Published in final edited form as:
Neuroscience. 2013 October 10; 250: 282–299. doi:10.1016/j.neuroscience.2013.07.013.

Minimal NF-κB activity in neurons

Samuel J. Listwak[#], **Priyanka Rathore**[#], and **Miles Herkenham**[*]
Section on Functional Neuroanatomy, National Institute of Mental Health, NIH, Bethesda, MD 20892, USA

[#] These authors contributed equally to this work.

Abstract

NF-κB is a ubiquitous transcription factor that regulates immune and cell-survival signaling pathways. NF-κB has been reported to be present in neurons wherein it reportedly responds to immune and toxic stimuli, glutamate, and synaptic activity. However, because the brain contains many cell types, assays specifically measuring neuronal NF-κB activity are difficult to perform and interpret. To address this, we compared NF-κB activity in cultures of primary neocortical neurons, mixed brain cells, and liver cells, employing Western blot of NF-κB subunits, EMSA of nuclear κB DNA binding, reporter assay of κB DNA binding, immunofluorescence of the NF-κB subunit protein p65, quantitative real-time PCR of NF-κB-regulated gene expression, and ELISA of produced proteins. Assay of p65 showed its constitutive presence in cytoplasm and nucleus of neurons at levels significantly lower than in mixed brain or liver cells. EMSA and reporter assays showed that constitutive NF-κB activity was nearly absent in neurons. Induced activity was minimal—many fold lower than in other cell types, as measured by phosphorylation and degradation of the inhibitor IκBα, nuclear accumulation of p65, binding to κB DNA consensus sites, NF-κB reporting, or induction of NF-κB-responsive genes. The most efficacious activating stimuli for neurons were the proinflammatory cytokines TNFα and IL-β. Neuronal NF-κB was not responsive to glutamate in most assays, and it was also unresponsive to hydrogen peroxide, lipopolysaccharide, norepinephrine, ATP, phorbol ester, and nerve growth factor. The chemokine gene transcripts CCL2, CXCL1, and CXCL10 were strongly induced via NF-κB activation by TNFα in neurons, but many candidate responsive genes were not, including the neuroprotective genes SOD2 and Bcl-xL. Importantly, the level of induced neuronal NF-κB activity in response to TNFα or any other stimulus was lower than the level of constitutive activity in non-neuronal cells, calling into question the functional significance of neuronal NF-κB activity.

INTRODUCTION

The transcription factor NF-κB is extensively studied for its role in regulating expression of genes related to immune and cell survival/cell death pathways. NF-κB functions are well studied in peripheral organs, but in the brain, understanding is complicated by the varied composition of brain cells, ranging from neurons to macroglia to microglia as well as supporting stromal cells. CNS responses to immune and pathogenic challenges are dominated by activity generated in non-neuronal cells, and neurons can be regarded as secondary targets of non-neuronal activity (Aarum et al., 2003, Ousman and Kubes, 2012).

[*]Correspondence should be addressed to Miles Herkenham, Section on Functional Neuroanatomy, NIMH, Bldg. 35, Rm. 1C913, 35 Convent Drive, Bethesda, MD 20892-3724 USA. herkenh@mail.nih.gov.

Neurons normally do not engage the intracellular pathways mediating immune and survival actions in part because they express relatively low levels of receptors for immune molecules such as cytokines and pathogens. Indeed, *in vitro* studies showed that neuronal NF-κB was largely unresponsive to cytokines and microbial pathogens that strongly triggered its activity in astrocytes (Jarosinski et al., 2001). Nevertheless, a considerable body of literature supports the presence of NF-κB activity in neurons, wherein it has been shown to play a role not only in neuroprotection (Fridmacher et al., 2003) and neurodegeneration (Zhang et al., 2005) but also neuronal development (Gutierrez et al., 2005), learning, memory, and synaptic plasticity (Boccia et al., 2007, Kaltschmidt and Kaltschmidt, 2009). These latter features assigned to neuronal NF-κB signaling suggest that the functional role of NF-κB in neurons is distinctly different than in other cells.

Neuronal NF-κB reportedly has a number of striking or unique features. One is that neurons possess substantial constitutive NF-κB activity. The earliest reports of this were based on constitutive immunohistochemical neuronal staining in brain sections by antibodies raised against the classical NF-κB subunits p65 and p50. Notably, an antibody against the "activated" form of p65 formed the basis for the findings in the early studies (Kaltschmidt et al., 1994). However, recent work showed that this antibody recognizes an undetermined protein that is not p65 (Herkenham et al., 2011). Similarly, many commercially available p65 and p50 antibodies have shown complex binding to multiple proteins in Western blot analyses (Pereira et al., 1996, Herkenham et al., 2011), making them unsuitable for immunohistochemistry.

Other claims for neuronal NF-κB activity were supported by data from assays in which neurons and non-neuronal brain cells were homogenized together (Clemens et al., 1997) or from studies in neuron-like cell lines (Lezoualc'h et al., 1998). Finally, several NF-κB reporter constructs and transgenic reporter mice have shown constitutive neuronal NF-κB reporting (Schmidt-Ullrich et al., 1996, Bhakar et al., 2002). However, different reporter mouse lines display qualitatively and quantitatively different patterns of neuronal reporting, and some NF-κB reporter lines show no constitutive CNS activity at all (Lernbecher et al., 1993, Carlsen et al., 2002). The reasons for differences in basal activity reporting have not been addressed.

The triggers for neuronal NF-κB activation are unique as well. Early studies proposed that a major activator is not cytokines or physical stressors, but rather glutamate and its analogs (Guerrini et al., 1995, Kaltschmidt et al., 1995) and, later, synaptic activity (Meffert et al., 2003). However, other studies showed that glutamate does not activate neuronal NF-κB at all (Lukasiuk et al., 1995, Mao et al., 1999).

Finally, the genes that are known to contain upstream κB DNA binding sites and to be regulated by NF-κB in immune cells are not significantly activated in neurons. For example, the prototypical NF-κB-responsive gene IκBα, whose expression is critical for the regulation of the NF-κB pathway, has been shown by *in situ* hybridization histochemistry (ISHH) to be induced in non-neuronal cells in the brain (Quan et al., 1997), but its mRNA induction has never been reported in neurons by ISHH. Overall, there is a lack of agreement about what genes are transcriptionally regulated by NF-κB in neurons, and traditional pro-inflammatory cytokine genes are not among the named genes (Kaltschmidt et al., 2002, Kassed et al., 2004, Kaltschmidt et al., 2006, Boersma et al., 2011, Schmeisser et al., 2012). Given the difficulty of working with brain tissue that contains non-neuronal cells with strong NF-κB activity levels or with neuron-like cell lines immortalized by fusion with cancer cells with strong NF-κB activity, we chose to examine primary cell culture, contrasting activity in neurons with that in mixed brain cells and liver cells.

Several kinds of assays were performed to address the presence and activation of neuronal NF-κB. In its inactive state in the cell cytoplasm, NF-κB exists as a dimer, typically the combination of the p50 and p65 subunits, bound with the inhibitor IκBα, which blocks the nuclear localization signal (NLS) present on p50 and p65. NF-κB activation is initiated by the enzymatic breakdown of the bound IκBα protein—IκBα is phosphorylated by the IκB kinase (IKK) complex and degraded through the ubiquitin/proteasome pathway. Removal of IκBα exposes the NLS, and the subunits are able to translocate to the nucleus where they can bind to κB DNA elements, typically represented by the consensus sequence GGGRNNYYCC, in gene promoters/enhancers and then initiate gene transcription. Measures of NF-κB activation include immunoblot (Western blot) assays of nuclear accumulation of subunits (typically p65, which has a transactivation domain) or disappearance of IκBα from the cytoplasm (or brief appearance of phosphorylated IκBα); microscopic tracking of nuclear translocation of immunolabeled subunits, usually p65; assays of κB DNA binding by electrophoretic mobility shift assay (EMSA), with identification of the protein binding partners done by supershift analysis; transgene reporting by constructs that contain the κB DNA sequences upstream of a reporter gene; and alterations in transcription levels of genes known to be regulated by NF-κB. In this study, all of the above-named assays for presence and activation have been employed.

EXPERIMENTAL PROCEDURES

Primary cell culture

Cortical *Neurons (CxN)*—Mouse neurons were cultured from gestational day-16 embryonic C57BL/6 mouse brains as described previously (Herkenham et al., 2011). Briefly, hippocampi or neocortices were dissected out in cold Hanks balanced salt solution (HBSS), trypsinized, triturated, strained and pelleted. The pellet was resuspended in Neurobasal medium supplemented with B27 (1X), Glutamax (2 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml) (all from Invitrogen) and then seeded onto poly-d-lysine coated coverslips at a density of 0.10×10^6 cells/well for immunocytochemistry, in 12-well poly-d-lysine coated plates at a density of 0.40×10^6 cells/well for ELISA analysis and in 6-well poly-d-lysine coated plates at 2×10^6 cells/well for protein or gene expression experiments. Neurons were maintained at 37°C in 5% CO_2/95% O_2. After four days in culture, the medium was changed to fresh supplemented Neurobasal medium containing cytosine arabinoside (10 mM) and 2-deoxycytidine (100 mM) (Sigma-Aldrich) to inhibit astrocyte growth. After 10 days in culture, cells were subjected to the various experimental conditions and processed for either immunostaining or immunoblotting.

Mixed brain cells (BRN)—Mouse brain cells comprising astrocytes, oligodendrocytes, microglia, neurons, and unidentified cells were cultured from the same 16-day embryos that produced the neuron cultures. Subcortical pieces were dissected out in cold HBSS, homogenized, trypsinized, triturated, strained, pelleted and resuspended in DMEM media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). The cells were plated onto poly-d-lysine coated coverslips for immunocytochemistry and/or in 6-well poly-d-lysine coated plates at 2×10^6 cells/well for protein or gene expression experiments. Experimental treatments were performed when the cells reached confluence.

Microglia—Microglial cells were obtained by shaking confluent mixed brain cell cultures overnight at 200 RPM and then collecting the culture supernatants containing the detached microglia. The supernatants were centrifuged (5 min at 200 × g), and the resulting pellet was re-suspended in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). The cells were plated onto poly-d-lysine coated coverslips and grown for 4–7 days prior to immunocytochemical analysis.

Liver cells (LVR)—Cells taken from fetal liver, including leukocytes, were cultured from the same 16-day embryos that produced the neuron and mixed brain cultures. Fetal liver pieces were dissected out in cold HBSS, homogenized, trypsinized, triturated, strained, pelleted, and resuspended in DMEM media supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml). The cells were seeded onto collagen (Sigma) coated 6-well plates at 2×10^6 cells/well and used for protein or gene expression experiments after 10 days in culture.

Drugs used for treatments

Lipopolysaccharide (LPS), L-glutamic acid (glutamate), (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA), ammonium pyrrolidinedithiocarbamate (PDTC), (E)-3-(4-methylphenylsulfonyl)-2-propenenitrile (BAY 11-7082), 5-(thien-3-yl)-3-aminothiophene-2-carboxamide (SC-514), 2-amino-5-phosphonopentanoate (AP5), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), phorbol 12-myristate 13-acetate (PMA), nimodipine, ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EDTA), and norepinephrine were obtained from Sigma; hydrogen peroxide (H_2O_2) was from Fisher Scientific; adenosine triphosphate (ATP) and nerve growth factor (NGF) were from Invitrogen; and brain-derived neurotrophic factor (BDNF), tumor necrosis factor α (TNFα) and IL-1β were from R&D Systems.

Antibodies used for Western blots, EMSA and Immunofluorescence

Primary antibodies used were: p65 for immunohistochemistry, Santa Cruz sc-372 (C-20); p65 for supershift, NIH#7057, gift of Ulrich Siebenlist, NIAID; IκBα, Cell Signaling #9242; PO_4-IκBα (ser 32), Cell Signaling #2859; p50, NCI #1263-TB7 (Nancy Rice, NCI, Fort Detrick, Frederick, MD); c-Rel, NCI #1266; RelB, NCI #1319; p52, NCI #1495; CREB, Cell Signaling #9192; PO_4-CREB, Cell Signaling #9191; PO_4-ERK (Thr202/Tyr204), Cell Signaling #9101; ERK, Cell Signaling #9102; tubulin β-III, Epitomics #2276; GFAP, Sigma #G3893 and Epitomics #2301; CD11b, Serotec #MAB377; GAPDH, Santa Cruz sc-25778 (FL-335); TBP, Santa Cruz #sc-204; and β-actin, Cell Signaling # 4970. Secondary antibodies used were: anti-Rb IgG-HRP, Promega #W401; anti-Rb IgG-IRDye 800CW, Licor #926-32211; anti-Ms IgG-HRP, Santa Cruz sc-2031; anti-Rb-IgG-AF-488, Invitrogen #A11008; anti-Rb-IgG-AF-555, Invitrogen #A21428; anti-Rat-IgG-AF-555, Invitrogen #A21434; anti-Mouse-IgG-AF-555, Invitrogen #A21422; and anti-Mouse-IgG-AF-488, Invitrogen #A11001.

Cell Extractions/Western blot

Cytosolic and nuclear extracts of cells from CxN, BRN, or LVR were prepared using the NE-PER kit (Pierce) according to the manufacturer's instructions. All buffers used in the cell preparations were supplemented with both a protease inhibitor cocktail (HALT, Pierce) and phosphatase inhibitor cocktail 1 (Sigma) at 1X concentration.

Western Blots were performed as previously described (Herkenham et al., 2011) or with the LI-COR Odyssey Imaging system (LI-COR Biosciences). Briefly, protein samples were loaded in equal amounts into individual wells of a 10% polyacrylamide-SDS gel and resolved using MOPS-SDS buffer. Proteins were transferred to a PVDF membrane (Millipore-Immobilon-P), and nonspecific sites on the membrane were blocked with either 5% dried milk in Tris-Buffered Saline containing 0.05% Tween-20 (TBST buffer) for X-ray film or with Odyssey blocking buffer (LI-COR) for Odyssey imaging. The membranes were incubated overnight at 4°C with optimized dilutions of primary antibody. After the overnight incubation, membranes were washed with TBST buffer and re-incubated with a goat anti-rabbit IgG-HRP secondary antiserum (Promega or LI-COR) diluted in either 5% dried milk-TBST or LI-COR blocking buffer at room temperature. The membranes were

washed with TBST and either reacted with the West Duro Chemiluminescent substrate (Pierce) and exposed to X-ray film or directly visualized on the Odyssey Imaging System. Approximate molecular weights of the target proteins were determined by comparison to a BenchMark prestained protein ladder (Invitrogen).

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts, prepared as mentioned previously, were incubated in EMSA binding buffer (10 mM Tris, pH 7.4, 20 mM KCL, 1 mM $MgCl_2$, 0.5 mM EDTA, 2.5% glycerol, 20 μg BSA, 0.5% NP40, 1 μg Poly dI:dC) for 30 min at room temperature in the presence of the double-stranded canonical (Zabel et al., 1991) κB oligonucleotide 5'-AGTTGAG**GGGACTTTCC**CAGGC-3' (Promega #E329A) end-labeled with ^{32}P. DNA-protein complexes were resolved on an 8% non-denaturing polyacrylamide gel in Tris-borate buffer. The gel was then dried and the DNA-protein complexes visualized by autoradiography. Pre-incubation of the extracts for 20 min at room temperature with unlabeled κB oligonucleotide, p50 antibody, or p65 antibody prior to the addition of the ^{32}P-labeled oligonucleotide determined the specificity of the binding reaction and the components of the DNA-protein complex. Gels and ^{14}C plastic standards containing known amounts of radioactivity (American Radiochemicals, St. Louis, MO) were placed in x-ray cassettes, apposed to film (BioMax MS, Kodak, Rochester, NY) for periods ranging from 1 to 20 days, and developed in an automatic processor (X-OMAT, Kodak). For quantification, autoradiographic images were digitized with a solid-state camera (CCD-72, Dage-MTI) and analyzed with Image J software (http://rsb.info.nih.gov/ij/). Transmittance measurements were converted to DPM/mg plastic using the calibration curve (Rodbard equation) generated from the standards.

Isolation of RNA/quantitative real-time PCR (qPCR)

Total RNA from the cultured cells was prepared by an initial extraction using the Trizol Reagent (Life Technologies, Grand Island, NY), then further purified using the Qiagen RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The total RNA was quantified, and 1 μg was converted into cDNA using Superscript II reverse transcriptase and Oligo dT primers (Life Technologies). Real-time PCR was performed using 2x SYBR Green Master Mix (Bio-Rad, Hercules, CA) and a MyIQ iCycler (Bio-Rad) with a three-step cycling program. Initial denaturation was for 3 min at 95° C, then the reaction was cycled 40 times using the following steps: 95°C for 15 sec, 58° C for 30 sec, and 72° C for 30 sec. The amplicons of each primer set for any target gene were validated by sequencing. Primer sequences are shown in Table 1. Levels of gene expression were calculated using the standard curve method by which the actual amount of PCR gene product generated was determined by comparison of the Ct values of a transcript in the sample to a standard curve of Ct values generated from serial dilutions of a known amount of that same gene product. A “housekeeping gene” in the same samples, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), analyzed in the same manor served to normalize differences in cDNA preparation between samples. Gene “dosage” values were presented as fg sample transcript/pg GAPDH.

kB5 NF-κB reporter mouse cells

Cortical neurons and mixed brain cells were prepared as above from transgenic mouse embryos whose transgene contained three copies of the canonical κB binding sequence from the immunoglobulin κ (Igκ) enhancer placed upstream of the β-globin reporter gene (Lernbecher et al., 1993). Total RNA and cDNA from the cultured cells was prepared as described above. Quantitative gene amplification of the kB5 transgene was performed using a custom TaqMan gene expression assay (Applied Biosystems, Foster City, CA) on a MyIQ

iCycler under the following cycling conditions, 50° C for 2 min, 95° C for 10 min, and finally, 40 cycles of 95° C for 15 sec then 60° C for 1 min. Primers for amplification and quantification of the kB5 transgene were: forward, (5')-AGCTGCATGTGGATCCTGAGA-(3'), reverse, (5')-GATAGGCAGCCTGCACTGGT-(3') and probe, (5')-(FAM)-CTGGTCTGTGTGCTGGCCCATCACT-(3'). Transgene amplicon size was 110 base pairs. Variation in sample preparation was corrected by quantification of GAPDH gene expression levels in the same sample using the primer set: forward (5')-CAAAATGGTGAAGGTCGGTGTG-(3'), reverse (5')-TGATGTTAGTGGGGTCTGGCTC-(3'). The transgene amplicon size was 110 bp. Changes in the levels of gene expression were quantified by comparing the Ct values obtained from any given sample against a standard curve of the target gene generated by measuring known amounts of the amplified inset under the exact same amplification conditions for that particular gene target.

Immunocytochemistry

Neurons or mixed cells grown on coverslips in 12-well plates were fixed in 4% PBS-paraformaldehyde for 30 min at room temperature, washed with PBS and then permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 15 min. The cells were blocked with PBS-0.1% Triton containing 5% Normal Goat Serum (NGS) for one h and incubated overnight at 4°C with anti-p65 antibody at a 1:1000 dilution in PBS-0.1% Triton-5% NGS. Cell types were identified by the co-incubation of the following antibodies with the p65 antiserum. Rabbit anti-βIII-tubulin at a 1:1000 dilution was used to mark neurons, mouse anti-GFAP at a 1:500 dilution was used to mark astrocytes and rat anti-CD11b at a 1:500 dilution was used to mark microglia. After washing in PBS-0.1% Triton, the cells were incubated for two h at room temperature with appropriate AlexaFlour labeled secondary antibodies, all used at a 1:1000 dilution, to mark the various cell types. Finally, after washing in PBS, the cells were incubated with DAPI for 5 min to mark nuclei. Coverslips were inverted and affixed to slides with ProLong Gold antifade reagent mounting buffer (Life Technologies). Photomicrographs of the cells were generated on a confocal microscope (LSM 510; Carl Zeiss).

Enzyme-linked immunosorbent assay (ELISA)

Neurons were plated at a density of 0.40×10^6 cells/well in 12-well poly-d-lysine coated plates. At day 10 (DIV10), the cells were stimulated for 24 h with various treatments in a constant volume of fresh NB/B27 media across all conditions. The media were then analyzed using ELISA kits (Peprotech) for CCL2, CXCL1, and CXCL10. Total cellular protein present in each well was determined by the Bradford method after removal of media and used to correct for any differences in cellular plating density from well to well. Between-assay percent coefficient of variance across multiple dose levels was 13.4% for CCL2, 11.7% for CXCL1, and 15.6 % for CXCL10.

Statistics

Data are shown as means ± SEM of at least three independent experiments. Student's *t*-test and one-way or two-way ANOVA was performed using JMP statistical software to determine statistical significance with the level of significance set at $P < 0.05$.

RESULTS

In neurons, p65 is present in low amounts in the cytoplasm and nucleus, and there is minimal constitutive NF-κB activity

We first prepared neocortical neuronal (CxN) cultures that had no measurable glial contamination. The cultures were examined for non-neuronal impurities by microscopic examination of glial cell types and by analysis of presence of the astrocyte marker GFAP by Western blot. Immunofluorescence staining with the neuronal marker βIII-tubulin showed that virtually all cells were stained, whereas CxN cultures treated with GFAP or Iba1 (a microglial marker) antibodies showed no staining (data not shown). Western blots showed strong GFAP bands in BRN but not CxN cultures (data not shown).

The previously validated p65 antibody sc-372 (Herkenham et al., 2011) was used in Western blots to quantify cytoplasmic and nuclear levels of p65 in CxN, BRN, and LVR. All three cell types displayed measurable p65 levels in both compartments, and in all cell types, the cytoplasmic compartment contained about four-fold higher levels than the nuclear compartment. The basal levels in both cytosolic and nuclear compartments were greatest in LVR, somewhat lower in BRN, and significantly lower in CxN (respectively, cytosolic: 2.58, 2.02, 1.15, nuclear: 0.62, 0.56, 0.24; P = N.S for LVR vs. BRN, and $p < 0.01$ for cytosolic LVR vs. CxN, cytosolic BRN vs. CxN, nuclear LVR vs. CxN, and nuclear BRN vs. CxN) (Fig. 1a, b). Measures of constitutive NF-κB activity in CxN showed that it was virtually undetectable by EMSA (Fig. 1c) and by kB5 reporting (Fig. 1d). Basal reporting levels in CxN were about 50-fold lower than basal levels in BRN.

The Rel family members were assayed for basal levels of protein by the Western blot/LI-COR Odyssey system and gene expression by qPCR (Table 2). In unstimulated CxN and BRN cytosolic proteins, p65, p50, and IκBα could be detected, whereas levels of RelB, c-Rel, p52, and IKK were too low and/or the antibodies used were not sufficiently sensitive to detect signal. For the proteins that could be detected, all were present at lower levels in CxN relative to BRN. Determination of basal gene expression levels in both CxN and BRN cells, converted to dose values, showed that 1) the transcript expression levels closely matched the respective protein levels, 2) in both CxN and BRN, the prototypical pathway members p65, p50, and IκBα were approximately 10-fold more abundant than the alternative or non-canonical pathway members c-Rel, relB, and p52, and 3) all NF-κB family genes except for IKK2 and c-Rel were expressed at approximately two-fold higher levels in BRN than CxN (Table 2).

In CxN, neurons expressed the type 1A (TNFR1; p55) TNF receptor at levels about 12% of those measured in BRN. Neuronal expression of the type 1B (TNFR2; p75) was barely detectable (Table 2). Presence of TNFR1 on neurons has been demonstrated in other studies by immunohistochemistry (Cheng et al., 1994), in situ hybridization histochemistry (Bette et al., 2003), and single-cell RT-PCR (Neumann et al., 2002).

TNFα activates NF-κB in neurons

In order to characterize neuronal NF-κB signaling, we first searched for an effective stimulus. We tested numerous candidate stimuli (see below), and TNFα proved to be the strongest and most reliable activator, though IL-1β was about as effective in several assays. Figures 2 and 3 and Table 3 show the consequence of activation of NF-κB in CxN by TNFα relative to activation in BRN and LVR by the same stimulus. TNFα dose-response studies revealed that the largest response was seen at the highest dose used, 100 ng/ml. TNFR1 are known to be coupled to NF-κB by canonical intracellular pathways leading to phosphorylation of IκBα by the IKK complex. In BRN and LVR, TNFα stimulation produced rapid and brief phosphorylation of IκBα, peaking at 5 min and returning to

baseline by 15 min ($p < 0.01$). In CxN, IκBα phosphorylation was much less strong but nevertheless detectable at 5 min ($p < 0.05$) (Fig. 2b). Levels of phospho-IκBα induction at 5 and 60 min in neurons were significantly less than in BRN and LVR ($p < 0.01$).

The TNFα-induced loss of IκBα in the cytoplasm occurred rapidly in a time-dependent fashion (Fig. 2c). In CxN, no change was seen at 15 min, and a 20% nonsignificant reduction of IκBα levels occurred at 30 min, with a return to baseline at 60 min of stimulation. Selected CxN protein samples ($n = 19$) were re-run using LI-COR technology, and at 30 min, IκBα levels were significantly reduced 35% compared to control ($p < 0.0001$). In BRN and LVR, a 90% reduction in levels occurred at 15 min and 30 min ($p < 0.001$), with a return towards baseline at 60 min.

The TNFα-induced appearance of p65 in the nucleus followed a similar time course for all cell types, but the increases at 15 and 30 min were much greater in BRN and LVR ($p < 0.01$) than in CxN cells ($p < 0.05$) (Fig. 2d). LI-COR analysis of CxN protein samples ($n = 20$) at 30 min showed a significant ~2-fold increase in nuclear levels ($p < 0.001$). In BRN samples ($N = 6$), the increase was ~12-fold ($p < 0.001$). Attempts to measure TNFα-induced movement of the alternative pathway proteins c-Rel, RelB, and p52 were unsuccessful due to low abundance and poor antibody detection.

IκBα degradation is accompanied by its resynthesis, so measures of IκBα mRNA are a reliable reflection of NF-κB activity. IκBα and the deubiquitinase A20 (TNFAIP3), a major component of the TNFα signaling pathway (Harhaj and Dixit, 2011), are genes that both regulate and are regulated by NF-κB. These genes were induced by TNFα in both CxN and BRN (Table 3), though the basal levels and degree of induction for both genes were lower in CxN than BRN. TNFα increased IκBα mRNA expression 2.6-fold to 143 fg/pg in CxN and 7.6-fold to 791 fg/pg in BRN, and it increased A20 mRNA 8.1-fold to 3.5 fg/pg in CxN and 31-fold to 117 fg/pg in BRN. The inductions were blocked by the IKK inhibitor TPCA (Podolin et al., 2005), indicating that they occur via NF-κB activation. The diminished neuronal response of both of the inhibitory genes IκBα and A20 rules out their overactivity as a candidate mechanism for recalcitrant neuronal NF-κB activity.

Cells grown on coverslips in the BRN culture were immunostained for p65 and co-stained with antibodies selective for neurons, astrocytes, or microglia. The morphology and immunostaining of BRN cells indicated that neurons, astrocytes, microglia, oligodendrocytes, and stromal cells were present in the culture. Microscopy showed varying relative amounts of p65 in the cytoplasm and nucleus of the various cell types in unstimulated cultures (Fig. 2e), generally in the same relative proportions revealed in Western blots. In TNFα-stimulated BRN cultures, nuclear translocation of p65 was not evident in neurons, whereas strong nuclear staining was seen in the cells not immunostained by the neuron-specific βIII-tubulin antibody (Fig. 2f). Many of the cells were determined to be astrocytes by GFAP counterstaining (not shown) or fibroblast-like cells based on their large nuclear size and overall morphology (Fig. 2f). Microglia showed a dramatic response to TNFα such that the p65 staining was almost completely confined to the nucleus of these cells, and the movement into the nucleus was completely blocked by TPCA pretreatment (Fig. 2g).

NF-κB activation was assessed by EMSA and kB5 reporter assays. In the EMSA blots, LVR extracts showed a strong shifted complex in response to TNFα stimulation (Fig. 3a). The complex was composed of p50:p65 heterodimers as indicated by the bands supershifted with p65 or p50 antibodies. A complex was also evident in BRN extracts at 15, 30, and 60 min, and continuous stimulation for 24 h led to a waning of the response (data not shown). TNFα at the optimal time point of 30 min produced a clear complex in BRN (Fig. 3b) that was

supershifted almost completely by p50 and p65 antibodies (data not shown) and attenuated by TPCA. In CxN extracts, a similar but weaker complex was formed after 30 min of TNFα, and the complex was supershifted by p65 and p50 antibodies (Fig. 3c).

The EMSA images inaccurately depict the differences in signal strength between CxN, BRN, and LVR because different exposure times were used and different amounts of nuclear protein loaded in the assays (see Fig. 3 legend). Quantitative densitometry performed on the bands showed that induction in CxN was several orders of magnitude lower than in BRN. The difference was dramatically shown in a single blot in which equal amounts of protein were loaded and co-exposed (Fig. 3d). The density measured up each lane is shown in Fig. 3e. Strikingly, stimulated activity in CxN did not reach basal activity levels in BRN.

The kB5 reporter assay (Fig. 3f) showed that 3 h of TNFα stimulation increased reporting in CxN 43-fold (from 1.36 to 54.5 fg/pg GAPDH) and 270-fold (from 54.9 to 14825 fg/pg GAPDH) in BRN. Thus, the level of TNFα-stimulated kB5 reporting in CxN only reached the same magnitude as the level of basal reporting in BRN.

Chemokines are strongly induced in neurons by TNFα via NF-κB activation

A very sensitive method of measuring TNFα-induced NF-κB activation in neurons was by real-time qPCR analysis of induction of gene expression. The neuronal source of the expression was assured by the fact that CxN cultures were unresponsive to LPS (see below), whereas non-neuronal cells showed a strong response to LPS. Validation that the induction was NF-κB-mediated was by addition of the selective IKK inhibitor TPCA (Podolin et al., 2005) (see Figs. 2g and 3b), which in pilot studies was more efficacious and consistent than PDTC or SC-514 and gave similar results as the IκBα phosphorylation inhibitor BAY 11-7082 (Pierce et al., 1997) (data not shown). Candidate responsive genes were selected on the basis of two criteria; 1) consensus κB DNA binding site(s) in the promoter/enhancer and 2) evidence of expression in neurons. Known neuronally expressed genes with upstream κB sites were identified by a number of means: 1) systematic search of conserved (in mouse, rat, and human) sequences in the range −10 kb to +100 bp for each transcription start site in the 2004 assembly of the mouse genome, accessed through the Genome Alignment and Annotation Database (GALA) and the UCSC Mouse genome browser, 2) search of a public database http://bioinfo.lifl.fr/NF-KB/, 3) directed BLAST analysis using canonical and consensus κB sequences to find matches, 4) and search of the literature for experimentally proven NF-κB binding sites. All basal and induced levels of gene expression were converted to “dose” levels by comparison with standard curves generated from log dose of amplicon vs. Ct response using GAPDH as a reference. The data for basal, TNFα-stimulated, and blocking experiments (addition of TPCA) to show mediation by NF-κB are provided in Table 3. Basal and TNFα-activated levels were compared in CxN and BRN. There was a large range of basal expression levels for the different categories of genes, which might be expected from the kinds of functions these genes normally subserve. For all gene transcripts except for CX3CL1, Cox2, Bcl-xL, and SOD2, basal expression levels in BRN were significantly greater than basal expression levels in CxN ($p < 0.05$ to $p < 0.0001$). For most but not all transcripts, TNFα significantly increased expression over baseline for BRN and CxN ($p < 0.05$ to $p < 0.0001$). In both cell types, TPCA completely blocked the inductions, and TPCA had no effect on its own. The genes with the largest mRNA induction were the three chemokines CCL2 (MCP-1), CXCL1 (Groα), and CXCL10 (IP-10). The size of the induced expression was much larger in BRN than in CxN, but because of the different basal values, the fold-levels of increases (all > 100-fold) were somewhat comparable between BRN and CxN. LCN2 was also strongly induced in both CxN (56-fold) and BRN (40-fold), though BRN stimulated levels were about 10-fold higher than CxN levels. Other tested genes were only modestly induced by TNFα in CxN, including IκBα, A20, IL-6, and

TIMP1. These transcripts were also induced in BRN. For example, IκBα was induced 2-fold in CxN and 7.6-fold in BRN, and A20 was induced 8-fold in CxN and 31-fold in BRN. Several genes showed no significant induction in CxN, including p65, CX3CL1, IGF2, Cox2, Bcl-xL, and SOD2. Slight induction of CX3CL1 and Cox2 but not p65, IGF2, Bcl-xL, and SOD2 was detected in BRN (Table 3).

Limited analysis by ELISA of proteins released into CxN culture media showed parallel increases for the chemokines CCL2 (unstim: 0.64 ± 0.16; TNFα stim: 20.64 ± 4.3 pg/ μg protein), CXCL1 (unstim: 0.10 ± 0.01; TNFα stim: 0.48 ± 0.14 pg/μg protein), and CXCL10 (unstim: 0.13 ± 0.04; TNFα stim: 3.25 ± 0.84 pg/μg protein), and these increases were completely blocked by TPCA (data not shown).

Glutamate is a very weak stimulus for NF-κB activation in neurons

Much of the literature on neuronal NF-κB indicates that glutamate activates NF-κB in neurons. We sought to demonstrate the relative degree of activation. To replicate methods used in published papers (Meffert et al., 2003, Mikenberg et al., 2007) and to avoid excitotoxic effects, glutamate was applied as a pulse for 10 min followed by washout and harvest 30–90 min (Western blot, immunofluorescence, EMSA) or 3 h later (qPCR). The analysis compared glutamate with TNFα administered continuously for comparable durations. For additional comparisons, responses in neurons and non-neuronal cells and responses to LPS in microglia were measured. In neurons, Western blots of p65 nuclear increases (Fig. 4a, b) were negative in all tests of glutamate stimulation. In some published studies (Meffert et al., 2003, Mikenberg et al., 2007), a positive response to glutamate occurred after pretreating the neuronal cultures with the synaptic activity blockers AP5, CNQX, and nimodipine prior to glutamate stimulation. We tested many combinations of dose and duration of the inhibitors, but they had no effect on either basal or stimulated activity (Fig. 4a). There was no change in IκBα levels and no detectable production of phospho-IκBα in the cytoplasmic fraction (data not shown). High doses and long durations post glutamate stimulation did not generate p65 movement into the neuronal nucleus (Fig. 4b). In contrast, glutamate strongly induced phospho-ERK and phospho-CREB (Fig. 4c, d), serving as positive control data assuring that glutamate was administered in the proper manner. The reduction in phospho-CREB at 1 h of glutamate simulation (Fig 4d) is consistent with published reports showing this inhibitory effect (Kopnisky et al., 2003).

The negative Western blot findings for p65 were consistent with the immunofluorescence data, which showed that glutamate produced no change in the appearance of p65 in the cytoplasmic processes or the nucleus of neurons (Fig. 4e), even using dosing and pretreatment conditions almost identical to published data showing disappearance of p65 in neuronal processes following glutamate stimulation (Mikenberg et al., 2007). A similar negative result was obtained with other p65 antibodies, including the sc-109 antibody (Santa Cruz) at the same 1:100 dilution used in that study (data not shown). A striking contrast to the negative neuronal response of p65 to glutamate was the rapid and massive movement of p65 from the cytoplasm into the nucleus of microglia following LPS stimulation (Fig. 4f), validating the immunocytochemical procedures.

Glutamate had no effect on kB5 reporting in CxN or BRN (Fig. 4i), the latter result verifying earlier findings that glutamate does not activate NF-κB in astrocytes (Guerrini et al., 1995, Lukasiuk et al., 1995, Moerman et al., 1999). In contrast, LPS massively increased kB5 reporting by 400-fold in BRN but had no effect at all in CxN, which confirms the purity of the CxN cultures (Fig. 4j). Cortical neurons are unresponsive to LPS because they do not express its receptor TLR4 (Chakravarty and Herkenham, 2005).

In contrast to the negative findings in three assays, the EMSA blots showed a small glutamate dose-dependent binding shift in CxN nuclear fractions (Fig. 4g), and the supershift analysis indicated that p65 and p50 were present in the shifted complex (Fig. 4h). Quantitative PCR analysis showed selective glutamate effects in CxN. Glutamate significantly elevated gene expression of CXCL1, IκBα, and TIMP-1, and these inductions except CXCL1were blocked by TPCA pretreatment. CCL2, IL-6, and PSD-95 transcripts were not induced by glutamate (Table 4).

In BRN, cursory examination showed that glutamate had restricted effects on gene expression. IL-6 and the chemokines CCL2 and CXCL1 were modestly increased, whereas CXCL10 and IκBα were unaffected (Table 5).

IL-1β and BRN-conditioned media also activate neuronal NF-κB

IL-1β activated neuronal NF-κB. It produced a two-fold increase in nuclear p65 by Western blot, induced a number of genes measured by qPCR, and induced three chemokines measured by ELISA (Table 6). The inductions were similar to those produced by TNFα but somewhat less for most transcripts. Conditioned medium (CM) collected from BRN cultures treated for 24 h with CxN growth media produced significant increases in p65 nuclear accumulation and target gene expression over control conditions ($P < 0.05$). Addition of LPS to the media (LPS-CM) resulted in significant increases in p65 nuclear accumulation and NF-κB target gene expression relative to basal levels but did not produce significant increases relative to CM treatment (Table 6). All inductions were blocked by the addition of TPCA (data not shown). CM-induced NF-κB-mediated gene expression suggests that the normal culture conditions support activated microglia that release inflammatory cytokines. Indeed, many microglia in the unstimulated cultures have an amoeboid appearance (e.g., Fig. 4f).

Other stimuli tested failed to activate NF-κB in neurons

LPS had no effect on kB5 reporting (Fig. 4j), target gene expression, or protein levels in CxN (Table 6). H_2O_2 was administered to assess stimulation by oxidative stress pathways. There was no response by EMSA or p65 Western blot. Norepinephrine produced a very weak signal in the Western blot, but it did not significantly induce target gene expression. ATP had no effect when assayed by Western blot or qPCR of induced genes. NGF had a very small effect on p65 by Western blot, but it did not induce any NF-κB-responsive genes. BDNF had no effect by EMSA or Western blot, but it did produce a sizeable elevation in chemokine mRNA and protein levels. The effect of TPCA on this small activation was not determined, so we cannot conclude that the activation was via NF-κB. The phorbol ester PMA had no effect shown by immunofluorescence of nuclear p65 accumulation in neurons grown in CxN and BRN cultures (data not shown) or by Western blot and ELISA of CxN nuclear fractions (Table 6).

DISCUSSION

This study afforded a comprehensive depiction of NF-κB properties in cultured primary neocortical and hippocampal neurons. Constitutive and induced NF-κB activity in cortical neurons (CxN) was detectable at very low levels in contrast with higher basal levels measured in mixed CNS-resident cell types (BRN) and in liver cells (LVR). In CxN, the basal cytoplasmic and nuclear levels of the NF-κB subunit p65 were significantly lower than in BRN and LVR cells, measured by Western blot analysis. In addition, there was minimal constitutive neuronal NF-κB activity assayed by protein binding to nuclear κB DNA sequences in EMSA analysis and by κB-driven reporter activity. In fact, constitutive kB5 reporting in neurons was 50-fold lower than constitutive reporting in mixed brain cells. NF-

κB was most effectively activated in neurons by the pro-inflammatory cytokines TNFα and IL-1β. However, the magnitude of response to TNFα in neurons was about 270-fold lower than in mixed brain cells measured in the kB5 reporter assay and about 100-fold lower in the EMSA analysis. In fact, induced activation in neurons only reached a level equivalent to constitutive activity levels in mixed brain cells. Glutamate was almost completely ineffective as a neuronal NF-κB activator. LPS, H_2O_2, NGF, BDNF, norepinephrine, ATP, and PMA were also ineffective, with isolated exceptions. The paucity of neuronal activation was seen at the earliest point of the NF-κB signaling pathway measured, i.e., at the stage of IκBα phosphorylation, though the limiting mechanism could be upstream of that, beginning with relatively low levels of receptors signaling to the IKK complex.

Neurons in culture lack appreciable constitutive NF-κB activity

Measures of basal levels of Rel proteins and Rel gene expression showed that the canonical members of the family were much more abundant than the alternative or non-canonical members in both CxN and BRN, and all members were much lower in neurons than mixed cells. The alternative Rel proteins were in such low abundance that we were unable to measure a response to TNFα stimulation (data not shown).

Although small amounts of p65 could be detected in the nucleus of unstimulated cells, evidence for basal NF-κB activity in neurons was lacking in the EMSA and κB reporter assays. This striking result can be contrasted with variable data in the literature based on EMSA analysis of basal activity in nuclear extracts of brain tissues and neuronal cells in control conditions. In brain tissues under basal conditions, the appearance of shifted bands ranges from strong bands to no bands at all. Of 36 published papers that we surveyed showing EMSAs performed with κB oligonucleotides that bind nuclear protein extracts from rat or mouse brain, 10 showed moderate-to-strong bands and 26 showed nonexistent-to-weak bands under basal conditions. In published studies of primary neuronal cell culture, 5 showed moderate-to-strong and 12 showed nonexistent-to-weak constitutive activity by EMSA. It is difficult to explain these different outcomes on the basis of biological differences. One explanation is that the levels are so low that it is difficult to distinguish signal from noise with EMSA analysis. We found that detecting a band in neurons required longer film exposure times and greater protein load amounts, and when these parameters were equated in neurons and mixed cells, and the values converted to standardized amounts, the differences in basal (and induced) activity between CxN and BRN were different by two orders of magnitude (Fig. 3d, e).

The large difference in activity found by EMSA analysis suggests that published data showing bands of nuclear fractions of dissected brain tissue overwhelmingly reflect activity levels present in the non-neuronal cells. However, a few reports suggest that a large portion of basal NF-κB activity in brain tissues is neuronal (Freudenthal et al., 2005, Schmeisser et al., 2012). Because these reports are based on assays of hippocampal extracts, the possibility remains that hippocampal neurons *in vivo* express a unique property of constitutive activity. We did not see that activity in cultured hippocampal neurons, so if true, the activity requires an intact brain environment.

Proinflammatory cytokines activate neuronal NF-κB

In immune cells, stimuli for strong NF-κB activation include cytokines acting through cytokine receptors, physical stressors, immunoglobulins, virally triggered intracellular messengers, and pathogen-associated molecular patterns (PAMPs) acting though toll-like receptors (TLRs). Neurons express low levels of receptors for these classes of stimuli, and the downstream intracellular pathways that transduce these signals are not strongly activated in neurons. Therefore, a critical question is, what is an adequate stimulus for neuronal NF-

κB activation? Based on the extensive literature on neuronal NF-κB activation, we tested the most-studied candidates. Supported by published data (Tamatani et al., 1999, Albensi and Mattson, 2000, Marchetti et al., 2004, Manuvakhova et al., 2011, Shim et al., 2011), the strongest and most consistent activator of NF-κB in neurons was TNFα. We employed multiple assays to demonstrate the response dynamics, and we compared the magnitude of the neuronal response to that in mixed brain cells. We found that qualitative aspects of the kinetics of response were similar between neurons and mixed cells, but the magnitude of TNFα-activated expression was about 300-fold lower in neurons. Neurons have about eight-fold lower TNF receptor levels by our measures, accounting in part for the reduced NF-κB response in neurons relative to mixed cells.

Glutamate minimally activates NF-κB

The most provocative and unique role assigned for NF-κB activation in neurons is in response to glutamate stimulation and to synaptic activity. Glutamate and its analogs were shown in early studies to activate neuronal NF-κB measured by EMSA in primary cerebellar granule cells (Guerrini et al., 1995, Kaltschmidt et al., 1995, Grilli et al., 1996). Similar results were shown in cortical neurons (Kaltschmidt et al., 1995, Pizzi et al., 2005, Mikenberg et al., 2007), assigning to NF-κB important normal neurophysiological functions that are unrelated to the immune-related functions usually associated with this transcription factor. In neuronal cultures from neocortex or hippocampus, we found that glutamate barely stimulated NF-κB activity when assayed by EMSA and targeted gene expression, and it did not induce any kB5 reporter activity in either CxN or BRN. Thus, neurons were unresponsive to glutamate either when simulated in isolation (CxN) or in mixed neuron-glia cultures (BRN). Likewise, other groups have shown no glutamate effect on NF-κB activity in primary cortical neurons (Mao et al., 1999, Marchetti et al., 2004, Mao et al., 2009).

In some studies of primary cortical neurons, the means to demonstrate activation by glutamate required silencing of purported constitutive activity by pharmacological pretreatments (Meffert et al., 2003, Mikenberg et al., 2007). We performed several kinds of pretreatments including pretreatment with the Ca^{++} chelator EGTA and inhibitors of synaptic activity (AP5 + CNQX + nimodipine). We showed by p65 Western blot, p65 immunofluorescence, and EMSA that pretreatments did not bring out detectable glutamate effects. This result is not surprising in light of our finding of very low levels of NF-κB activity in resting states.

Some studies have shown glutamate agonist-induced p65 movement in dendrites by employing sensitive markers, i.e., a p65-GFP fusion protein that serves as a proxy for p65 movement (Wellmann et al., 2001, Meffert et al., 2003). We found cytoplasmic and dendritic presence of p65, but we were unable to show movement into the nucleus by traditional p65 Western blot or immunofluorescence, nor could we show p65 disappearance from dendrites by immunofluorescence even when using inhibitor and glutamate treatment and microscopic visualization conditions almost identical to those used in one publication to show dendritic p65 disappearance (Mikenberg et al., 2007). It may be the case that p65 movement in dendrites is so minimal that it can only be inferred from the movement of the surrogate p65-GFP construct. Alternatively, it is possible that p65-GFP behaves differently than authentic p65, or its presence in the cell overcomes normal inhibition by IκB proteins.

Other stimuli: Oxidative stress

Reactive oxygen species (ROS) and oxygen radicals were originally suggested to be the major source of NF-κB activation in cells (Schreck and Baeuerle, 1994), but this claim was later refuted (Hayakawa et al., 2003). A recent study (Riquelme et al., 2011) showed that

H_2O_2 stimulated NF-κB activity about two-fold in primary neurons, which we and others (Bowie and O'Neill, 2000, Oliveira-Marques et al., 2009) could not show.

Other stimuli: Neurotransmitters and neurotrophic factors

NE, which activates NF-κB in PC12 cells (Minneman et al., 2000), had no effect in primary neurons. NGF did not activate NF-κB in our assays. Reports of NF-κB activation by NGF have been in cell lines (Bui et al., 2001), mixed cell populations (Wood, 1995), or glia (Carter et al., 1996), but not in primary neuronal cells. For BDNF as a stimulus, we found only an NF-κB-mediated induction of chemokine mRNA expression, which may have functional significance in neuronal development (Gavalda et al., 2009, Gutierrez and Davies, 2011).

Other stimuli: Phorbol ester

The phorbol ester PMA, a diacylglycerol mimetic that triggers protein kinase C (PKC) signaling, has been reported to activate NF-κB in several cell lines (Nelsen et al., 1988, Holden et al., 2008). We found no evidence of NF-κB activation by PMA in primary cortical neurons.

The neuronal NF-κB response triggers induction of chemokines, lipocalin 2 (LCN2), IL-6, and TIMP-1, but not Cox2, IGF2, or PSD-95

When the NF-κB pathway is activated by TNFα in neurons, chemokines represent the major class of responsive genes, similar to that seen in cell lines (Tian et al., 2005). Induced chemokine gene expression was large in neurons, almost as great as in mixed cells when measured as fold-induction. CCL2, CXCL1, and CXCL10 are known to attract circulating leukocytes into the brain (Wilson et al., 2010). In addition, CCL2 acting through the CCR2 receptor has been proposed to be a neuromodulator (Rostene et al., 2007). Neuronal CXCL10 acting through the CXCR3 receptor is thought to affect microglial function (Rappert et al., 2004). CCL2 and CXCL10 have been associated with motility of newborn neurons (Liu et al., 2007) and neuronal longevity in aging (Villeda et al., 2011). CXCL1 has been shown to inhibit proliferation of neural progenitor cells (Choi et al., 2008) and promote proliferation of oligodendrocyte precursors (Robinson et al., 1998).

The failure of induction of CX3CL1 (fractalkine) expression in neurons was surprising because this is a chemokine produced by neurons and thought to hold CX3CR1 receptor-bearing microglia in a quiescent state (Cardona et al., 2006). Unlike the other chemokines examined, CX3CL1 showed a high level of constitutive expression, supporting an important role in neuronal function. Further work is need to determine how it is regulated in neurons.

LCN2 mRNA was upregulated in an NF-κB-dependent fashion by TNFα in neurons, in agreement with a recent publication (Naude et al., 2012). LCN2 has been reported to be a chemokine inducer in the CNS, notably of CXCL10 (Lee et al., 2011). Thought to be expressed mainly in glia, it has recently been shown to exist in neurons and influence dendritic spine shape changes associated with psychological stress (Mucha et al., 2011).

IL-6 mRNA was induced in CxN neurons by TNFα and by BRN-conditioned media, supporting findings of neuronal IL-6 production by TNFα and IL-1β (Ringheim et al., 1995). IL-6 has been reported to play a role in psychological stress-induced changes in behavior through an NF-κB signaling pathway (Monje et al., 2011), but it has not been shown that neuronal IL-6 is a key player, keeping in mind the facts that non-neuronal cells express IL-6 at higher levels than neurons and that we have no evidence that IL-6 is activated in neurons by stimuli other than TNFα and conditioned media.

TIMP-1 mRNA was induced in CxN by a number of stimuli, notably glutamate. Its role in neurite outgrowth has been emphasized (Ould-yahoui et al., 2009), further implicating NF-κB as an important player in neuronal development, especially dendritic modeling.

We found no induction of Cox2 mRNA by TNFα in neurons. An earlier study showed activation of the Cox2 promoter via κB interactions and induction of an NF-κB reporter construct by TNFα in HeLa and neuroblastoma cells and concluded but did not directly demonstrate that Cox2 is a neuronal target gene of NF-κB (Kaltschmidt et al., 2002).

We also failed to find mRNA induction of PSD-95 by glutamate and IGF2, two genes that have been reported to be regulated in neurons via NF-κB activation and to serve important functional roles related to dendritic spine morphology (Boersma et al., 2011) (Schmeisser et al., 2012). More characterization is needed to resolve these discrepancies.

In summary, cortical neurons compared to mixed brain and liver cells showed qualitatively similar though quantitatively diminished NF-κB activation and NF-κB–mediated transcriptional regulation. The activation in neurons was mediated predominantly by the binding of p50 and p65 subunits to the κB DNA recognition sequence. Pro-inflammatory cytokines TNFα and IL-1β were the most effective stimuli of those we examined, whereas other agents, notably glutamate, in several diverse biological categories did not appreciably activate neuronal NF-κB. TNFα-induced NF-κB activation in neurons was greater than two orders of magnitude lower than in non-neuronal cell types. The genes most strongly induced via NF-κB in neurons code for secreted immune molecules that could affect intercellular communication, cell migration, proliferation, and remodeling in the CNS. The limited response in neurons may be due in part to low levels of TNFR and other receptors that target the IKK complex, reduced kinase activity and efficacy, post-translational modifications of NF-κB subunits, and epigenetic inhibitory factors that serve to block adverse consequences of the signaling pathway. The minimal activity of neuronal NF-κB suggests that the functional role of κB DNA binding in neurons is minor, which may in part explain the reported apparent contradictory effects of NF-κB in neuronal cell death or survival (Mincheva-Tasheva and Soler, 2013).

Acknowledgments

The work was supported by the Intramural Research Program, NIMH, NIH. We thank Thomas Wirth, Ulm, Germany, for the gift of kB5 reporter mice and Laura Elnitski, NHGRI, NIH, for help with GALA database search.

References

Aarum J, Sandberg K, Haeberlein SL, Persson MA. Migration and differentiation of neural precursor cells can be directed by microglia. Proc Natl Acad Sci USA. 2003; 100:15983–15988. [PubMed: 14668448]

Albensi BC, Mattson MP. Evidence for the involvement of TNF and NF-κB in hippocampal synaptic plasticity. Synapse. 2000; 35:151–159. [PubMed: 10611641]

Bette M, Kaut O, Schafer MK, Weihe E. Constitutive expression of p55TNFR mRNA and mitogen-specific up-regulation of TNFα and p75TNFR mRNA in mouse brain. J Comp Neurol. 2003; 465:417–430. [PubMed: 12966565]

Bhakar AL, Tannis LL, Zeindler C, Russo MP, Jobin C, Park DS, MacPherson S, Barker PA. Constitutive nuclear factor-κB activity is required for central neuron survival. J Neurosci. 2002; 22:8466–8475. [PubMed: 12351721]

Boccia M, Freudenthal R, Blake M, de la Fuente V, Acosta G, Baratti C, Romano A. Activation of hippocampal nuclear factor-κB by retrieval is required for memory reconsolidation. J Neurosci. 2007; 27:13436–13445. [PubMed: 18057202]

Boersma MC, Dresselhaus EC, De Biase LM, Mihalas AB, Bergles DE, Meffert MK. A requirement for nuclear factor-κB in developmental and plasticity-associated synaptogenesis. J Neurosci. 2011; 31:5414–5425. [PubMed: 21471377]

Bowie A, O'Neill LA. Oxidative stress and nuclear factor-κB activation: a reassessment of the evidence in the light of recent discoveries. Biochem Pharmacol. 2000; 59:13–23. [PubMed: 10605930]

Bui NT, Livolsi A, Peyron JF, Prehn JH. Activation of nuclear factor κB and Bcl-x survival gene expression by nerve growth factor requires tyrosine phosphorylation of IκBα. J Cell Biol. 2001; 152:753–764. [PubMed: 11266466]

Cardona AE, Pioro EP, Sasse ME, Kostenko V, Cardona SM, Dijkstra IM, Huang D, Kidd G, Dombrowski S, Dutta R, Lee JC, Cook DN, Jung S, Lira SA, Littman DR, Ransohoff RM. Control of microglial neurotoxicity by the fractalkine receptor. Nat Neurosci. 2006; 9:917–924. [PubMed: 16732273]

Carlsen H, Moskaug JO, Fromm SH, Blomhoff R. In vivo imaging of NF-κB activity. J Immunol. 2002; 168:1441–1446. [PubMed: 11801687]

Carter BD, Kaltschmidt C, Kaltschmidt B, Offenhauser N, Bohm-Matthaei R, Baeuerle PA, Barde YA. Selective activation of NF-κB by nerve growth factor through the neurotrophin receptor p75. Science. 1996; 272:542–545. [PubMed: 8614802]

Chakravarty S, Herkenham M. Toll-like receptor 4 on nonhematopoietic cells sustains CNS inflammation during endotoxemia, independent of systemic cytokines. J Neurosci. 2005; 25:1788–1796. [PubMed: 15716415]

Cheng B, Christakos S, Mattson MP. Tumor necrosis factors protect neurons against metabolic-excitotoxic insults and promote maintenance of calcium homeostasis. Neuron. 1994; 12:139–153. [PubMed: 7507336]

Choi SH, Veeraraghavalu K, Lazarov O, Marler S, Ransohoff RM, Ramirez JM, Sisodia SS. Non-cell-autonomous effects of presenilin 1 variants on enrichment-mediated hippocampal progenitor cell proliferation and differentiation. Neuron. 2008; 59:568–580. [PubMed: 18760694]

Clemens JA, Stephenson DT, Smalstig EB, Dixon EP, Little SP. Global ischemia activates nuclear factor-kappa B in forebrain neurons of rats. Stroke. 1997; 28:1073–1080. discussion 1080–1071. [PubMed: 9158652]

Estin C, Vernadakis A. Primary glial cells and brain fibroblasts: interactions in culture. Brain Res Bull. 1986; 16:723–731. [PubMed: 2874876]

Freudenthal R, Boccia MM, Acosta GB, Blake MG, Merlo E, Baratti CM, Romano A. NF-κB transcription factor is required for inhibitory avoidance long-term memory in mice. Eur J Neurosci. 2005; 21:2845–2852. [PubMed: 15926932]

Fridmacher V, Kaltschmidt B, Goudeau B, Ndiaye D, Rossi FM, Pfeiffer J, Kaltschmidt C, Israel A, Memet S. Forebrain-specific neuronal inhibition of nuclear factor-κB activity leads to loss of neuroprotection. J Neurosci. 2003; 23:9403–9408. [PubMed: 14561868]

Gavalda N, Gutierrez H, Davies AM. Developmental switch in NF-κB signalling required for neurite growth. Development. 2009; 136:3405–3412. [PubMed: 19762427]

Grilli M, Goffi F, Memo M, Spano P. Interleukin-1beta and glutamate activate the NF-kappaB/Rel binding site from the regulatory region of the amyloid precursor protein gene in primary neuronal cultures. J Biol Chem. 1996; 271:15002–15007. [PubMed: 8663145]

Guerrini L, Blasi F, Denis-Donini S. Synaptic activation of NF-κB by glutamate in cerebellar granule neurons in vitro. Proc Natl Acad Sci USA. 1995; 92:9077–9081. [PubMed: 7568076]

Gutierrez H, Davies AM. Regulation of neural process growth, elaboration and structural plasticity by NF-κB. Trends Neurosci. 2011; 34:316–325. [PubMed: 21459462]

Gutierrez H, Hale VA, Dolcet X, Davies A. NF-κB signalling regulates the growth of neural processes in the developing PNS and CNS. Development. 2005; 132:1713–1726. [PubMed: 15743881]

Harhaj EW, Dixit VM. Deubiquitinases in the regulation of NF-κB signaling. Cell Res. 2011; 21:22–39. [PubMed: 21119682]

Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, Kikugawa K. Evidence that reactive oxygen species do not mediate NF-κB activation. EMBO J. 2003; 22:3356–3366. [PubMed: 12839997]

Herkenham M, Rathore P, Brown P, Listwak SJ. Cautionary notes on the use of NF-κB p65 and p50 antibodies for CNS studies. Journal of neuroinflammation. 2011; 8:141. [PubMed: 21999414]

Holden NS, Squires PE, Kaur M, Bland R, Jones CE, Newton R. Phorbol ester-stimulated NF-κB-dependent transcription: roles for isoforms of novel protein kinase C. Cell Signal. 2008; 20:1338–1348. [PubMed: 18436431]

Jarosinski KW, Whitney LW, Massa PT. Specific deficiency in nuclear factor-κB activation in neurons of the central nervous system. Lab Invest. 2001; 81:1275–1288. [PubMed: 11555675]

Kaltschmidt B, Kaltschmidt C. NF-κB in the nervous system. Cold Spring Harb Perspect Biol. 2009; 1:a001271. [PubMed: 20066105]

Kaltschmidt B, Linker RA, Deng J, Kaltschmidt C. Cyclooxygenase-2 is a neuronal target gene of NF-κB. BMC Mol Biol. 2002; 3:16. [PubMed: 12466023]

Kaltschmidt B, Ndiaye D, Korte M, Pothion S, Arbibe L, Prullage M, Pfeiffer J, Lindecke A, Staiger V, Israel A, Kaltschmidt C, Memet S. NF-κB Regulates Spatial Memory Formation and Synaptic Plasticity through Protein Kinase A/CREB Signaling. Mol Cell Biol. 2006; 26:2936–2946. [PubMed: 16581769]

Kaltschmidt C, Kaltschmidt B, Baeuerle PA. Stimulation of ionotropic glutamate receptors activates transcription factor NF-κB in primary neurons. Proc Natl Acad Sci U S A. 1995; 92:9618–9622. [PubMed: 7568184]

Kaltschmidt C, Kaltschmidt B, Neumann H, Wekerle H, Baeuerle PA. Constitutive NF-κB activity in neurons. Mol Cell Biol. 1994; 14:3981–3992. [PubMed: 8196637]

Kassed CA, Butler TL, Patton GW, Demesquita DD, Navidomskis MT, Memet S, Israel A, Pennypacker KR. Injury-induced NF-κB activation in the hippocampus: implications for neuronal survival. Faseb J. 2004; 18:723–724. [PubMed: 14766792]

Kopnisky KL, Chalecka-Franaszek E, Gonzalez-Zulueta M, Chuang DM. Chronic lithium treatment antagonizes glutamate-induced decrease of phosphorylated CREB in neurons via reducing protein phosphatase 1 and increasing MEK activities. Neuroscience. 2003; 116:425–435. [PubMed: 12559097]

Lee S, Kim JH, Seo JW, Han HS, Lee WH, Mori K, Nakao K, Barasch J, Suk K. Lipocalin-2 Is a chemokine inducer in the central nervous system: role of chemokine ligand 10 (CXCL10) in lipocalin-2-induced cell migration. J Biol Chem. 2011; 286:43855–43870. [PubMed: 22030398]

Lernbecher T, Muller U, Wirth T. Distinct NF-κB/Rel transcription factors are responsible for tissue-specific and inducible gene activation. Nature. 1993; 365:767–770. [PubMed: 7692309]

Lezoualc'h F, Sagara Y, Holsboer F, Behl C. High constitutive NF-κB activity mediates resistance to oxidative stress in neuronal cells. J Neurosci. 1998; 18:3224–3232. [PubMed: 9547231]

Liu XS, Zhang ZG, Zhang RL, Gregg SR, Wang L, Yier T, Chopp M. Chemokine ligand 2 (CCL2) induces migration and differentiation of subventricular zone cells after stroke. J Neurosci Res. 2007; 85:2120–2125. [PubMed: 17510981]

Lukasiuk K, Kaczmarek L, Condorelli DF. Inducible and constitutive transcription factor NF-κB-like DNA binding activities in rat brain cells cultured in vitro. Neurochem Int. 1995; 26:173–178. [PubMed: 7599537]

Manuvakhova MS, Johnson GG, White MC, Ananthan S, Sosa M, Maddox C, McKellip S, Rasmussen L, Wennerberg K, Hobrath JV, White EL, Maddry JA, Grimaldi M. Identification of novel small molecule activators of nuclear factor-κB with neuroprotective action via high-throughput screening. J Neurosci Res. 2011; 89:58–72. [PubMed: 21046675]

Mao X, Moerman AM, Lucas MM, Barger SW. Inhibition of the activity of a neuronal κB-binding factor by glutamate. J Neurochem. 1999; 73:1851–1858. [PubMed: 10537043]

Mao XR, Moerman-Herzog AM, Chen Y, Barger SW. Unique aspects of transcriptional regulation in neurons--nuances in NFκB and Sp1-related factors. Journal of neuroinflammation. 2009; 6:16. [PubMed: 19450264]

Marchetti L, Klein M, Schlett K, Pfizenmaier K, Eisel UL. Tumor necrosis factor (TNF)-mediated neuroprotection against glutamate-induced excitotoxicity is enhanced by N-methyl-D-aspartate receptor activation. Essential role of a TNF receptor 2-mediated phosphatidylinositol 3-kinase-dependent NF-kappa B pathway. J Biol Chem. 2004; 279:32869–32881. [PubMed: 15155767]

Meffert MK, Chang JM, Wiltgen BJ, Fanselow MS, Baltimore D. NF-κB functions in synaptic signaling and behavior. Nat Neurosci. 2003; 6:1072–1078. [PubMed: 12947408]

Mikenberg I, Widera D, Kaus A, Kaltschmidt B, Kaltschmidt C. Transcription factor NF-κB is transported to the nucleus via cytoplasmic dynein/dynactin motor complex in hippocampal neurons. PLoS ONE. 2007; 2:e589. [PubMed: 17622342]

Mincheva-Tasheva S, Soler RM. NF-κB signaling pathways: role in nervous system physiology and pathology. Neuroscientist. 2013; 19:175–194. [PubMed: 22785105]

Minneman KP, Lee D, Zhong H, Berts A, Abbott KL, Murphy TJ. Transcriptional responses to growth factor and G protein-coupled receptors in PC12 cells: comparison of α1-adrenergic receptor subtypes. J Neurochem. 2000; 74:2392–2400. [PubMed: 10820200]

Moerman AM, Mao X, Lucas MM, Barger SW. Characterization of a neuronal κB-binding factor distinct from NF-κB. Brain Res Mol Brain Res. 1999; 67:303–315. [PubMed: 10216229]

Monje FJ, Cabatic M, Divisch I, Kim EJ, Herkner KR, Binder BR, Pollak DD. Constant darkness induces IL-6-dependent depression-like behavior through the NF-κB signaling pathway. J Neurosci. 2011; 31:9075–9083. [PubMed: 21697358]

Mucha M, Skrzypiec AE, Schiavon E, Attwood BK, Kucerova E, Pawlak R. Lipocalin-2 controls neuronal excitability and anxiety by regulating dendritic spine formation and maturation. Proc Natl Acad Sci U S A. 2011; 108:18436–18441. [PubMed: 21969573]

Naude PJ, Nyakas C, Eiden LE, Ait-Ali D, van der Heide R, Engelborghs S, Luiten PG, De Deyn PP, den Boer JA, Eisel UL. Lipocalin 2: novel component of proinflammatory signaling in Alzheimer's disease. Faseb J. 2012; 26:2811–2823. [PubMed: 22441986]

Nelsen B, Hellman L, Sen R. The NF-κB-binding site mediates phorbol ester inducible transcription in nonlymphoid cells. Mol Cell Biol. 1988; 8:3526–3531. [PubMed: 3145412]

Neumann H, Schweigreiter R, Yamashita T, Rosenkranz K, Wekerle H, Barde YA. Tumor necrosis factor inhibits neurite outgrowth and branching of hippocampal neurons by a rho-dependent mechanism. J Neurosci. 2002; 22:854–862. [PubMed: 11826115]

Oliveira-Marques V, Marinho HS, Cyrne L, Antunes F. Role of hydrogen peroxide in NF-κB activation: from inducer to modulator. Antioxid Redox Signal. 2009; 11:2223–2243. [PubMed: 19496701]

Ould-yahoui A, Tremblay E, Sbai O, Ferhat L, Bernard A, Charrat E, Gueye Y, Lim NH, Brew K, Risso JJ, Dive V, Khrestchatisky M, Rivera S. A new role for TIMP-1 in modulating neurite outgrowth and morphology of cortical neurons. PLoS ONE. 2009; 4:e8289. [PubMed: 20011518]

Ousman SS, Kubes P. Immune surveillance in the central nervous system. Nat Neurosci. 2012; 15:1096–1101. [PubMed: 22837040]

Pereira DS, Kushner DB, Ricciardi RP, Graham FL. Testing NF-κB1-p50 antibody specificity using knockout mice. Oncogene. 1996; 13:445–446. [PubMed: 8710386]

Pierce JW, Schoenleber R, Jesmok G, Best J, Moore SA, Collins T, Gerritsen ME. Novel inhibitors of cytokine-induced IκBα phosphorylation and endothelial cell adhesion molecule expression show anti-inflammatory effects in vivo. J Biol Chem. 1997; 272:21096–21103. [PubMed: 9261113]

Pizzi M, Sarnico I, Boroni F, Benarese M, Steimberg N, Mazzoleni G, Dietz GP, Bahr M, Liou HC, Spano PF. NF-κB factor c-Rel mediates neuroprotection elicited by mGlu5 receptor agonists against amyloid beta-peptide toxicity. Cell Death Differ. 2005; 12:761–772. [PubMed: 15818410]

Podolin PL, Callahan JF, Bolognese BJ, Li YH, Carlson K, Davis TG, Mellor GW, Evans C, Roshak AK. Attenuation of murine collagen-induced arthritis by a novel, potent, selective small molecule inhibitor of IκB Kinase 2, TPCA-1 (2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide), occurs via reduction of proinflammatory cytokines and antigen-induced T cell Proliferation. J Pharmacol Exp Ther. 2005; 312:373–381. [PubMed: 15316093]

Quan N, Whiteside M, Kim L, Herkenham M. Induction of inhibitory factor κBα mRNA in the central nervous system after peripheral lipopolysaccharide administration: an in situ hybridization histochemistry study in the rat. Proc Natl Acad Sci U S A. 1997; 94:10985–10990. [PubMed: 9380746]

Rappert A, Bechmann I, Pivneva T, Mahlo J, Biber K, Nolte C, Kovac AD, Gerard C, Boddeke HW, Nitsch R, Kettenmann H. CXCR3-dependent microglial recruitment is essential for dendrite loss after brain lesion. J Neurosci. 2004; 24:8500–8509. [PubMed: 15456824]

Ringheim GE, Burgher KL, Heroux JA. Interleukin-6 mRNA expression by cortical neurons in culture: evidence for neuronal sources of interleukin-6 production in the brain. J Neuroimmunol. 1995; 63:113–123. [PubMed: 8550808]

Riquelme D, Alvarez A, Leal N, Adasme T, Espinoza I, Valdes JA, Troncoso N, Hartel S, Hidalgo J, Hidalgo C, Carrasco MA. High-frequency field stimulation of primary neurons enhances ryanodine receptor-mediated Ca2+ release and generates hydrogen peroxide, which jointly stimulate NF-kappaB activity. Antioxid Redox Signal. 2011; 14:1245–1259. [PubMed: 20836702]

Robinson S, Tani M, Strieter RM, Ransohoff RM, Miller RH. The chemokine growth-regulated oncogene-α promotes spinal cord oligodendrocyte precursor proliferation. J Neurosci. 1998; 18:10457–10463. [PubMed: 9852583]

Rostene W, Kitabgi P, Parsadaniantz SM. Chemokines: a new class of neuromodulator? Nat Rev Neurosci. 2007; 8:895–903. [PubMed: 17948033]

Schmeisser MJ, Baumann B, Johannsen S, Vindedal GF, Jensen V, Hvalby OC, Sprengel R, Seither J, Maqbool A, Magnutzki A, Lattke M, Oswald F, Boeckers TM, Wirth T. IκB kinase/nuclear factor κB-dependent insulin-like growth factor 2 (Igf2) expression regulates synapse formation and spine maturation via Igf2 receptor signaling. J Neurosci. 2012; 32:5688–5703. [PubMed: 22514330]

Schmidt-Ullrich R, Memet S, Lilienbaum A, Feuillard J, Raphael M, Israel A. NF-κB activity in transgenic mice: developmental regulation and tissue specificity. Development. 1996; 122:2117–2128. [PubMed: 8681793]

Schreck R, Baeuerle PA. Assessing oxygen radicals as mediators in activation of inducible eukaryotic transcription factor NF-κB. Methods Enzymol. 1994; 234:151–163. [PubMed: 7808288]

Shim DJ, Yang L, Reed JG, Noebels JL, Chiao PJ, Zheng H. Disruption of the NF-κB/IκBalpha Autoinhibitory Loop Improves Cognitive Performance and Promotes Hyperexcitability of Hippocampal Neurons. Mol Neurodegener. 2011; 6:42. [PubMed: 21663635]

Tamatani M, Che YH, Matsuzaki H, Ogawa S, Okado H, Miyake S, Mizuno T, Tohyama M. Tumor necrosis factor induces Bcl-2 and Bcl-x expression through NFκB activation in primary hippocampal neurons. J Biol Chem. 1999; 274:8531–8538. [PubMed: 10085086]

Tian B, Nowak DE, Jamaluddin M, Wang S, Brasier AR. Identification of direct genomic targets downstream of the nuclear factor-κB transcription factor mediating tumor necrosis factor signaling. J Biol Chem. 2005; 280:17435–17448. [PubMed: 15722553]

Villeda SA, Luo J, Mosher KI, Zou B, Britschgi M, Bieri G, Stan TM, Fainberg N, Ding Z, Eggel A, Lucin KM, Czirr E, Park JS, Couillard-Despres S, Aigner L, Li G, Peskind ER, Kaye JA, Quinn JF, Galasko DR, Xie XS, Rando TA, Wyss-Coray T. The ageing systemic milieu negatively regulates neurogenesis and cognitive function. Nature. 2011; 477:90–94. [PubMed: 21886162]

Wellmann H, Kaltschmidt B, Kaltschmidt C. Retrograde transport of transcription factor NF-κB in living neurons. J Biol Chem. 2001; 276:11821–11829. [PubMed: 11096106]

Wilson EH, Weninger W, Hunter CA. Trafficking of immune cells in the central nervous system. J Clin Invest. 2010; 120:1368–1379. [PubMed: 20440079]

Wood JN. Regulation of NF-κB activity in rat dorsal root ganglia and PC12 cells by tumour necrosis factor and nerve growth factor. Neurosci Lett. 1995; 192:41–44. [PubMed: 7675306]

Zabel U, Schreck R, Baeuerle PA. DNA binding of purified transcription factor NF-κB. Affinity, specificity, Zn2+ dependence, and differential half-site recognition. J Biol Chem. 1991; 266:252–260. [PubMed: 1985897]

Zhang W, Potrovita I, Tarabin V, Herrmann O, Beer V, Weih F, Schneider A, Schwaninger M. Neuronal activation of NF-κB contributes to cell death in cerebral ischemia. J Cereb Blood Flow Metab. 2005; 25:30–40. [PubMed: 15678110]

In neurons, constitutive levels of the NF-κB subunit p65 were lower than in mixed brain cells or liver cells.

TNF was the most effective stimulus but levels of NF-κB activation by TNF were far lower in neurons than in other cells.

Glutamate was not an effective activator of neuronal NF-κB.

Other reported activators did not stimulate neuronal NF-κB activity.

Chemokine mRNAs were the most NF-κB-responsive genes in neurons.

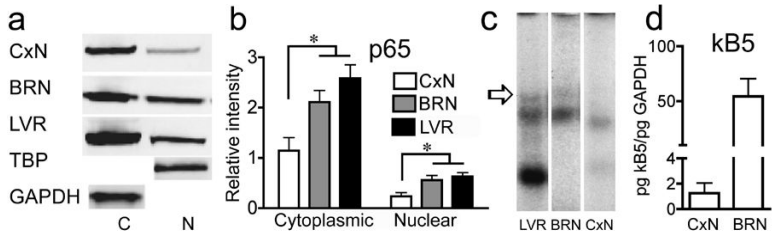

Fig. 1.

Constitutive NF-κB presence and activity in neurons is low compared to other cell types. The levels of immunoreactive p65 in the cytoplasm (C) and nucleus (N) from cortical neurons (CxN), mixed brain cells (BRN), and liver cells (LVR), are shown as Western blot images from the LI-COR system (a) and graphically (b). *, $p < 0.01$. Protein loads, 5 μg c, Control (unstimulated) lanes were extracted from autoradiographs of EMSA blots shown in later figures. d, kB5 reporting levels for CxN and BRN in unstimulated conditions, $p < 0.01$. GAPDH, cytoplasmic glyceraldehyde 3-phosphate dehydrogenase; TBP, Nuclear Tata binding protein.

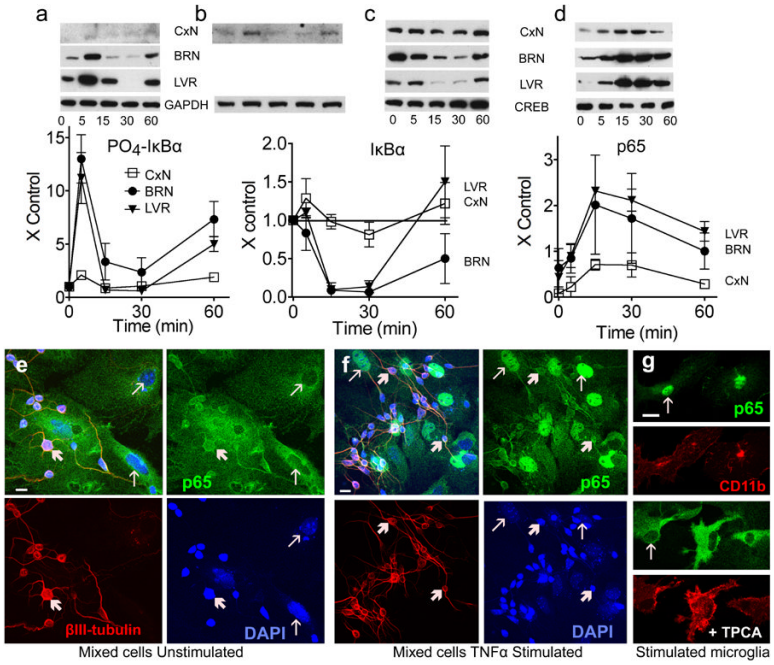

Fig. 2.

TNFα induces neuronal NF-κB p65 movement into the nucleus. a, Western blot analysis of phospho-IκBα protein induction in the cell cytoplasm of three tissue types at various times of TNFα stimulation performed under similar blotting conditions. Protein loads, 10 μg. b, phospho-IκBα protein in CxN cells in a more sensitive assay than in a. Protein loads, 40 μg. c, IκBα in the cytoplasm was strongly reduced in BRN and LVR but not CxN. Protein loads, 20 μg. d, p65 was increased in the nucleus of the three cell types, but much more strongly in BRN and LVR than CxN. Representative blots are shown for b, c and d. Protein loads, 5 μg. e, Photomicrograph of a cluster of neurons (red; βIII-tubulin antibody) growing on a mixed-cell bed with p65 (green; sc-372 antibody) present in the cytoplasm and nucleus of neurons and unidentified cells, with nuclei marked by DAPI (blue). Unstimulated cells showed p65 predominantly in the cytoplasm. Large, sheet-like cells contained p65 throughout, and neurons contained p65 in the nucleus, cytoplasm, and processes. f, Stimulation by TNFα (100 ng/ml) for 30 min produced strong nuclear localization in the mixed cells without demonstrably changing the p65 distribution in neurons. Thin arrows point to non-neuronal nuclei that were weakly p65-positive in the unstimulated condition and strongly p65 positive after TNFα stimulation. The shape and large nuclear diameter (15–20 μm) of the illustrated responsive cells suggests that they are fibroblasts derived from brain vasculature and meninges (Estin and Vernadakis, 1986). Thick arrows point to neuronal nuclei that were weakly p65-positive in both unstimulated and TNFα-stimulated conditions. g, Microglia 30 min after TNFα stimulation showed strong nuclear p65 staining (top). Cells that were treated with 10 μM TPCA prior to stimulation showed no nuclear accumulation (bottom). Arrows point to cell nuclei. Magnification bars = 10 μm at left and 5 μm for the microglia at right.

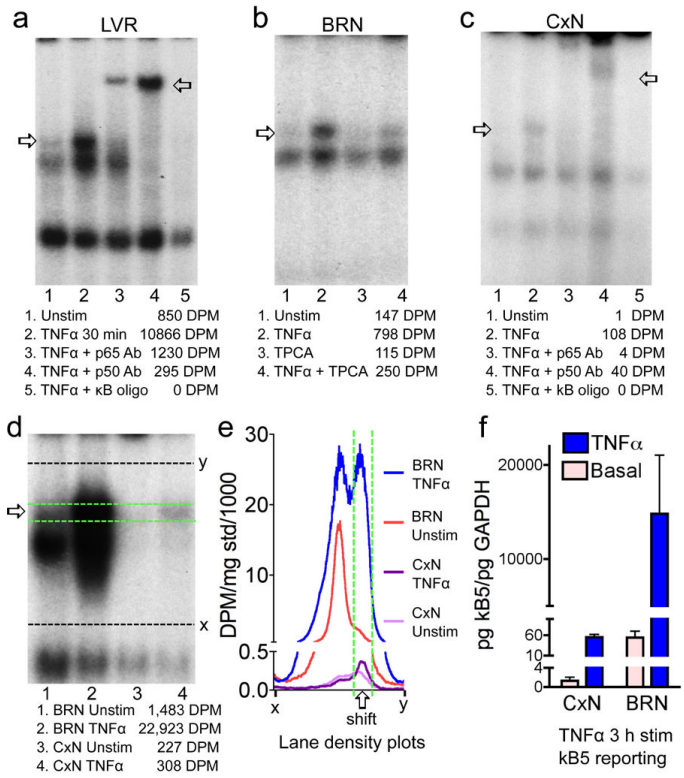

Fig. 3.

TNFα induces neuronal NF-κB activity in two functional assays. a, EMSAs of LVR (10 μg protein loaded per well, 36-h exposure); b, BRN (5 μg protein loaded per well, 5-day exposure, j); and c, CxN (10 μg protein loaded per well, 5-day exposure). TNFα was given at 100 ng/ml. Radioactive densities of the shifted bands (arrows on left) are reported for each lane after transmittance levels were converted to DPM by the radioactive standards exposed for the same duration. Arrows on the right mark supershifts in liver cells (a) and neurons (c) complexed with antibodies against p65 (NIH #7057) and p50 (NCI-Rice #1263-TB7). TPCA (1 μM) is an inhibitor of IKK activity. d, BRN and CxN nuclear proteins were loaded in equal amounts (10 μg) onto the same gel and run for 7 days; e, quantifies the densities, converted to DPM, between points x and y in the four lanes shown in d. f, TNFα-induced kB5 reporting in CxN and BRN. All differences between basal and stimulated reporting and between CxN and BRN were significant at $p < 0.01$.

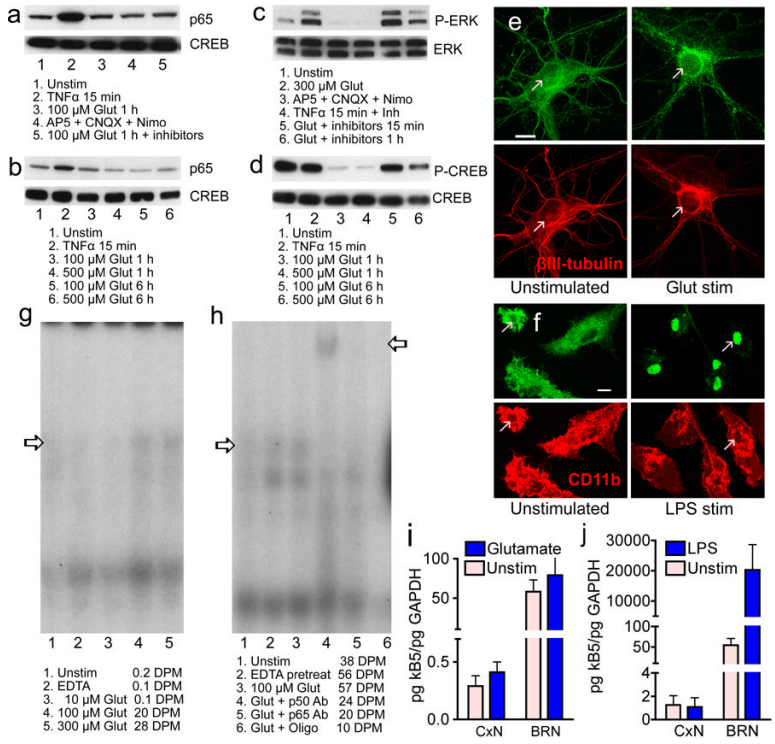

Fig. 4.

Four assays show that glutamate either minimally activates or does not activate neuronal NF-κB. a-d, CxN cultures. a, Western blot of nuclear p65 showed that glutamate has no effect on protein levels relative to the positive TNFα control when administered alone or after pre-treatment with activity inhibitors (AP5, CNQX, and nimodipine) b, Glutamate had no effect on levels of nuclear p65 in CxN at two doses and at two time points. c, In a positive control experiment, glutamate but not TNFα strongly and rapidly activated phospho-ERK in neurons. Inhibitors were without effect. d, Glutamate but not TNFα strongly reduced phospho-CREB at two doses at 1 h, with a return toward baseline at 6 h. e and f, Cells in culture were immunostained for p65 and βIII-tubulin (e), or CD11b (f). e, In neurons, immunofluorescence showed p65 in cytoplasm and nucleus, with an unchanged pattern following stimulation by 100 μM glutamate for 5 or 15 min followed by washout, stopping at 90 min, as was done by Mikenberg et al. (2007). Identical results were obtained with continuous 100 μM glutamate stimulation and stimulation with 300 μM glutamate, and prior treatment with inhibitors had no effect (data not shown). f, In microglial cells, immunofluorescence showed strong LPS-induced movement of p65 into the nucleus. The response in astrocytes to LPS was minimal (data not shown). Arrows point to cell nuclei. g, EMSA of CxN nuclear proteins showed a small glutamate effect at 100 and 300 μM (15 μg protein, 7 day exposure). h. EMSA of CxN nuclear proteins showed that the glutamate stimulation effect was super-shifted with p65 and p50 antibodies (20 μg protein, 20 day exposure). i, kB5 reporting showed no effect of glutamate on activity in CxN or BRN cells. j, LPS (1 μg/ml for 3 h) had no effect of kB5 reporting in CxN, but LPS caused a massive increase in BRN.

Table 1

List of primers used for qPCR

Gene Name	NCBI Accession Number	Amplicon Size	Sense Primer (5'–3')	Antisense Primer (5'–3')
CCL2	NM_011333	281	ATCCCAATGAGTAGGCTGGAGAC	GGTGGTTGTGGAAAAGGTAGTGG
CXCL1	NM_008176	169	GCTGGGATTCACCTCAAGAA	TGGGGACACCTTTTAGCATC
CXCL10	NM_021274	115	CAGTGAGAATGAGGGCCATAGG	CGGATTCAGACATCTCTGCTCAT
CX3CL1	NM_009142	287	ATCCCAGTGGCTTTGCTCATC	AAGGTCTTCCAATGTGGCGG
Nfkbia (IκBα)	NM_010907	243	TGTCAACAGGGTAACCTACCA	ACATTCTTTTTGCCACTTTCC
Rela (p65)	NM_009045	279	GCCTACCCGAAACTCAACTTC	CTCTTTGGAACAGGTGCAGAC
Nfkb1 (p50)	NM_008689	196	TCCACTGTCTGCCTCTCTCGTC	GCCTTCAATAGGTCCTTCCTGC
Nfkb2 (p52)	NM_019408	219	TGACCTCACTTGCAGTACCA	GTATGTGTCCACCAGGCTTC
Crel (C-rel)	NM_009044	184	GAAACTGCTGCAAGATTGTGGTC	CCCGCATGAAGAATAGTAAGGTTC
Relb (RelB)	NM_009046	269	TGATGTCCTTGGAGAGTTGAG	GCAGGTCCAACATAGTGAAGA
Ikbkb (IKK2)	NM_010546	248	GCTGCTGCTTCAGGCAATCC	TGTCTGGGCTTCCACTCACG
Tnfaip3 (A20)	NM_009397	268	CAGAAAACAAGGGCTTTTGCAC	GTTCGAGGCATGTCTCTATGCTG
TIMP1	NM_011593	211	GCATCTGGCATCCTCTTGTT	CATTTCCCACAGCCTTGAAT
IL-6	NM_031168	259	TCTGGGAAATCGTGGAAATGAG	TCTCTGAAGGACTCTGGCTTTGTC
Bcl-xL	NM_009743	221	TGGAAAGCGTAGACAAGGAGATG	CAGAACCACACCAGCCACAGTC
Cox2	NM_011198	229	TGTACAAGCAGTGGCAAAGG	CCCCAAAGATAGCATCTGGA
SOD2	NM_013671	192	CCAAAGGAGAGTTGCTGGAG	TAAGGCCTGTTGTTCCTTGC
PSD95	NM_007864	254	ACTGCATCCTTGATGTCTCAG	TTCGATGACACGTTTCACTTT
LCN2	NM_008491	274	TCTGGGAAATATGCACAGGT	ACACTCACCACCCATTCAGT
TNFRsf1A	NM_011609	173	GGCTGCAGTCCACGCACTGG	TGTGAAAAGGGCACCTTTACGGC
TNFRsf1B	NM_011610	229	ACTCCAAGCATCCTTACATCGT	CTACTGCATCCTGGGATTTCTC
GAPDH	NM_008084	256	CAAAATGGTGAAGGTCGGTGTG	TGATGTTAGTGGGGTCTCGCTC

Table 2

Basal levels of Rel family and TNF Receptor proteins and mRNA expression in neurons (CxN) and mixed brain cells (BRN); means ± SEM from 8–28 separate determinations.

a. Levels of un-stimulated NF-κB-related cytosolic proteins (relative infrared intensity units corrected for gel load ((β-actin) from the Li-Cor Odyssey system).

Protein	CxN	BRN	Significance CxN vs. BRN
p65	1.15 ± 0.26	2.02 ± 0.29	$p < 0.01$
IκBα	0.27 ± 0.04	1.76 ± 0.36	$p < 0.001$
p50	1.34 ± 0.19	4.94 ± 0.60	$p < 0.0001$

b. Expression levels of un-stimulated NF-κB-related genes determinbed by qPCR, expressed as fg gene target/pg GAPDH

Gene	CxN	BRN	Significance CxN vs. BRN
Rela (p65)	48.4 ± 5.1	88.3 ± 8.7	$p < 0.001$
Nfkbia (IκBα)	51.8 ± 5.8	116.0 ± 23.3	$p < 0.005$
Nfkbl (p50)	28.4 ± 2.0	88.3 ± 16.7	$p < 0.001$
Crel (c-Rel)	1.17 ± 0.17	1.40 ± 0.24	p = N.S.
Relb (RelB)	3.62 ± 0.55	11.0 ± 1.2	$p < 0.0001$
Nfkb2 (p52)	2.06 ± 0.31	4.15 ± 0.92	$p < 0.05$
Ikk2 (IKKβ)	11.0 ± 1.3	16.2 ± 2.6	p = N.S.
TNFR1	62.7 ± 14.7	509.0 ± 89.2	$p < 0.0001$
TNFR2	0.02 ± 0.004	5.58 ± 2.22	$p < 0.005$

Table 3

TNFα (100 ng/ml for 3 h) effects on gene expression, by qPCR, in CxN and BRN cells represented as converted mean dose values (fg gene target/pg GAPDH) ± SEM. Induction via NF-κB was determined by addition of the selective IKK inhibitor TPCA (10 μM).

Cell type	Gene	Unstim	TNFα Stim	TPCA	Stim + TPCA
CxN	CCL2	0.14 ± 0.04	41.75 ± 8.79	0.13 ± 0.07	0.02 ± 0.01
	CXCL1	0.27 ± 1.08	9.93 ± 2.98	0.22 ± 0.13	0.34 ± 0.10
	CXCL10	0.94 ± 0.24	185.1 ± 28.3	0.29 ± 0.10	7.97 ± 3.50
	CX3CL1	50.4 ± 8.28	65.2 ± 15.5	39.3 ± 7.46	29.1 ± 6.12
	IκBα	54.7 ± 6.04	143.4 ± 20.9	50.0 ± 7.43	27.6 ± 1.41
	A20	0.43 ± 0.07	3.53 ± 1.28	0.15 ± 0.05	0.09 ± 0.03
	LCN2	0.21 ± 0.06	11.69 ± 3.67	0.03 ± 0.01	0.01 ± 0.01
	IL-6	8.64 ± 1.10	32.6 ± 11.6	9.22 ± 2.31	8.78 ± 2.10
	TIMP1	0.43 ± 1.07	1.18 ± 0.26	0.08 ± 0.03	0.05 ± 0.01
	IGF2	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.02 ± 0.01
	p65	50.6 ± 5.1	46.1 ± 6.1	N.D.	N.D.
	Cox2	110.1 ± 25.6	106.2 ± 27.7	N.D.	N.D.
	Bcl-xL	19.3 ± 3.82	19.6 ± 4.37	N.D.	N.D.
	SOD2	148.6 ± 31.8	102.7 ± 17.9	N.D.	N.D.
BRN	CCL2	2.52 ± 1.08	1465 ± 186	0.44 ± 0.17	3.49 ± 1.53
	CXCL1	2.15 ± 0.67	86.6 ± 25.4	0.69 ± 0.30	1.84 ± 0.60
	CXCL10	4.30 ± 0.90	1201 ± 167	1.56 ± 0.62	37.2 ± 9.61
	CX3CL1	48.3 ± 20.5	156.4 ± 23.4	47.0 ± 3.0	61.2 ± 13.6
	IκBα	102.9 ± 18.6	790.7 ± 88.2	41.6 ± 7.2	112.8 ± 34.3
	A20	3.76 ± 0.52	117.3 ± 24.1	0.48 ± 0.10	1.60 ± 0.42
	LCN2	15.5 ± 7.08	627.1 ± 46.2	21.4 ± 12.1	17.9 ± 8.67
	IL-6	102.9 ± 21.5	806.4 ± 111.1	51.0 ± 9.98	122.3 ± 26.4
	TIMP1	22.4 ± 6.29	114.0 ± 70.7	6.21	52.4
	IGF2	0.31 ± 0.09	0.24 ± 0.05	0.36 ± 0.01	0.44 ± 0.29
	p65	88.3 ± 8.7	125.1 ± 25.5	N.D.	N.D.
	Cox2	152.0 ± 35.2	384.4 ± 63.5	N.D.	N.D.
	Bcl-xL	22.0 ± 7.38	32.3 ± 11.7	N.D.	N.D.
	SOD2	70.3 ± 28.2	111.6 ± 33.7	N.D.	N.D.

N.D., Not Determined

For all gene transcripts except for CX3CL1, Cox2, Bcl-xL, and SOD2, basal expression levels in BRN were significantly greater than basal expression levels in CxN ($p < 0.05$ to $p < 0.0001$). For all transcripts except for CX3CL1 (CxN only), Cox2 (CxN only), IGF2, p65, Bcl-xL, and SOD2, TNFα significantly increased expression over baseline for BRN and CxN ($p < 0.05$ to $p < 0.0001$). Total numbers of determinations ranged from 3 to 28 (except, 1 determination each for TPCA and Stim + TPCA for TIMP1 in BRN).

Table 4

Quantitative PCR of genes induced by glutamate (Glut) stimulation of CxN neurons, expressed as fold increase over basal levels.

Gene	# Experiments Stim/Stim+TPCA	100 μM Glut	Glut + TPCA
CCL2	5/3	1.49	0.35
CXCL1	5/3	3.90 *	2.61
CXCL10	5/3	2.77	0.65
IκBα	5/3	1.57 *	0.92 **
IL-6	5/0	1.04	N.D.
TIMP-1	5/3	15.21 *	1.82 **
PSD-95	5/3	0.95	0.73

Conditions: pre-incubation for 24 h with 10 μM Nimodipine/40 μM CNQX/100 μM AP5, wash, 10 min 100 μM glutamate stimulation (with or without 10 μM TPCA), wash, and 3-h recovery prior to harvest for RNA preparation.

* Significant increase over basal, $p < 0.05$;

** significant decrease from stimulated, $p < 0.05$

Table 5

Quantitative PCR of genes induced by glutamate stimulation of BRN cells, expressed as fold increase over basal levels.

Gene	Stimulation condition	Stimulation (X-fold)
CCL2	a	2.5, 2.8
	b	1.3, 2.0
CXCL1	a	1.4, 2.0
	b	2.7, 3.6
CXCL10	a	1.3, 1.1
	b	0.9, 2.2
CX3CL1	a	2.2, 1.6
	b	1.8, 2.2
IκBα	a	1.2, 1.2
	b	0.8, 1.1
IL-6	a	7.4, 8.7
	b	1.5, 0.8

a, 100 or 300 μM glutamate treatment for 10 min, then 3 h washout.

b, 300 μM glutamate continuous treatment for 3 or 6 h, respectively.

Stimulation values represent n = 1.

Table 6

Other Stimuli tested in CxN neurons

Treatment	EMSA (NF-κB binding at 30 min)	Western Blot (Fold increase in nuclear p65 at 30 min)	qPCR (Fold increase at 3 h)	ELISA (Fold increase at 24 h)
IL-1β (50 ng/ml)	weak increase	2.0 (2)	CXCL1: 204 (2) CXCL10: 7.8 (2) CX3CL1: 1.5 (2) IκBα: 1.6 (2) IL-6: 3.1 (2) TIMP1: 2.8 (2) Cox2: 3.3 (2) Bcl-xL: 1.9 (2)	CCL2: 33.8 (2) CXCL1: 46.6 (2) CXCL10: 17.6 (2)
Unstimulated BRN-conditioned media	N.D.	3.0 (3)	CCL2: 124 (5) CXCL1: 36.0 (6) CXCL10: 168 (6) IκBα: 2.1 (6) IL-6: 24.6 (4) TIMP1: 25.6 (6)	N.D.
Stimulated (1 μg/ml LPS) BRN-conditioned media	N.D.	4.0 (3)	CCL2: 246 (5) CXCL1: 41.3 (5) CXCL10: 300 (5) IκBα: 3.1 (5) IL-6: 45.8 (4) TEMP1: 39.1 (5)	N.D.
LPS (1 μg/ml)	No effect	1.1 (4)	CCL2: 1.1 (6) CXCL1: 1.6 (7) CXCL10: 0.7 (7) CX3CL1: 1.2 (4) IκBα: 1.0 (7) IL-6: 1.3 (6) TIMP1: 0.7 (7) Bcl-xL: 1.0 (4)	CCL2: 0.8 (4) CXCL1: 1.1 (5) CXCL10: 2.3 (5)
H_2O_2 (300 μM)	No effect (2)	1.0 (1)	N.D.	N.D.
Norepinephrine (1 μM)	N.D.	1.4 (3)	CCL2: 0.8 (2) CXCL1: 2.0 (2) CXCL10: 1.3 (2) IκBα: 0.8 (2) IL-6: 1.5 (2)	N.D.

Treatment	EMSA (NF-κB binding at 30 min)	Western Blot (Fold increase in nuclear p65 at 30 min)	qPCR (Fold increase at 3 h)	ELISA (Fold increase at 24 h)
			TIMP1: 2.9 (2)	
ATP (100 μM)	N.D.	1.1 (1)	CXCL1: 0.8 (1) CXCL10: 2.2 (1) IκBα: 1.0 (1) IL-6: 1.3 (1) TIMP1: 2.0 (1) Cox2: 1.5 (1) Bcl-xL: 0.8 (1)	N.D.
NGF (100 ng/ml)	N.D.	1.7 (3)	CCL2: 1.0(2) CXCL1: 1.0 (2) CXCL10: 1.4 (2) CX3CL1: 0.5 (1) IκBα: 1.2 (2) IL-6: 1.2 (2) TIMP1: 0.8 (1) Cox2: 0.7 (1) Bcl-xL: 1.0 (2)	N.D.
BDNF (100 ng/ml)	N.D.	0.5 (2)	CCL2: 1.4 (1) CXCL1: 3.2 (2) CXCL10: 1.9 (2) IκBα: 0.9 (2) IL-6: 0.5 (1) TIMP1: 1.9 (2) Cox2: 0.8 (1)	CCL2: 7.45 (1) CXCL1: 46.6 (2) CXCL10: 17.6 (2)
PMA (10 or 100 nM)		0.7 (3)		CCL2: 4.1 (2) CXCL1: 1.0 (2) CXCL10: 1.1 (2)

Values in parenthesis indicate experimental replications.

N.D.: Not Determined.