Published in final edited form as:
J Neuroimmune Pharmacol. 2011 December ; 6(4): 617–625. doi:10.1007/s11481-011-9279-2.

Orphanin FQ/Nociceptin Activates Nuclear Factor Kappa B

Courtney L. Donica,
Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave Suite 323, Oklahoma City, OK 73117, USA

Oklahoma Center for Neuroscience, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave Suite 323, Oklahoma City, OK 73117, USA

Vanessa I. Ramirez,
Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave Suite 323, Oklahoma City, OK 73117, USA

Hibah O. Awwad,
Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave Suite 323, Oklahoma City, OK 73117, USA

Nurulain T. Zaveri,
Astraea Therapeutics, LLC, 320 Logue Avenue, Suite 142, Mountain View, CA 94043, USA

Lawrence Toll, and
SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025, USA

Kelly M. Standifer
Department of Pharmaceutical Sciences, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave Suite 323, Oklahoma City, OK 73117, USA

Oklahoma Center for Neuroscience, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave Suite 323, Oklahoma City, OK 73117, USA

Department of Cell Biology, University of Oklahoma Health Sciences Center, 1110 N. Stonewall Ave Suite 323, Oklahoma City, OK 73117, USA

University of Oklahoma College of Pharmacy, 1110 N. Stonewall Ave Suite 326, Oklahoma City, OK 73117, USA

Kelly M. Standifer: kelly-standifer@ouhsc.edu

Abstract

Endogenous neuropeptide orphanin FQ/nociceptin (OFQ/N) and its receptor, nociceptin orphanin FQ peptide receptor (NOPr), play a modulatory role throughout the body including nociceptive sensitivity, motor function, spatial learning, and the immune system. NOPr is an inhibitory G protein coupled receptor (GPCR) that modulates expression and release of inflammatory mediators from immune cells and in the CNS. Inhibitory GPCRs have been shown to activate the immune and central nervous system regulator, nuclear factor kappa B (NFκB), whose family consists of several subunits. When activated, NFκB translocates to the nucleus and can modify transcription. To determine if OFQ/N modulates NFκB activity, SH-SY5Y human neuroblastoma cells were treated with OFQ/N and assessed for changes in nuclear accumulation, DNA binding,

Correspondence to: Kelly M. Standifer, kelly-standifer@ouhsc.edu.

Guarantors: Courtney L. Donica and Kelly M. Standifer.

Disclaimers The authors have no conflict of interest to disclose.

and transcriptional activation. For the first time, we show that OFQ/N increases the nuclear accumulation (1.9–2.8-fold) and the DNA binding of NFκB (2.9-fold) by 2 h as determined by immunoblotting and electromobility shift assay, respectively. OFQ/N induction of NFκB binding to DNA is protein kinase C-dependent and NOPr-specific. OFQ/N stimulated binding of both NFκB p50 and p65 subunits to their consensus binding site on DNA. OFQ/N also induces transcriptional activation of an NFκB reporter gene 2.2-fold by 2 h with an EC_{50} of 6.3 nM. This activation of NFκB by OFQ/N suggests a likely mechanism for its modulation of the central nervous and immune systems.

Keywords

NOP receptor; Orphanin FQ/nociceptin; NFκB; Immune; Opioid; Cytokine

Introduction

Orphanin FQ/nociceptin (OFQ/N) is a 17-amino acid, endogenous neuropeptide with high affinity for its receptor, the nociceptin/orphanin FQ peptide receptor (NOPr). NOPr is a $G_{i/o}$-coupled receptor that inhibits adenylate cyclase and voltage-gated calcium channels and activates inwardly rectifying potassium channels, with an overall effect of inhibiting neurotransmission. OFQ/N activation of NOPr has many functions throughout the body, including modulating nociception, locomotion, smooth muscle contraction, learning, memory, and cardiovascular function, as noted in a recent review (Lambert 2008). Of particular interest is discovery of the effect of OFQ/N in modulating the immune system.

OFQ/N and NOPr play an important immunomodulatory role as can be seen by their distribution and activity in the immune system. NOPr is expressed in lymphocytes, monocytes, and peripheral blood mononuclear cells, as well as T cell and B cell lines (Halford et al. 1995; Wick et al. 1995; Peluso et al. 1998; Arjomand et al. 2002). Both NOPr and OFQ/N are expressed in human peripheral blood neutrophils, where OFQ/N induces neutrophil chemotaxis (Serhan et al. 2001; Fiset et al. 2003). OFQ/N blocks antibody formation in vivo and in vitro in rodent spleen cells (Anton et al. 2010), as well as inhibits T cell proliferation. It also blocks synthesis of pro-inflammatory cytokine interleukin (IL)-2 in rat splenocytes (Miller and Fulford 2007) and inhibits complete Freund's adjuvant-induced increases in pro-inflammatory IL-6, IL-1β, and tumor necrosis factor-α (TNFα) mRNA in cultured astrocytes (Fu et al. 2007). OFQ/N has been shown to increase IκB kinase (IKK) phosphorylation, leading to the phosphorylation and degradation of IκB in SH-SY5Y human neuroblastoma cells (Liu and Wong 2005), suggesting that OFQ/N may modulate nuclear factor kappa B (NFκB) by promoting the degradation of IκB.

NFκB is a transcription factor known to be a critical regulator of the immune system, modulating more than 100 genes. The NFκB family is composed of five subunits (RelA/p65, Rel B, c-rel, p50, and p52) which form homo- and hetero-dimers in a cell- and tissue-specific manner. NFκB is typically bound by an inhibitory protein, IκB, which restricts NFκB to the cytoplasm. Upon phosphorylation of IκB by IKK, NFκB is released and translocates to the nucleus, binds to DNA promoter regions expressing the NFκB response element, and modulates gene transcription. All NFκB subunits contain a Rel homology domain, which enables the subunits to dimerize, target the nucleus, and bind the DNA. However, only p65, Rel B, and c-rel contain a trans-activation domain that allows activation of transcription. Therefore, NFκB can function as a stimulator or repressor of transcription, depending on the particular subunit combination that is bound to the DNA (Gilmore 2006). Although the above studies implicate a relationship between OFQ/N, NFκB, and cytokine expression, a direct link between OFQ/N and NFκB has not been demonstrated to date.

NFκB also has been shown to play important roles within the central nervous system. In neurons specifically, NFκB can be activated by numerous factors, including glutamate, growth factors, calcium, reactive oxygen species, and protein kinases. In the nervous system under normal conditions, NFκB modulates synaptic plasticity, memory formation, cognition, development, and neuronal survival (reviewed in Levenson et al. 2006). OFQ/N has been implicated in regulating spatial learning and memory as well as development and neuronal differentiation (Neal et al. 2001; Zaveri et al. 2006; Kuzmin et al. 2009). Interestingly, it has been shown that immune factors such as TNFα and IL-6, both of which are modulated by OFQ/N (Fu et al. 2007), are critical for proper formation and maintenance of neural development and synaptic plasticity (Boulanger 2009). Additionally, NFκB has been shown to be constitutively active in certain neurons. Recent findings have led to the suggestion that NFκB in the CNS acts as both a signal transducer at the synapse as well as a transcriptional regulator in the nucleus (reviewed in Memet 2006). Neurons have also been found to modulate immune responses within the central nervous system, including evidence that brain derived cytokines may play a role in neuronal plasticity, as well as in the pathology underpinning psychiatric disorders such as major depressive disorder (Khairova et al. 2009; Tian et al. 2009).

Given the important and widespread roles of NFκB and OFQ/N in the nervous as well as immune system and the ability of OFQ/N to facilitate depression-like symptoms in animal models, we utilized human neuroblastoma SH-SY5Y cells to analyze the effect of OFQ/N on NFκB. These cells endogenously express NOPr and OFQ/N at levels comparable to monocytes and T lymphocytes (Peluso et al. 2001). Our study is the first to show this important mechanism of action of OFQ/N-induced NFκB activation by the NOPr. OFQ/N induces NFκB nuclear translocation, binding to DNA and activation of transcription in a time- and concentration-dependent manner. Protein kinase C (PKC) inhibition, as well as NOPr antagonism, blocks OFQ/N-induced NFκB binding to DNA. This activation of NFκB suggests a mechanism for the modulation of the neuroimmune system by OFQ/N.

Materials and methods

Cell culture

Human neuronal SH-SY5Y cells were maintained in a 1:1 mixture of Eagle's minimum essential media with nonessential amino acids and Ham's nutrient mixture F12 (MEM/F12; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA, USA). Cells were grown in a 6% CO_2–94% air humidified atmosphere at 37°C. Experiments were performed on cells >80% confluency in 6- or 12-well plates or 60 mm dishes (Thakker and Standifer 2002a, b). SH-SY5Y cells endogenously express the NOPr at 6–13 fmol/mg, which is comparable to NOPr expression in monocytes and T lymphocytes (Peluso et al. 2001; Mandyam and Standifer, unpublished data).

Drug treatment

Cells were incubated for 15 min in fresh MEM/F12 media containing 0.1% protease-free bovine serum albumin and 25 mg/ml bacitracin (vehicle) prior to the addition of drug treatment to prevent OFQ/N degradation and adhesion to the culture dish. Basal groups received only vehicle. For antagonist experiments, NOPr antagonist SB612111 (3 or 30 nM, Astraea Therapeutics, LLC, Mountain View, CA, USA) or PKC inhibitors chelerythrine chloride (Che; 1 μM; Sigma Aldrich, St. Louis, MO, USA) and Gö6976 (3 μM; Calbiochem, Gibbstown, NJ, USA) were added 15 min prior to the addition of OFQ/N. Cells were treated with varying concentrations of OFQ/N (100 pM–1 μM) for the time indicated.

Drug treatments were terminated by three rapid, gentle washes with ice-cold phosphate buffered saline (PBS; pH 7.4).

Nuclear extraction

After termination of drug treatment, the nuclear fraction was extracted using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce Biotechnology, Rockford, IL, USA) according to manufacturer's protocol, in the presence of protease and phosphatase inhibitors (2 mM Na_3VO_4, 10 μM NaP_2O_7, 250 μM phenylmethylsulfonyl fluoride, Complete Protease Inhibitor Cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA)). Aliquots of the cytoplasmic and nuclear fractions used for BCA protein estimation and samples were stored at −80°C.

Immunoblotting

Nuclear extracts were solubilized in 4× Laemmli buffer and stored at −80°C. Pre-heated samples (10 min at 95°C) were resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis on 8–15% Tris–glycine gels (~20 μg total protein/well) and transferred to polyvinylidene fluoride membranes. After blocking with 5% milk in TBS-T (20 mM Tris, 140 mM NaCl, 0.05% Tween 20 (Sigma Aldrich)), primary antibodies were incubated overnight at 4°C in the following sequence: rabbit anti-p65 (1:1,000; sc-372), rabbit anti-p50 (1:500; sc-7178), and rabbit anti-nucleolin (1:1,000, sc-13057). Secondary antibody goat anti-rabbit (1:2,000, sc-2301) conjugated to horseradish peroxidase was incubated for 1 h at RT in 5% milk in TBS-T. Immunoreactive bands were visualized by chemiluminescence (Pierce ECL Substrate), captured with the Ultralum Omega Imaging System, and densitometry was performed using Ultra Quant 6.0. Nuclear extract immunoblots were normalized to nucleolin. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Electromobility shift assay

Single-stranded DNA oligonucleotides mimicking the NFκB DNA binding site (5′ AGT-TGA-GGG-GAC-TTT-CCC-AGG-C) were synthesized with or without 5′ biotin label and were annealed according to Pierce Technical Bulletin in a solution of 10 mM Tris, 1 mM EDTA, and 50 mM NaCl (pH 8.0). DNA Retardation Gels (6%; Invitrogen, Carlsbad, CA, USA) were flushed and pre-run for 50 min at 100 V in 0.5× Tris/boric acid/EDTA (45 mM Tris–borate, 1 mM EDTA) buffer. Binding reactions were prepared on ice in microcentrifuge tubes in the following order for a final volume of 20 μL: ultrapure water, binding buffer (10 mM Tris, 50 mM KCl, 1 mM DTT, pH 7.5), 50 ng/μL Poly (dI·dC), 4 μg of nuclear extract protein, and 40 fmol biotin-labeled Target DNA oligo. For competition or supershift assay, 200× excess of unlabeled target DNA or 2 μL antibody (p65, sc-732x; p50, sc-7178x), respectively, was added 20 min prior to the addition of nuclear extract at RT. After all components of binding reaction were added, the mixture was incubated for 20 min at RT. Loading buffer was then added and samples were electrophoresed for 85 min at 100 V and transferred onto zeta probe blotting membrane (Bio-Rad, Hercules, CA, USA) for 45 min at 100 V. Membranes were cross-linked for 15 min under UV light using the UltraLum Imaging System. Biotin-labeled DNA was detected using the Pierce Chemiluminescent Nucleic Acid Detection Module. Bands were quantified by normalizing the bound fraction to the unbound fraction.

Calcium phosphate transfection

SH-SY5Y cells were plated onto 150 mm plates to ~60% confluency. Culture media were replaced at least 1 h prior to transfection with 5 μg pGL4.32 [*luc2P*/NF-κB-RE/Hygro] DNA Vector (Firefly; Promega, Madison, WI, USA) and 1 μg pGL4.74[*hRluc*/TK] DNA

Vector (Renilla; Promega) using the calcium phosphate transfection method. According to the protocol, DNA for both Renilla and Firefly vectors were added to a 2 M calcium chloride solution in distilled water. The DNA mixture was then added to 2× HEPES buffered saline (50 mM HEPES, 280 mM NaCl, and 1.5 mM Na_2HPO_4) and incubated for 20 min at RT. The transfection mixture was then added to the cells dropwise. After 18–24 h, the cells were washed once with PBS and then split into multi-well dishes. The cells were treated with the indicated drug and harvested 24 h later.

Dual-Luciferase Reporter Assay for NFκB transcriptional activation

SH-SY5Y cells were co-transfected with both NFκB/Firefly and TK/Renilla vectors and treated with OFQ/N as described above. As a positive control, 10 ng/ml TNFα was used for NFκB activation. NFκB transcriptional activation was determined with the Promega Dual-Luciferase Reporter Assay System. Cells were harvested in 100 μL 1× passive lysis buffer and collected into 1.5 ml tubes. Samples were homogenized for 10 s and then subjected to two freeze–thaw cycles to ensure sufficient cell lysis. Reagents for measurement of Firefly and Renilla signals were prepared according to manufacturer's technical manual. Luciferase activation was measured using the TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA, USA). Firefly signal was normalized to Renilla signal, which accounts for transfection efficiency.

Statistical analysis

Data were analyzed using one-way ANOVA with Dunnett's multiple comparison test on GraphPad Prism 5 for Windows and were considered statistically significant (*) $p<0.05$ compared to basal.

Results

To understand the impact of OFQ/N treatment on NFκB pathways, we studied the initial steps in NFκB activation, targeting of the subunit complex to the nucleus in SH-SY5Y cells that natively express the NOPr. Given that p65/p50 is the predominant NFκB complex, we examined the nuclear accumulation of NFκB p65 and p50 with OFQ/N (Fig. 1). OFQ/N treatment caused a gradual increase in NFκB p65 nuclear accumulation, ranging from 1.5- to 1.9-fold over 4 h in the presence of OFQ/N ($p<0.05$). This same treatment also increased p50 NFκB 1.5- to 2.8-fold during the same 4-h period ($p<0.05$).

Upon entering the nucleus, NFκB dimers must bind the DNA to regulate gene transcription. The time course for NFκB binding to DNA oligonucleotides after OFQ/N treatment was examined using an electromobility shift assay (EMSA; Fig. 2). A 2.2-fold increase in binding of NFκB was seen after 1 h of OFQ/N treatment that was maintained over 4 h ($p<0.01$). The specificity of NFκB activation was confirmed in our system, since the shift of the biotin-labeled oligonucleotide probe in the absence of the nuclear extract was undetectable, as expected for a nuclear extract-free control (far left lane, Fig. 2). Competition with excess (200×), unlabeled DNA oligonucleotide blocked NFκB binding at each time point, confirming the specificity of the probe and the direct activation of NFκB by OFQ/N treatment in our cell line (Fig. 2). The highlighted band was chosen for analysis given that it was always present and completely blocked by the excess unlabeled probe. Conversely, the other bands present were not always visible and not completely blocked by excess, unlabeled probe and were thus excluded from examination. Altogether, this demonstrates that OFQ/N treatment in SH-SY5Y cells activated the NFκB pathway, specifically by increasing NFκB binding to DNA in a time-dependent manner.

Furthermore, the potency of NFκB activation by OFQ/N was determined via a concentration response curve using EMSA (Fig. 3). OFQ/N significantly increased NFκB binding in SH-SY5Y cells by 2.5-fold compared to basal levels ($p<0.01$), with an EC_{50} of 24 nM. Maximal NFκB binding was noted with 1 μM OFQ/N, so subsequent experiments were performed with that concentration.

OFQ/N activates conventional PKC isoforms through NOPr signaling (Lou et al. 1997; Mandyam et al. 2002; Thakker and Standifer a, b; Thakker et al. 2007), and NFκB and IKK can be activated by PKC (Mut et al. 2010; Duran et al. 2003; Catley et al. 2004; Matsubara et al. 2005; Shinohara et al. 2005; Viatour et al. 2005; Li et al. 2009; Lu et al. 2009; Happel et al. 2011); therefore, it was essential to understand whether PKC was involved in the mechanism of NFκB activation via OFQ/N. SH-SY5Y cells were incubated with inhibitors of PKC for 15 min prior to the addition of OFQ/N; Che inhibits all PKC isoforms and Gö6976 inhibits only conventional isoforms (α, β, γ; Martiny-Baron et al. 1993). Che completely blocked OFQ/N-induced NFκB binding compared to OFQ/N-treated cells in the absence of inhibitor ($p<0.01$), whereas Gö6976 reduced OFQ/N-induced NFκB binding by approximately 50% (Fig. 4, $p<0.05$). This indicates that both conventional and non-conventional PKC isoforms are involved in OFQ/N-induced NFκB activation. The specificity of OFQ/N acting at the NOPr was confirmed by utilizing NOPr antagonist SB612111 (3 or 30 nM). SB612111 blocked OFQ/N-induced NFκB binding even at concentrations as low as 3 nM ($p<0.01$), confirming that OFQ/N actions were mediated through the NOP receptor. NOPr antagonist and PKC inhibitor treatments alone were without effect on NFκB binding compared to basal levels.

Given that NFκB is capable of binding DNA without activating transcription depending on the subunits involved, the identity of the subunits was investigated by conducting supershift EMSA experiments (Fig. 5). This assay utilized antibodies against the NFκB p65 or p50 subunits in order to confirm the presence of the subunit in the binding complex. Specific binding of the antibody causes a “supershift” as the molecular weight of the complex increases. Alternatively, the antibody can block a critical binding site on the transcription factor, resulting in inhibition of the probe-NFκB complex and absence of the band on the membrane (Smith and Delbary-Gossart 2001; Perdew et al. 2006). OFQ/N-treated nuclear extracts were incubated with two concentrations of antibodies specific for either NFκB p65 or p50. The ability of both antibodies to block OFQ/N-induced increase in the NFκB/DNA binding complex indicates that OFQ/N induces binding of both p50 and the transcription activating p65 subunit to DNA (Fig. 5).

Given that a particular combination of p50 (homodimer) and p65/p50 (heterodimer) subunits renders NFκB a transcriptional stimulator or repressor, the ability of OFQ/N to induce NFκB transcriptional activity was determined by a Dual-Luciferase Reporter Assay (Fig. 6). SH-SY5Y cells were transfected and treated as described in the “Materials and methods” section. OFQ/N increased NFκB transcriptional activity 2.2-fold over 4 h (Fig. 6a; $p<0.05$). Luciferase activity was increased by OFQ/N with an EC_{50} of 6.3 nM (Fig. 6b), confirming the ability of OFQ/N to activate NFκB-dependent transcription. TNFα, a robust activator of NFκB, was used as a positive control.

Discussion

OFQ/N regulates multiple aspects of immune function, but the mechanism of modulation has been unclear. NFκB is a core transcriptional factor in weighing whether an immune response results in a net pro-inflammatory response or anti-inflammatory response. Our findings in this study are the first to establish this direct link between OFQ/N and NFκB. It has been known for some time that opioid receptors, such as mu opioid receptor (MOR) and

kappa opioid receptor (KOR), modulate NFκB in a cell type-specific manner. While KOR agonists inhibit NFκB activation, MOR agonists activate or repress NFκB activation in a time- and concentration-dependent manner, as discussed in recent reviews (Bidlack et al. 2006; Chen et al. 2006; Finley et al. 2008). Based upon this knowledge and a single report that OFQ/N increased IKK phosphorylation in SH-SY5Y cells (Liu and Wong 2005), we posited that OFQ/N treatment would result in NFκB activation. Using a step-wise approach in that same cell line, we clearly demonstrate that OFQ/N activates NFκB nuclear translocation, DNA binding, and activation of NFκB-mediated transcription. This study provides an important means by which OFQ/N may modulate expression of immune factors such as cytokines and chemokines. OFQ/N specifically increased nuclear accumulation of both p50 and p65 subunits and maximally increased the binding of NFκB to DNA within 2 h. Further, supershift experiments confirmed the binding of p50 and p65 subunits to DNA after OFQ/N treatment. Since binding of both subunits to DNA would enable NFκB to repress (p50 homodimer) or stimulate (p50/p65 heterodimer) transcription, this supports the hypothesis that OFQ/N could modulate immune function through NFκB by repressing as well as stimulating transcriptional activity. The OFQ/N-induced increase of NFκB DNA binding activity was concentration-dependent, NOPr-specific, and PKC-dependent. SH-SY5Y cells are known to express PKC α, β, δ, ε, and ζ isoforms (Turner et al. 1994; Zeidman et al. 1999). Since OFQ/N-induced NFκB binding was completely inhibited by Che but inhibited only 50% by Gö6976 (selective for α, β, and γ isoforms), it appears that OFQ/N-mediated NFκB DNA binding involves activation of both conventional and atypical PKC isoforms. The βγ subunits of GPCRs have been shown to utilize kinases such as PKC, protein kinase A, and AKT in the modulation of NFκB (reviewed in Ye 2001). Activated PKC leads to IKK activation, IκB degradation, and activation of NFκB. This is consistent with our findings of OFQ/N-mediated increases in NFκB nuclear accumulation and DNA binding in a PKC-dependent manner, along with another study showing that a 10-min treatment with OFQ/N increased IKK phosphorylation (Liu and Wong 2005). Finally, OFQ/N increased NFκB transcriptional activity over the same time frame and similar potency as binding to DNA. The increased transcriptional activity demonstrated by the dual-luciferase assay is particularly important, confirming that the OFQ/N-induced NFκB dimer contains at least the p65 subunit trans-activation domain, which will further translate into NFκB-mediated gene transcription.

NFκB is a critical transcription factor that affects over 100 genes in the CNS as well as the immune system. By increasing NFκB transcriptional activity, OFQ/N can provide a potential mechanism for many of the immune system effects, including OFQ/N modulation of T cell function (Waits et al. 2004; Easten et al. 2009), induction of neutrophil chemotaxis (Serhan et al. 2001), inhibition of antibody formation (Anton et al. 2010), and downregulation of pro-inflammatory cytokines in the spinal cord, astrocytes, and splenocytes (Fu et al. 2007; Miller and Fulford 2007).

Pro- and anti-inflammatory cytokines modulate the nociceptive sensitivity in neurons, learning, memory, neuronal development, differentiation, proliferation, and survival (McAfoose and Baune 2009; Uceyler et al. 2009; Casazza et al. 2010; Spooren et al. 2011). NFκB has also been shown to stimulate either apoptosis or neuronal survival in SH-SY5Y cells (Bian et al. 2001; Bui et al. 2001). NFκB has been implicated to play an important role in both the physiology and pathophysiology of the brain through the action of cytokines, such as IL-6, IL-1, IL-10, and TNFα (Khalaf et al. 2010; Park and Bowers 2010; Levenson et al. 2006). Altered expression of TNFα and IL-6 has been found in both Alzheimer's and Parkinson's disease (Boulanger 2009; Park and Bowers 2010; Spooren et al. 2011). Elevated levels of TNFα and IL-1 have been found in major depressive disorder patients and other severe mood disorders. Abnormal levels of these cytokines have also been associated with cognitive deficits and an increased risk for depression (Khairova et al. 2009).

IL-6 modulates astrocyte proliferation, chemotaxis, and secretion of inflammatory factors such as chemokines, cytokines, and prostaglandins (reviewed in Spooren et al. 2011). OFQ/N has been shown to downregulate IL-6 (Fu et al. 2007), in a PKC-dependent manner (Feng et al. 2010; Hao et al. 2010). These studies are consistent with our findings that OFQ/N-induced activation of NFκB is PKC-dependent (Fig. 4).

Interestingly, OFQ/N and NOPr are expressed in the brain as early as 16 weeks gestation in humans (Neal et al. 2001), potentially enabling OFQ/N to modulate brain development through cytokines in an NFκB-dependent manner. The potential effect of OFQ/N and NFκB on immune function becomes even more important given the developments in brain-immune interactions, as well as cross-talk between neurons and neuroimmune cells, such as astrocytes and glia. Therefore, OFQ/N activation of NFκB may be one mechanism by which OFQ/N modulates processes in the immune and central nervous systems.

Acknowledgments

The research results discussed in this publication were made possible, in part, by the OHRS award for project number HR08-152 from the Oklahoma Center for the Advancement of Science and Technology, grant number R01DA017380 from the National Institute on Drug Abuse, and the Department of the Army (W81XWH-09-1-0443; The U.S. Army Medical Research Acquisition Activity, 820 Chandler Street, Fort Detrick MD 21702-5014 is the awarding and administering acquisition office). The information and interpretations included in this manuscript do not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred. The authors would like to thank Andrew Mansour and Ryan Webb for excellent technical and statistical assistance, respectively.

References

Anton B, Leff P, Meissler JJ, Calva JC, Acevedo R, Salazar A, Matus M, Flores A, Martinez M, Adler MW, Gaughan JP, Eisenstein TK. Nociceptin/orphanin FQ suppresses adaptive immune responses in vivo and at picomolar levels in vitro. J Neuroimmune Pharmacol. 2010; 5:143–154. [PubMed: 20119853]

Arjomand J, Cole S, Evans CJ. Novel orphanin FQ/nociceptin transcripts are expressed in human immune cells. J Neuroimmunol. 2002; 130:100–108. [PubMed: 12225892]

Bian X, McAllister-Lucas LM, Shao F, Schumacher KR, Feng Z, Porter AG, Castle VP, Opipari AW Jr. NF-kappa B activation mediates doxorubicin-induced cell death in N-type neuroblastoma cells. J Biol Chem. 2001; 276:48921–48929. [PubMed: 11679590]

Bidlack JM, Khimich M, Parkhill AL, Sumagin S, Sun B, Tipton CM. Opioid receptors and signaling on cells from the immune system. J Neuroimmune Pharmacol. 2006; 1:260–269. [PubMed: 18040803]

Boulanger LM. Immune proteins in brain development and synaptic plasticity. Neuron. 2009; 64:93–109. [PubMed: 19840552]

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

Casazza K, Hanks LJ, Alvarez JA. Role of various cytokines and growth factors in pubertal development. Med Sport Sci. 2010; 55:14–31. [PubMed: 20956857]

Catley MC, Cambridge LM, Nasuhara Y, Ito K, Chivers JE, Beaton A, Holden NS, Bergmann MW, Barnes PJ, Newton R. Inhibitors of protein kinase C (PKC) prevent activated transcription: role of events downstream of NF-kappaB DNA binding. J Biol Chem. 2004; 279:18457–18466. [PubMed: 14976190]

Chen YL, Law PY, Loh HH. Nuclear factor kappaB signaling in opioid functions and receptor gene expression. J Neuroimmune Pharmacol. 2006; 1:270–279. [PubMed: 18040804]

Duran A, Diaz-Meco MT, Moscat J. Essential role of RelA Ser311 phosphorylation by zetaPKC in NF-kappaB transcriptional activation. EMBO J. 2003; 22:3910–3918. [PubMed: 12881425]

Easten KH, Harry RA, Purcell WM, McLeod JD. Nociceptin-induced modulation of human T cell function. Peptides. 2009; 30:926–934. [PubMed: 19428771]

Feng W, Song Y, Chen C, Lu ZZ, Zhang Y. Stimulation of adenosine A(2B) receptors induces interleukin-6 secretion in cardiac fibroblasts via the PKC-delta-P38 signalling pathway. Br J Pharmacol. 2010; 159:1598–1607. [PubMed: 20050850]

Finley MJ, Happel CM, Kaminsky DE, Rogers TJ. Opioid and nociceptin receptors regulate cytokine and cytokine receptor expression. Cell Immunol. 2008; 252:146–154. [PubMed: 18279847]

Fiset ME, Gilbert C, Poubelle PE, Pouliot M. Human neutrophils as a source of nociceptin: a novel link between pain and inflammation. Biochemistry. 2003; 42:10498–10505. [PubMed: 12950177]

Fu X, Zhu ZH, Wang YQ, Wu GC. Regulation of proinflammatory cytokines gene expression by nociceptin/orphanin FQ in the spinal cord and the cultured astrocytes. Neuroscience. 2007; 144:275–285. [PubMed: 17069983]

Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. Oncogene. 2006; 25:6680–6684. [PubMed: 17072321]

Halford WP, Gebhardt BM, Carr DJ. Functional role and sequence analysis of a lymphocyte orphan opioid receptor. J Neuroimmunol. 1995; 59:91–101. [PubMed: 7797625]

Hao F, Tan M, Wu DD, Xu X, Cui MZ. LPA induces IL-6 secretion from aortic smooth muscle cells via an LPA1-regulated, PKC-dependent, and p38alpha-mediated pathway. Am J Physiol Heart Circ Physiol. 2010; 298:H974–H983. [PubMed: 20044439]

Happel C, Kutzler M, Rogers TJ. Opioid-induced chemokine expression requires NF-{kappa}B activity: the role of PKC {zeta}. J Leukoc Biol. 2011; 89:301–309. [PubMed: 20952659]

Khairova RA, Machado-Vieira R, Du J, Manji HK. A potential role for pro-inflammatory cytokines in regulating synaptic plasticity in major depressive disorder. Int J Neuropsychopharmacol. 2009; 12:561–578. [PubMed: 19224657]

Khalaf H, Jass J, Olsson PE. Differential cytokine regulation by NF-kappaB and AP-1 in Jurkat T-cells. BMC Immunol. 2010; 11:26. [PubMed: 20507572]

Kuzmin A, Madjid N, Johansson B, Terenius L, Ogren SO. The nociceptin system and hippocampal cognition in mice: a pharmacological and genetic analysis. Brain Res. 2009; 1305 Suppl:S7–S19. [PubMed: 19782658]

Lambert DG. The nociceptin/orphanin FQ receptor: a target with broad therapeutic potential. Nat Rev Drug Discov. 2008; 7:694–710. [PubMed: 18670432]

Levenson, J.; Pizzi, M.; Sweatt, JD. NF-KB/Rel transcription factor family. Austin: Landes Bioscience; 2006.

Li S, Wang L, Dorf ME. PKC phosphorylation of TRAF2 mediates IKKalpha/beta recruitment and K63-linked polyubiquitination. Mol Cell. 2009; 33:30–42. [PubMed: 19150425]

Liu AM, Wong YH. Mu-opioid receptor-mediated phosphorylation of IkappaB kinase in human neuroblastoma SH-SY5Y cells. Neurosignals. 2005; 14:136–142. [PubMed: 16088228]

Lou LG, Ma L, Pei G. Nociceptin/orphanin FQ activates protein kinase C, and this effect is mediated through phospholipase C/Ca2+pathway. Biochem Biophys Res Commun. 1997; 240:304–308. [PubMed: 9388473]

Lu ZG, Liu H, Yamaguchi T, Miki Y, Yoshida K. Protein kinase Cdelta activates RelA/p65 and nuclear factor-kappaB signaling in response to tumor necrosis factor-alpha. Cancer Res. 2009; 69:5927–5935. [PubMed: 19549902]

Mandyam CD, Thakker DR, Christensen JL, Standifer KM. Orphanin FQ/nociceptin-mediated desensitization of opioid receptor-like 1 receptor and mu opioid receptors involves protein kinase C: a molecular mechanism for heterologous cross-talk. J Pharmacol Exp Ther. 2002; 302:502–509. [PubMed: 12130708]

Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D, Schachtele C. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem. 1993; 268:9194–9197. [PubMed: 8486620]

Matsubara M, Tamura T, Ohmori K, Hasegawa K. Histamine H1 receptor antagonist blocks histamine-induced proinflammatory cytokine production through inhibition of Ca2+-dependent protein kinase C, Raf/MEK/ERK and IKK/I kappa B/NF-kappa B signal cascades. Biochem Pharmacol. 2005; 69:433–449. [PubMed: 15652235]

McAfoose J, Baune BT. Evidence for a cytokine model of cognitive function. Neurosci Biobehav Rev. 2009; 33:355–366. [PubMed: 18996146]

Memet S. NF-kappaB functions in the nervous system: from development to disease. Biochem Pharmacol. 2006; 72:1180–1195. [PubMed: 16997282]

Miller TR, Fulford AJ. Regulation of nociceptin/orphaninFQ secretion by immune cells and functional modulation of interleukin-2. Peptides. 2007; 28:2243–2252. [PubMed: 17950489]

Mut M, Amos S, Hussaini IM. PKC alpha phosphorylates cytosolic NF-kappaB/p65 and PKC delta delays nuclear translocation of NF-kappaB/p65 in U1242 glioblastoma cells. Turk Neurosurg. 2010; 20:277–285. [PubMed: 20669099]

Neal CR Jr, Akil H, Watson SJ Jr. Expression of orphanin FQ and the opioid receptor-like (ORL1) receptor in the developing human and rat brain. J Chem Neuroanat. 2001; 22:219–249. [PubMed: 11719021]

Park KM, Bowers WJ. Tumor necrosis factor-alpha mediated signaling in neuronal homeostasis and dysfunction. Cell Signal. 2010; 22:977–983. [PubMed: 20096353]

Peluso J, LaForge KS, Matthes HW, Kreek MJ, Kieffer BL, Gaveriaux-Ruff C. Distribution of nociceptin/orphanin FQ receptor transcript in human central nervous system and immune cells. J Neuroimmunol. 1998; 81:184–192. [PubMed: 9521621]

Peluso J, Gaveriaux-Ruff C, Matthes HW, Filliol D, Kieffer BL. Orphanin FQ/nociceptin binds to functionally coupled ORL1 receptors on human immune cell lines and alters peripheral blood mononuclear cell proliferation. Brain Res Bull. 2001; 54:655–660. [PubMed: 11403992]

Perdew, GH.; Vanden Heuval, JP.; Peters, JM. Regulation of gene expression: molecular mechanisms. Humana, Totowa: 2006.

Serhan CN, Fierro IM, Chiang N, Pouliot M. Cutting edge: nociceptin stimulates neutrophil chemotaxis and recruitment: inhibition by aspirin-triggered-15-epi-lipoxin A4. J Immunol. 2001; 166:3650–3654. [PubMed: 11238602]

Shinohara H, Yasuda T, Aiba Y, Sanjo H, Hamadate M, Watarai H, Sakurai H, Kurosaki T. PKC beta regulates BCR-mediated IKK activation by facilitating the interaction between TAK1 and CARMA1. J Exp Med. 2005; 202:1423–1431. [PubMed: 16301747]

Smith, M.; Delbary-Gossart, S. Electrophoretic mobility shift assay (EMSA). Humana, Totowa: 2001.

Spooren A, Kolmus K, Laureys G, Clinckers R, De Keyser J, Haegeman G, Gerlo S. Interleukin-6, a mental cytokine. Brain Res Rev. 2011 (in press).

Thakker DR, Standifer KM. Induction of G protein-coupled receptor kinases 2 and 3 contributes to the cross-talk between mu and ORL1 receptors following prolonged agonist exposure. Neuropharmacology. 2002a; 43:979–990. [PubMed: 12423667]

Thakker DR, Standifer KM. Orphanin FQ/nociceptin blocks chronic morphine-induced tyrosine hydroxylase upregulation. Brain Res Mol Brain Res. 2002b; 105:38–46. [PubMed: 12399106]

Thakker DR, Altememi GF, Mandyam CD, Ibrahim H, Ramirez VI, Standifer KM. Naloxone benzoylhydrazone activates extracellular signal-regulated protein kinases and modulates nociceptin opioid peptide receptor activity. Am J Pharmacol Toxicol. 2007; 2:51–59.

Tian L, Rauvala H, Gahmberg CG. Neuronal regulation of immune responses in the central nervous system. Trends Immunol. 2009; 30:91–99. [PubMed: 19144568]

Turner NA, Rumsby MG, Walker JH, McMorris FA, Ball SG, Vaughan PF. A role for protein kinase C subtypes alpha and epsilon in phorbol-ester-enhanced K(+)- and carbachol-evoked noradrenaline release from the human neuroblastoma SH-SY5Y. Biochem J. 1994; 297(Pt 2):407–413. [PubMed: 8297348]

Uceyler N, Schafers M, Sommer C. Mode of action of cytokines on nociceptive neurons. Exp Brain Res. 2009; 196:67–78. [PubMed: 19290516]

Viatour P, Merville MP, Bours V, Chariot A. Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation. Trends Biochem Sci. 2005; 30:43–52. [PubMed: 15653325]

Waits PS, Purcell WM, Fulford AJ, McLeod JD. Nociceptin/orphanin FQ modulates human T cell function in vitro. J Neuroimmunol. 2004; 149:110–120. [PubMed: 15020071]

Wick MJ, Minnerath SR, Roy S, Ramakrishnan S, Loh HH. Expression of alternate forms of brain opioid ‘orphan’ receptor mRNA in activated human peripheral blood lymphocytes and lymphocytic cell lines. Brain Res Mol Brain Res. 1995; 32:342–347. [PubMed: 7500847]

Ye RD. Regulation of nuclear factor kappaB activation by G-protein-coupled receptors. J Leukoc Biol. 2001; 70:839–848. [PubMed: 11739545]

Zaveri NT, Waleh N, Toll L. Regulation of the prepronociceptin gene and its effect on neuronal differentiation. Gene. 2006; 384:27–36. [PubMed: 16935438]

Zeidman R, Pettersson L, Sailaja PR, Truedsson E, Fagerstrom S, Pahlman S, Larsson C. Novel and classical protein kinase C isoforms have different functions in proliferation, survival and differentiation of neuroblastoma cells. Int J Cancer. 1999; 81:494–501. [PubMed: 10209967]

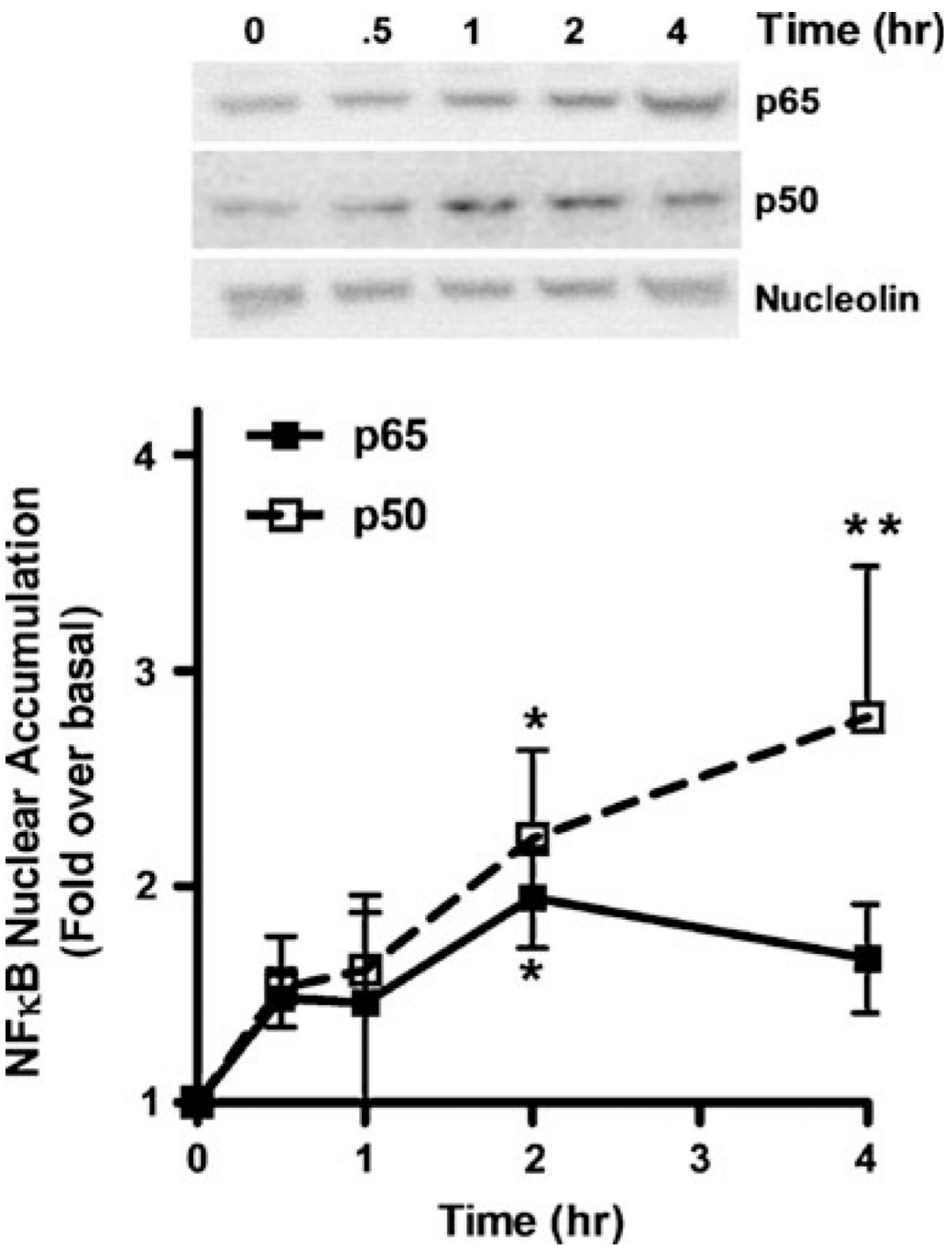

Fig. 1.

OFQ/N increases NFκB p65 and p50 nuclear accumulation over time. SH-SY5Y cells were treated with 1 μM OFQ/N for the indicated time. Nuclear fractions were extracted and protein levels determined by immunoblotting with antibodies specific for NFκB p65, p50, or nucleolin. NFκB immunoreactivity was normalized to nucleolin (loading control) and expressed as fold over basal. Basal values were 0.9±0.4 for p50 and 0.4±0.2 for p65. OFQ/N increases nuclear accumulation of p65 and p50 by 2 h (1.9- and 2.2-fold, respectively; *p<0.05, **p<0.01, compared to basal). Representative blot and mean densitometric analysis ± SEM of three to five experiments are shown

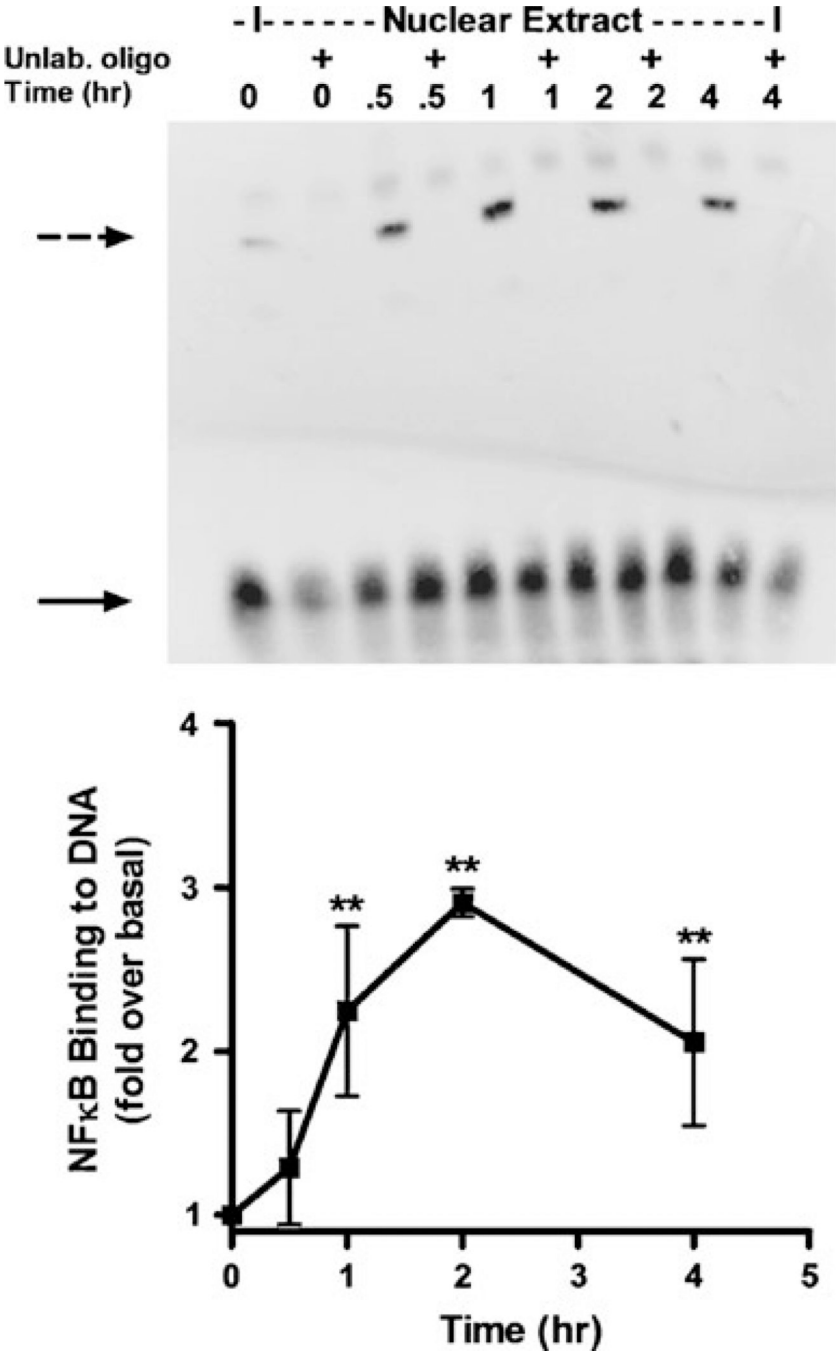

Fig. 2.

OFQ/N increases NFκB binding to DNA. SH-SY5Y cells were treated with 1 μM OFQ/N for the indicated times and nuclear fractions extracted. Binding of the biotinylated oligonucleotides (ODN) to targets in the nuclear extract expressing the NFκB consensus sequence is indicated by the *dashed arrow*; free probe is indicated by the *solid arrow*. Bound probe was normalized to free probe and expressed as fold over basal (basal values=0.2±0.1). OFQ/N treatment maximally increases NFκB binding to DNA 2.9-fold by 2 h (**p<0.01, compared to basal). Excess, unlabeled ODN (200×) completely blocks NFκB binding to DNA. Representative EMSA blot and mean densitometric analysis±SEM of three to six experiments are shown

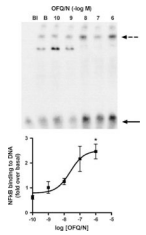

Fig. 3.

OFQ/N treatment increases NFκB binding to DNA in a concentration-dependent manner. SH-SY5Y cells were treated for 2 h with various concentrations of OFQ/N and nuclear fractions extracted. Bound fraction and free probe are labeled by *dashed* and *solid arrows*, respectively. Bound probe was normalized to free probe and expressed as fold over basal (basal values=0.3±0.1). OFQ/N treatment increases NFκB binding to DNA, with E_{max} at 1 μM (**p<0.01, compared to basal), with an EC_{50} of 24 nM ($logEC_{50}$=−7.6±0.5). Representative EMSA blot and mean densitometric analysis±SEM of three to five experiments are shown. *BL* probe in the absence of nuclear extract, *B* basal

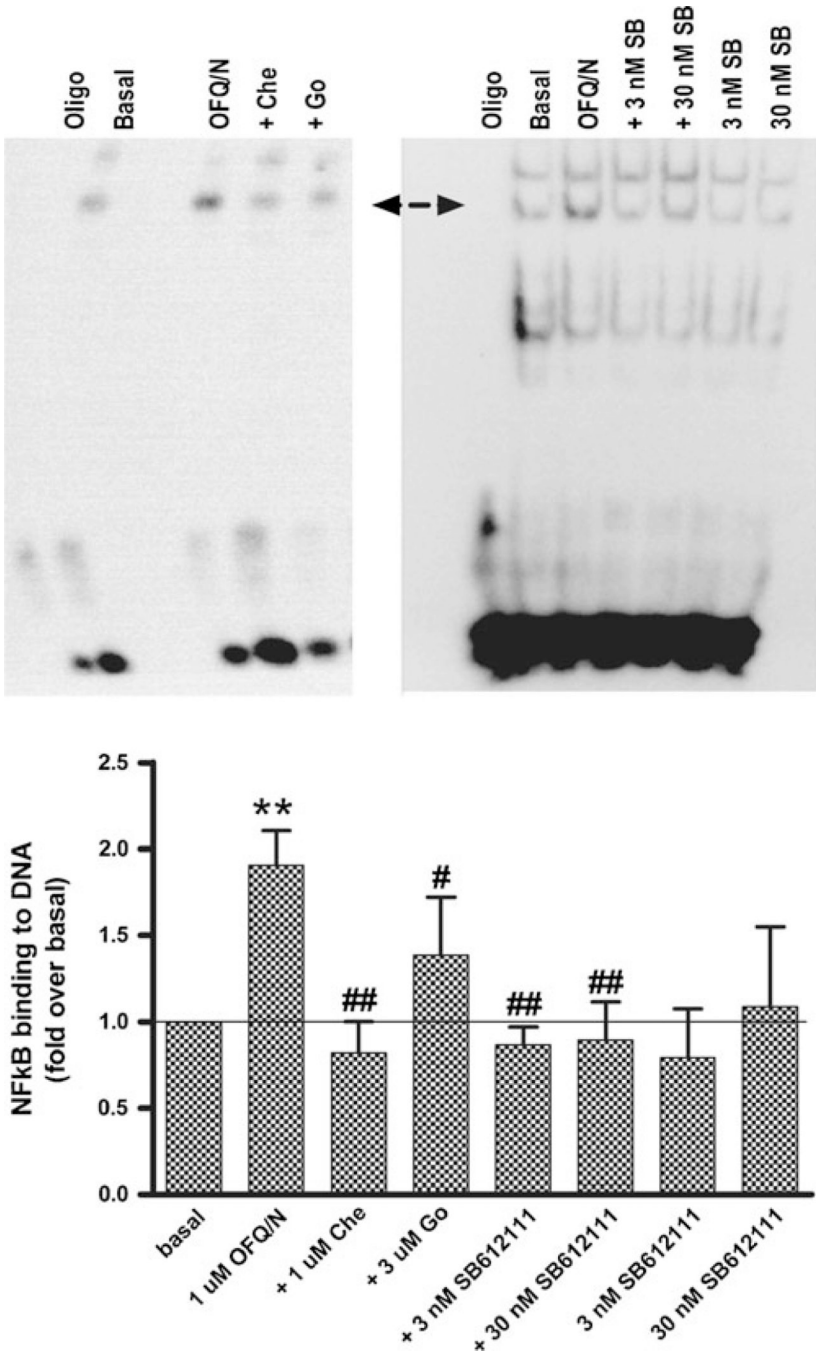

Fig. 4.

PKC inhibitors and NOPr antagonist block OFQ/N-induced NFκB binding. SH-SY5Y cells were treated for 2 h with 1 μM OFQ/N in the presence or absence of PKC inhibitors Che (1 μM) or Gö6976 (3 μM) or NOPr antagonist SB612111 (3 or 30 nM). Bound fraction is labeled by *dashed arrow*. Bound probe was normalized to free probe and expressed as fold over basal (basal values=1.0±0.4). Both PKC inhibitors reduced NFκB binding to DNA in nuclear extracts, as did the NOPr antagonist (**p*<0.01 compared to basal; #*p*<0.05, ##*p*<0.01 compared to OFQ/N). Representative EMSA blot and mean densitometric analysis±SEM of three to seven experiments are shown

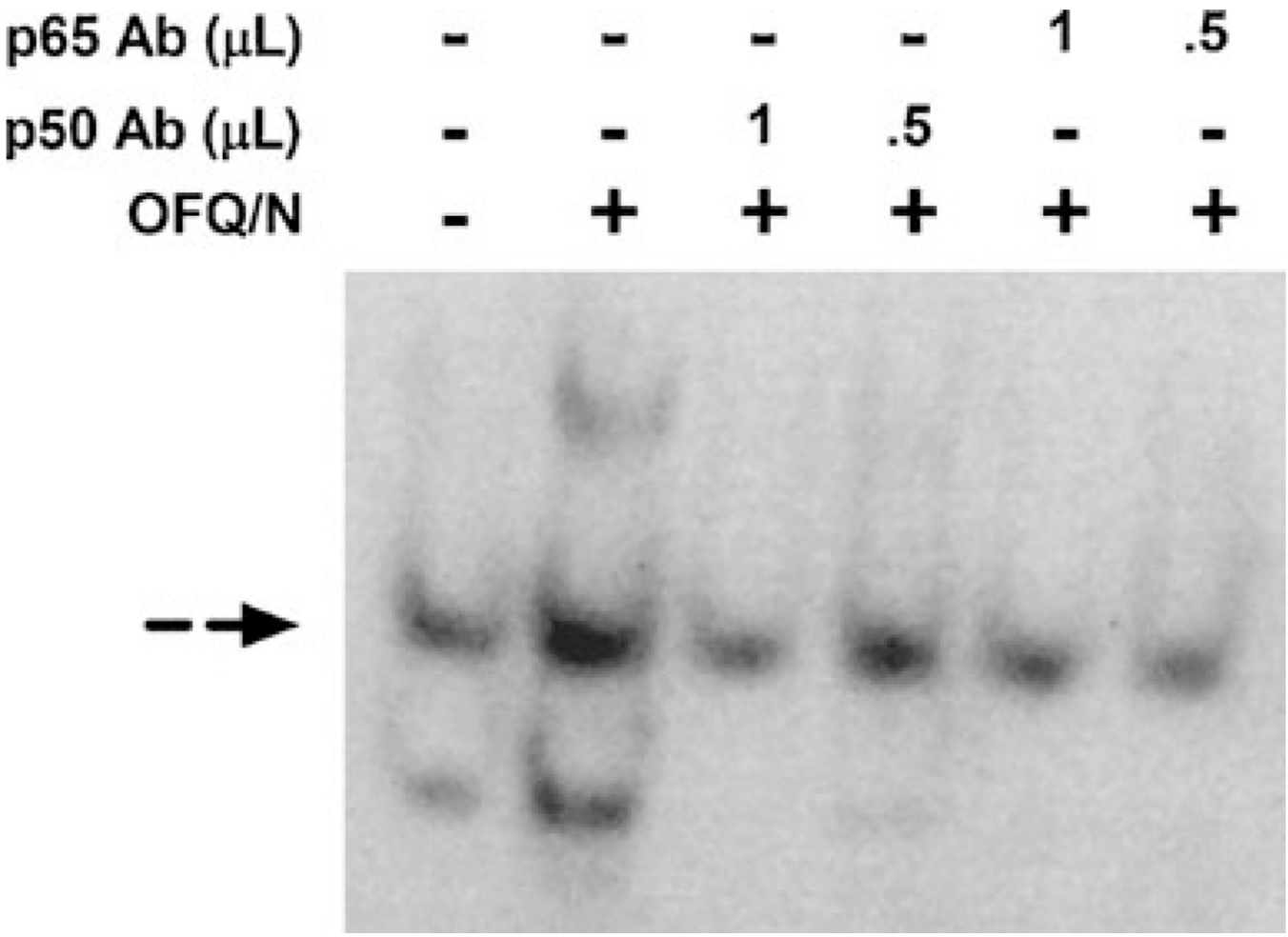

Fig. 5.

OFQ/N induces NFκB p65 and p50 subunits binding to DNA. SH-SY5Y cells were treated with 1 μM OFQ/N for 2 h and nuclear fractions extracted. The indicated NFκB subunit antibodies were added to the biotinylated DNA ODN. Bound fraction that undergoes supershift in the presence of the antibodies is indicated by the *dashed arrow*. Both p50 and p65 antibodies blocked OFQ/N-induced binding of NFκB to DNA. Representative EMSA blot of three experiments is shown

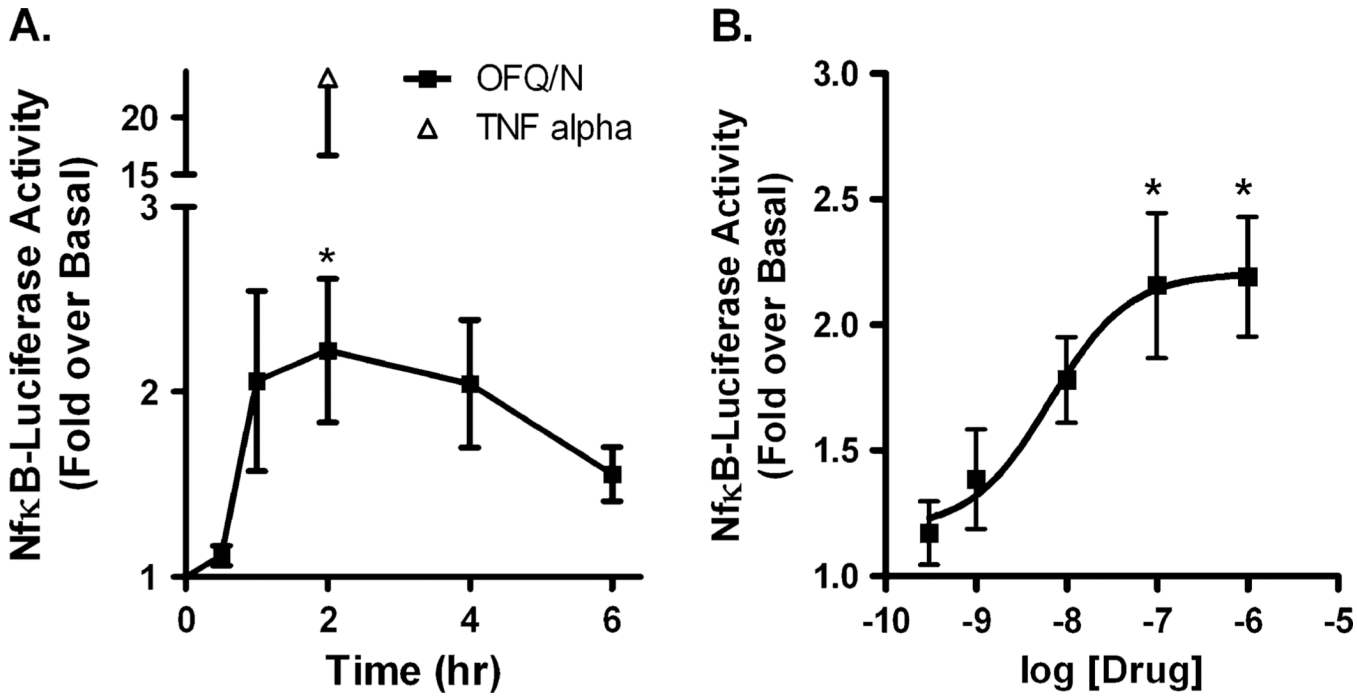

Fig. 6.

OFQ/N induces transcriptional activation of an NFκB reporter gene. SH-SY5Y cells were transfected as described in methods. Luminescence measured from NFκB/Firefly Luciferase activity was normalized to internal control thymidine kinase/Renilla Luciferase activity and expressed as fold over basal (basal values=0.03±0.01). TNFα, a robust activator of NFκB, served as the positive control. OFQ/N treatment increases transcriptional activity of NFκB reporter gene in **a** a time-dependent (*p<0.05, compared to basal) and **b** a concentration-dependent manner (logEC_{50}=−8.2±0.5; *p<0.05, compared to 300 pM). Data expressed as mean ± SEM of three to eight experiments