Neuroscience 250 (2013) 282–299

MINIMAL NF-κB ACTIVITY IN NEURONS

S. J. LISTWAK,[†] P. RATHORE[†] AND M. HERKENHAM[*]

Section on Functional Neuroanatomy, National Institute of Mental Health, NIH, Bethesda, MD 20892-3724, USA

Abstract—Nuclear factor-kappa B (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, electrophoretic mobility shift assay (EMSA) of nuclear κB DNA binding, reporter assay of κB DNA binding, immunofluorescence of the NF-κB subunit protein p65, quantitative real-time polymerase chain reaction (PCR) of NF-κB-regulated gene expression, and enzyme-linked immunosorbent assay (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 pro-inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin-beta (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. Published by Elsevier Ltd. on behalf of IBRO.

Key words: NF-κB, transcription factor, plasticity, neuroprotection, TNF.

INTRODUCTION

The transcription factor nuclear factor-kappa B (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). 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

*Corresponding author. Address: Section on Functional Neuroanatomy, NIMH, Building 35, Room 1C913, 35 Convent Drive, Bethesda, MD 20892-3724, USA.
E-mail address: herkenh@mail.nih.gov (M. Herkenham).
[†] These authors contributed equally to this work.

Abbreviations: AP5, 2-amino-5-phosphonopentanoate; BDNF, brain-derived neurotrophic factor; BRN, mixed brain cells; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CxN, cortical neurons; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HBSS, Hanks balanced salt solution; HRP, horseradish peroxidase; IgG, immunoglobulin G; IKK, IκB kinase; IL, interleukin; ISHH, *in situ* hybridization histochemistry; LCN2, lipocalin 2; LPS, lipopolysaccharide; LVR, liver cells; NF-κB, nuclear factor-kappa B; NGF, nerve growth factor; NGS, normal goat serum; NLS, nuclear localization signal; PBS, phosphate-buffered saline; PDTC, ammonium pyrrolidinedithiocarbamate; PMA, phorbol 12-myristate 13-acetate; qPCR, quantitative real-time polymerase chain reaction; SDS, sodium dodecyl sulfate; TBP, Tata binding protein; TBST, Tris-Buffered Saline containing 0.05% Tween-20; TLR, toll-like receptor; TNFα, tumor necrosis factor α; TPCA, 2-[(aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide.

0306-4522/13 $36.00 Published by Elsevier Ltd. on behalf of IBRO.
http://dx.doi.org/10.1016/j.neuroscience.2013.07.013

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 NF-κB inhibitor, α 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, 2006; Kassed et al., 2004; 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 (1×), Glutamax (2 mM), penicillin (100 U/ml), and streptomycin (100 mg/ml) (all from Invitrogen, Carlsbad, CA, USA) 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 enzyme-linked immunosorbent assay (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 4 days in culture, the medium was changed to fresh supplemented Neurobasal medium containing cytosine arabinoside (10 mM) and 2-deoxycytidine (100 mM) (Sigma–Aldrich, St. Louis, MO, USA) 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