s) of Ca\textsuperscript{2+} influx with cytokine treatment was observed. In aggregate, these data demonstrated that an alteration in Ca\textsuperscript{2+} influx was not responsible for the enhanced signals following TNF-\(\alpha\) treatment.

Others have suggested previously that increased RyR-mediated Ca\textsuperscript{2+} release may account for enhanced muscarinic signals via the process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (18). Even though enhanced RyR signals were not observed with cytokine addition, changes in functional coupling between the two ER receptors could underlie the TNF-\(\alpha\) effect. To disrupt any coupling between the IP\textsubscript{3}R and RyR, the RyR was inhibited by a 30-min preincubation with 100 \(\mu\)M ryanodine, and again CCh-induced Ca\textsuperscript{2+} signals were examined following TNF-\(\alpha\) treatment. Ryanodine treatment blocked caffeine-induced Ca\textsuperscript{2+} release completely (Fig. 3E), and, as previously reported, a reduction in fluorescence due to caffeine binding to the Fura-2 dye was observed (23). Nevertheless, blocking the RyR had no effect on the TNF-\(\alpha\)-enhanced muscarinic signals (Fig. 3F).

Because a reduction in Ca\textsuperscript{2+} clearance could also increase \([\text{Ca}\textsuperscript{2+}]_{i}\) after exposure to an agonist, Ca\textsuperscript{2+} clearance parameters were examined following TNF-\(\alpha\) treatment. To examine this process, the decay of Ca\textsuperscript{2+} signals recorded in the caffeine-imaging experiments was fit to a single exponential (Fig. 4A). This decay parameter accounts for Ca\textsuperscript{2+} clearance at both the plasma membrane (PMCA) and endoplasmic reticulum (SERCA) as well as any Ca\textsuperscript{2+} uptake by cellular organelles, including mitochondria. No significant alteration in the clearance of Ca\textsuperscript{2+} from neuronal soma was observed at any of the time points assayed (Fig. 4B). In addition, experiments were also performed in the presence of the selective mitochondrial calcium uniporter inhibitor, Ru360 (24). The average values for the CCh responses in the presence of this inhibitor, which has been previously shown in the literature and within our laboratory to effectively block mitochondrial Ca\textsuperscript{2+} uptake (25), did not deviate from the previously observed trend with cytokine addition (peak Con = 0.18 \(\pm\) 0.5 ratio units, TNF-\(\alpha\) = 0.32 \(\pm\) 0.1 ratio unit). Furthermore, the steady-state expression levels of both PMCA and SERCA were analyzed by Western blotting with respective pan antibodies and determined to be unchanged (Fig. 4C), demonstrating that altered clearance was not responsible for the enhanced muscarinic and purinergic signals.

IP\textsubscript{3}R-mediated Ca\textsuperscript{2+} release could also be enhanced by increasing the Ca\textsuperscript{2+} loading of the ER after cytokine exposure. To determine the steady-state Ca\textsuperscript{2+} levels, we utilized a reversible SERCA pump inhibitor, cyclopiazonic acid (CPA), in the presence of La\textsuperscript{3+} block and measured the peak height and peak area of the response (26). CPA addition led to a transient increase in Ca\textsuperscript{2+} that was observed to be equal in both TNF-\(\alpha\) and control treated neurons (Fig. 4D). The total Ca\textsuperscript{2+} released, as indicated by the area of the response, was also of equal magnitude for each treatment group. Cumulatively, these experiments indicated that the TNF-\(\alpha\) effect on muscarinic signaling was independent of Ca\textsuperscript{2+} influx, Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release, Ca\textsuperscript{2+} clearance, and ER Ca\textsuperscript{2+} loading, and likely

FIGURE 3. Elimination of Ca\textsuperscript{2+} influx and functional RyR Coupling. Ca\textsuperscript{2+}-imaging experiments were performed on 24-h TNF-\(\alpha\)-treated C57BL/6 primary neurons in reduced Ca\textsuperscript{2+}-La\textsuperscript{3+}-containing perfusion buffer (0.5 mM Ca\textsuperscript{2+}, 300 \(\mu\)M La\textsuperscript{3+}) and in the presence of 100 \(\mu\)M RyR to elucidate the components of the muscarinic cascade affected by cytokine treatment.

A. sample traces are shown to demonstrate the solution had no effect on the Ca\textsuperscript{2+}-mediated signals but completely blocked KCl-induced Ca\textsuperscript{2+} entry. B, CCh responses averaged for each coverslip are shown as a diamond in the box plots (for parameters see Fig. 1). C and D, Ca\textsuperscript{2+} entry was also directly measured by the reperfusion of the neurons with a La\textsuperscript{3+} free (normal) physiological saline solution. C, representative traces from one control and one TNF-\(\alpha\)-treated neuron are shown to demonstrate the lack of effect on the peak and kinetics of Ca\textsuperscript{2+} entry. D, peak values for Ca\textsuperscript{2+} entry are shown in box plot format. E and F, the possibility of enhanced RyR coupling leading to enhanced muscarinic signals through Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release was tested directly by inhibiting the RyR with 100 \(\mu\)M ryanodine. E, sample traces are depicted showing the ability of the drug treatment to completely inhibit caffeine-induced Ca\textsuperscript{2+} signals, whereas the CCh signal was preserved. F, average CCh signals from nine control and nine 24-h TNF-\(\alpha\)-treated coverslips are shown in box plot format.
Enhanced IP3R Levels by TNF-α

A Ca2⁺ Clearance After Agonist Exposure

![Graph showing Ca2⁺ clearance after agonist exposure with time points at 24h, 48h, and 72h for control and TNF-α treatment.]

B Decay Constant (1/sec) vs. Time (h)

![Graph showing decay constant over time with control and TNF-α treatment.]

C 100 kD 100 kD 37 kD

![Western blot image showing WB 24h with bands for Ca2⁺ ATPase/β-Actin, SERCA, PMCA, and β-Actin.]

D CPA (50 μM) in La3⁺ Block Solution

![Graph showing Δ 340/380 Ratio vs. Time with control and TNF-α treatment.]

FIGURE 4. Ca2⁺ clearance and ER Ca2⁺ homeostasis. Primary neurons were analyzed for the possible contribution of decreased Ca2⁺ clearance or enhanced ER Ca2⁺ stores on the elevated Gq-coupled Ca2⁺ signals. A and B, the clearance of Ca2⁺ from the soma was measured by fitting the decay of caffeine-induced Ca2⁺ transients to a single exponential. C, arrows indicate the decay of the Ca2⁺ signals fit by a single exponential function, which is illustrated in the inset. H, the average time constants obtained by the mathematical fitting of the decrease in 340/380 ratio (Ca2⁺ clearance) after each treatment over time are shown. C, total neuronal homogenates were subjected to SDS-PAGE and Western blotting for SERCA, PMCA, and β-actin. Optical density ratios of each Ca2⁺ ATPase versus β-actin are shown for both control and TNF-α-treated cultures. D, ER Ca2⁺ levels were analyzed using the reversible SERCA inhibitor CPA in the presence of La3⁺ block. CPA induced a transient increase in [Ca2⁺]i that was analyzed for both peak height (left panel) and area of the response (right panel). p values were obtained from two-tailed Student’s t test.

pointed to molecular alterations in the IP3-mediated Ca2⁺ release pathway.

TNF-α Mediated Modulation of Type-1 IP3R mRNA and Protein Levels—Because TNF receptor signaling is linked to two major transcriptional pathways, JNK/activator protein-1 and NFκB (27), the mechanism of TNF-α enhancement of CCh signals could involve transcriptional regulation and/or stabilization of the one or more IP3R gene transcripts. To elucidate possible increases in steady-state mRNA, total RNA was extracted from neuronal cultures exposed to recombinant TNF-α and quantitative real-time PCR (qRT-PCR) was performed. The expression of monocyte chemoattractant protein-1 (MCP-1) mRNA, whose expression is known to be regulated by TNF-α (28), served as a positive control, and increased by 6 h following TNF-α application and remained at elevated levels through 12 h (Fig. 5A). This increase in MCP-1 message level was significant (p < 0.05) versus RNA derived from non-treated control cultures.

TaQMan primer/probe sets specific to each of the three types of IP3R were used to monitor transcript levels of the receptors in untreated neurons or neurons exposed to TNF-α for 0, 6, 12, and 18 h. The level of transcript for the predominant neuronal type of the IP3R, the type-1 IP3R, was increased significantly at 12 h (p = 0.05, Fig. 5C). The type-2 IP3R followed a similar trend but did not reach significance (p = 0.12, Fig. 5E), whereas the transcript level for the type-3 IP3R remained unchanged over time with TNF-α exposure (Fig. 5G). SERCA transcript was used as an internal control for input RNA and for continuity with subsequent Western blot experiments, because its expression did not change in response to TNF-α treatment (see Fig. 4C).

Western blotting was performed to determine whether enhanced levels of type-1 IP3R transcript would lead to higher steady-state levels of protein. Consistent with the qRT-PCR analyses, the levels of the type-1 IP3R at 24 and 48 h post-TNF-α exposure were enhanced (Fig. 5B) and the ratio of receptor to SERCA protein was significantly elevated (Fig. 5F, 24 h: p = 0.03 and 48 h: p = 0.01) following TNF-α exposure. SERCA protein expression was used as a loading control, because its expression was abundant in these ER-enriched preparations and its large molecular weight allowed it to be observed on the protein blot (Fig. 5, B and F). Western blotting was also undertaken for the type-2 IP3R (Fig. 5, F and H), which, similar to the qRT-PCR data, showed no significant change in protein after cytokine addition. Hence, the elevation in type-1 IP3R mRNA at 12 h and subsequent protein elevation at 24 and 48 h correlated with the observed increase in Ca2⁺ signals at the 24- and 48-h time points.

Resolution of IP3R Protein Levels and Ca2⁺ Signaling—A time course of TNF-α influence on IP3R protein expression and CCh-induced Ca2⁺ signal alterations was observed with elevations at 24 and 48 h and a return to baseline at the 72-h time point. In an effort to determine the reason for signal termination, TNF-α peptide concentration and activity was assessed by an enzyme-linked immunosorbent assay and an in vitro activity assay in which a reporter plasmid construct was transiently transfected into BHK cells (Fig. 6B). The concentration of TNF-α in the treated supernatants was unchanged over the 72-h
period (Fig. 6A). However, the activity of the TNF-α in the culture media diminished over time. Supernatants from primary neuronal cultures treated for 24 h exhibited a significant increase in luciferase activity over control, whereas samples from cultures treated for 48 and 72 h failed to activate the NFκB reporter construct (Fig. 6C).

These data indicate exogenously added TNF-α was biologically active during the initial 24 h of incubation, and offers an explanation as to why CCh-induced Ca^{2+} signal enhancement and increased type-1 IP{\textsubscript{3}}R protein levels were lost by 72 h in primary neuronal cultures treated with TNF-α.

FIGURE 5. IP{\textsubscript{3}}R mRNA and protein levels following treatment with murine TNF-α. 11-Day in vitro primary neuronal cultures were treated with 100 ng/ml mouse recombinant TNF-α. RNA was extracted from 12 wells (n = 6) containing 6 × 10{\textsuperscript{5}} cells per two wells using the TRIzol phenol/chloroform method, and 2 μg was archived as cDNA. A, a quantitative real-time RT-PCR primer/probe set specific for monocyte chemoattractant protein-1 (MCP-1) was used as a positive TNF-α target control. C, E, and G, the steady-state levels of the type-1/2/3 IP{\textsubscript{3}}R transcripts over an 18-h period were observed by independent primer probe sets and normalized to SERCA mRNA at each time point. B, protein was extracted from 24-, 48-, and 72-h TNF-α- and control-treated neurons enriched for the ER membrane. 25 μg of total protein was submitted to SDS-PAGE and type-1 IP{\textsubscript{3}}R, and SERCA as a loading control, protein levels were monitored by Western blotting. Band intensities were quantified using Labworks software and presented as optical density ratios of IP{\textsubscript{3}}R to SERCA for control (D, lanes 1–6) and TNF-α-treated groups (D, lanes 7–12). F and H, 24-h protein levels were also examined for the type-2 IP{\textsubscript{3}}R in a similar fashion. p values were obtained from two-tailed Student’s t testing. *, indicates a p value of <0.05.
Enhanced IP$_3$R Levels by TNF-α

**FIGURE 6.** TNF-α activity in primary neuronal culture supernatants. TNF-α peptide levels at 24, 48, and 72 h following addition to primary neuronal cultures were measured by enzyme-linked immunosorbent assay (A), and the ability of this cytokine to activate NFκB signaling following prolonged culture incubation times was assessed using an in vitro bioactivity assay. B, functional activity of TNF-α in the culture medium from treated neuronal cultures was measured by adding aliquots of these media samples to BHK cells transiently transfected with a plasmid construct harboring a tandem NFκB binding element array and minimal promoter driving the expression of the firefly luciferase gene. C, luciferase activity was measured using a luminometer. Error bars indicate one standard deviation from the mean.

**DISCUSSION**

The present study elucidates a novel neuronal pathway in which the expression of the IP$_3$R can be regulated by TNF-α through the activation of a JNK signaling cascade. Regardless of microglial presence, neuronal exposure to murine recombinant TNF-α resulted in an increase in type-1 IP$_3$R mRNA and protein levels, and was shown to enhance Ca$_{2+}$ signals downstream of Gq-coupled receptor stimulation. Furthermore, this increase in [Ca$_{2+}$], was determined to be independent of possible alterations in Ca$_{2+}$ influx, Ca$_{2+}$-induced Ca$_{2+}$ release, and Ca$_{2+}$ clearance.

Our data indicate that TNF-α-mediated enhancement of type-1 IP$_3$R protein levels underlies the alterations observed in muscarinic signals. This occurs after TNF-α binds to either the type-I or II TNF R, activates a JNK-related cascade, which results in greater steady-state levels of type-1 IP$_3$R mRNA at 12 h and protein at 24 and 48 h post-cytokine addition. We further proposed that the return of IP$_3$R protein levels at 72 h occurs due to metabolism of recombinant TNF-α in the culture media. However, it is also plausible that alterations in TNF receptor coupling and surface expression could be playing a role in the loss of this signal. We also cannot completely rule out the contribution of other IP$_3$R post-translational modifications such as phosphorylation (32–34), enhanced IP$_3$ binding (35), modulation of ATP binding, and/or modulation by binding partners (reviewed in Ref. 36), because these have all been shown to affect IP$_3$-mediated Ca$_{2+}$ release. It is also possible that TNF-α may regulate the production of IP$_3$ through the modulation of phospholipase C activity. These alternative mechanisms serve as the foci of future research endeavors.
Nevertheless, the Ca\textsuperscript{2+} signal enhancement temporally correlated with increased IP\textsubscript{3}R protein levels, and changes in steady-state levels of IP\textsubscript{3}R protein have been shown previously to directly alter Ca\textsuperscript{2+} responses without additional modifications to the receptor (37, 38).

Neuroinflammation is a complex process, which involves not only neurons, but also central nervous system-resident inflammatory cells (e.g. microglia). We sought to determine if the presence of microglia could affect the signaling of TNF-\alpha within the brain in such a way so as to either amplify the signal or dampen its pro-inflammatory effects. Our experiments suggested, in this specific inflammatory pathway, microglia may not be playing a pivotal role. However, in other immunological cascades, microglia may act as regulators of inflammation or
Enhanced IP$_3$R Levels by TNF-$\alpha$

produce secondary signals that may modulate neuronal Ca$^{2+}$ signaling.

Camandola and colleagues (39) previously demonstrated that genetic ablation of the RelA gene in mouse embryonic fibroblasts increased IP$_3$R expression and Ca$^{2+}$ signaling, which they proposed was the result of a release of an inhibitory effect imparted by the NF$\kappa$B pathway. We submit that this increase in IP$_3$R expression may actually be a result of a release in JNK inhibition by the NF$\kappa$B pathway; a possibility that these authors also suggested because they observed an increase in JNK activity following RelA knockdown. The ability of TNF-$\alpha$ to induce a chimeric response depending on differential receptor coupling may lead to a dynamic regulation of Ca$^{2+}$ signals during neuroinflammatory events.

Our in vitro study has suggested that metabolism and inactivation of TNF-$\alpha$ may cause the enhancement of IP$_3$R-mediated Ca$^{2+}$ signals to be short-lived. However, prolonged production of TNF-$\alpha$ within the brain may cause a more chronic enhancement of muscarinic signals. Furthermore, cytokine-induced alterations in coupling of the receptor to downstream proteins may play a role in regulating the inflammatory signal to create an acute or chronic response. Future research will seek to uncover specific transcription or message stabilization factors lying downstream of JNK that are responsible for the enhancement of IP$_3$R levels. Previous work done by Deelman et al. (40) has suggested two sites within the type-1 IP$_3$R promoter that the activator protein-1 transcription factor may act upon. The functional characterization of these sites will be the source of future undertakings. With this knowledge a better understanding of exogenous factors that can activate this pathway, besides TNF-$\alpha$, can be obtained, which may elucidate the physiological role of this signaling cascade during inflammatory events within the brain.

The long term physiological consequences of the dynamic control of TNF-$\alpha$ over IP$_3$R expression have yet to be determined. It is plausible that the enhancement of muscarinic signals represents a single step in a process of preparing the neuron for programmed cell death. It is also possible that another inflammatory signal, possibly another cytokine, or an excitatory amino acid, could cause the neuron to pass a threshold toward an irreversible pathway to death. If this is the case, the enhancement of IP$_3$R steady-state levels could facilitate the changes in Ca$^{2+}$ signals necessary for initiation of apoptosis. Consistent with our study, Floden and colleagues (41) have shown that TNF-$\alpha$ alone does not induce neuronal cell death. However, in the combined presence of glutamate agonists, the cytokine can mediate apoptosis via enhanced inducible nitric-oxide synthase expression. Conversely, the increase in IP$_3$R expression could represent more of a physiological “braking” mechanism instead of a pathophysiological alteration. An increase in the local concentration of muscarinic agonists could lead to a more quiescent neuron and allow it to bypass excitotoxic death caused by a proximal dysfunctioning neuron. It is conceivable that both of these mechanisms could occur depending on the secondary signal evoked, with the neuron poised to save itself or initiate its own death to preserve the overall neural circuit. Differences in TNF receptor type expression and the activity of receptor-associated factors may hold the key for understanding why certain neuronal populations are more at risk for cellular dysfunction and demise in neurological diseases exhibiting underlying chronic neuroinflammation.

Acknowledgments—We thank Maria Frazer, Jason Hamilton, and Landa Prifti (University of Rochester) for animal husbandry, Rita Giuliano (University of Rochester) for tissue culture advice, Sanjay Maggiirwar for the NF$\kappa$B construct, and Matthew Betzenhauser (University of Rochester) for advice on IP$_3$R protein enrichment and for assistance on editing the manuscript.

REFERENCES

1. McGeer, E. G., Kligeris, A., and McGeer, P. L. (2005) Neurobiol. Aging 26, Suppl. 1, 94–97
2. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N., and Williamson, B. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 3666–3670
3. Ortaldo, J. R., Mason, L. H., Mathieson, B. J., Liang, S. M., Flick, D. A., and Herberman, R. B. (1986) Nature 321, 700–702
4. Laster, S. M., Wood, J. G., and Gooding, L. R. (1988) J. Immunol. 141, 2629–2634
5. Hanisch, U. K. (2002) Glia 40, 140–155
6. Kinouchi, K., Brown, G., Pasternak, G., and Donner, D. B. (1991) Biochem. Biophys. Res. Commun. 181, 1532–1538
7. Arntt, H. A., Mason, J., Marino, M., Suzuki, K., Matsuohma, G. K., and Ting, I. P. (2001) Nat. Neurosci. 4, 1116–1122
8. Barger, S. W., Horster, D., Furukawa, K., Goodman, Y., Kriegstein, J., and Mattson, M. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9328–9332
9. Gelbard, H. A., Dzenko, K. A., DiLoreto, D., del Cerro, C., del Cerro, M., and Epstein, L. G. (1993) Dev. Neurosci. 15, 417–422
10. Ogoshi, F., Yin, H. Z., Kuppambatty, Y., Song, B., Aminardi, S., and Weiss, J. H. (2005) Exp. Neurol. 193, 384–393
11. Jara, J. H., Singh, B. B., Floden, A. M., and Combs, C. K. (2007) J. Neurochem. 100, 1407–1420
12. Stutzmann, G. E., Smith, I., Caccamo, A., Oddo, S., Laferla, F. M., and Parker, I. (2006) J. Neurosci. 26, 5180–5189
13. Dieckmann-Schuppert, A., and Schmittler, H. J. (1997) Cell Tissue Res. 288, 119–126
14. Sanchez, I. F., Sneideran, L. F., Williamson, A. L., Fan, S., Chakraborty-Sett, S., and Maggirwar, S. B. (2003) Mol. Cell. Biol. 23, 4649–4662
15. Detrait, E. R., Bowers, W. J., Halterman, M. W., Giuliano, R. E., Bennent, L., Federoff, H. I., and Richfield, E. K. (2002) Mol. Ther. 5, 723–730
16. Smith, I. F., Hitt, B., Green, K. N., Oddo, S., and Laferla, F. M. (2005) J. Neurochem. 94, 1711–1718
17. Tokuyama, Y., Harma, M., Jones, E. M., Fan, Z., and Bell, G. I. (1995) Bioch. Biophys. Res. Commun. 211, 211–218
18. Weiss, G. B. (1974) Annu. Rev. Pharmacol. 14, 343–354
19. Mattson, M. P., and Kater, S. B. (1987) J. Neurosci. 7, 94, 981–987
20. Laster, S. M., Wood, J. G., and Gooding, L. R. (1988) J. Neurochem. 5180–5189
21. Dolmetsch, R. E., Xu, K., and Lewis, R. S. (1991) Nature 343, 354–364
22. Langer, J. B. (1990) J. Gen. Physiol. 95, 1417–1422
23. Muschol, M., Dasgupta, B. R., and Salzberg, B. M. (1999) Biophys. J. 77, 577–586
24. Ying, W. L., Emerson, J., Clarke, M. J., and Sanadi, D. R. (1991) Biochemistry 30, 4949–4952
25. Bruce, J. J., Giovannucci, D. R., Blinder, G., Shuttleworth, T. J., and Yule, D. I. (2004) J. Biol. Chem. 279, 12909–12917
26. Goeger, D. E., Riley, R. T., Dorner, J. W., and Cole, R. J. (1988) Bioch. Biophys. Res. Commun. 171, 978–981
27. Song, H. Y., Regnier, C. H., Kirschning, C. I., Goeddel, D. V., and Rothe, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 9792–9796
28. Sica, A., Wang, J. M., Colotta, F., Dejana, E., Mantovani, A., Oppenheim, J. J., Larsen, C. G., Zachariae, C. O., and Matsuohma, K. (1990) J. Immunol. 144, 3034–3038
29. Bennett, B. L., Sasaki, D. T., Murray, B. W., O’Leary, E. C., Sakata, S. T., Xu, W., Leisten, J. C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S. S., Man-
ning, A. M., and Anderson, D. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13681–13686
30. Edwards, D. R., and Denhardt, D. T. (1985) Exp. Cell Res. 157, 127–143
31. Waetzig, V., Czeloth, K., Hidding, U., Mielke, K., Kanzow, M., Brecht, S., Goetz, M., Lucius, R., Herdegen, T., and Hanisch, U. K. (2005) Glia 50, 235–246
32. Joseph, S. K., and Ryan, S. V. (1993) J. Biol. Chem. 268, 23059–23065
33. Wagner, L. E., 2nd, Li, W. H., and Yule, D. I. (2003) J. Biol. Chem. 278, 45811–45817
34. Tang, T. S., Tu, H., Wang, Z., and Bezprozvanny, I. (2003) J. Neurosci. 23, 403–415
35. Joseph, S. K., and Rice, H. L. (1989) Mol. Pharmacol. 35, 355–359
36. Bezprozvanny, I. (2005) Cell Calc. 38, 261–272
37. McGowan, T. A., and Sharma, K. (2000) Kidney Int. Suppl. 77, S99–S103
38. Bokkala, S., Rubin, E., and Joseph, S. K. (1999) Alcohol. Clin. Exp. Res. 23, 1875–1883
39. Camandola, S., Cutler, R. G., Gary, D. S., Milhavet, O., and Mattson, M. P. (2005) J. Biol. Chem. 280, 22287–22296
40. Deelman, L. E., Jonk, L. J., and Henning, R. H. (1998) Gene (Amst.) 207, 219–225
41. Floden, A. M., Li, S., and Combs, C. K. (2005) J. Neurosci. 25, 2566–2575