Early Growth Response 2 (Egr2) Induction by Neuronal Activity-dependent Nuclear Factor Kappa B (NF-κB) Activation

By

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A thesis submitted to the Faculty of Graduate Studies
in partial fulfilment of the requirements for the degree of

Master of Science

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Abstract

Nuclear factor kappa B (NF-κB) mediated signalling is complex and plays a critical role in many biological processes. Investigators have reported that NF-κB is activated during the induction of long term potentiation (LTP), a proposed mechanism for memory encoding, and may be a requirement for synaptic plasticity and memory. In this study, mRNA extracted from hippocampal slices of NF-kB p50 knockout mice and its littermate before and after induction of LTP was analyzed using DNA microarray analysis (Affymetrix GeneChip® Mouse Genome 430 2.0) to explore candidate target genes of NF-kB in LTP. The early growth response 2 (*Egr-2*) was identified as one putative NF-kB target gene.

Egr-2 mRNA and protein analysis of primary cortical neurons and HeLa cells chemically stimulated with Tumor Necrosis Factor α (TNFα) to activate the NF-kB signalling pathway confirmed the microarray results. In addition, examination of the *Egr-2* promoter sequence for NF-kB binding sites using chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assays (EMSA) confirmed promoter occupancy and specificity of binding *in vivo*, respectively. These data suggest that *Egr-2* expression level is controlled by direct transcriptional activity of the NF-kB transcription factor.

Acknowledgments

I would like to thank my supervisor, Dr. Benedict Albensi and the members of my advisory committee, Dr. Don Smyth, Dr. David Eisenstat and Dr. Gordon Glazner for the mentorship they have provided for me over the course of my studies. Their guidance and has been invaluable in the development of my research skills. I am specifically grateful to Dr. Eisenstat for his generosity in sharing his laboratory equipment and his insight into the world of molecular research.

My special thanks go to Dr. Kensuke Oikawa, who has been instrumental in teaching me how to properly plan and execute my experiments. I appreciate the significant amount of time he has spent mentoring me. Thank you to the technical staff of Dr. Albensi and Dr. Eisenstat's labs whose efforts made working in the lab more enjoyable.

I would like to thank the University of Manitoba, Manitoba Health Research Council, the Alzheimer's Society of Manitoba, Scottish Rite Charitable Foundation, and the Canadian Institute of Health Research for their financial support.

Last, but not least, thanks to my wonderful husband and best friend, Farhoud. His positive attitude and support have always kept me motivated. Thank you for encouraging me to be the best person I could possibly be. Without your love and support, I could not have been what I am.

Dedication

This thesis is dedicated to my family and friends

for their unconditional love and support

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List of Abbreviations

α-HRP	*Anti-Horse radish peroxidase*
®	*Registered sign*
°C	*Degree Celsius*
µg	*Microgram*
µl	*Microlitre*
ACPD	*Aminocyclopentano-l,3-dicarboxylic acid*
ATP	*Adenosine triphosphate*
bp	*Basepair*
cAMP	*Cyclic Adenosine Monophosphate*
cDNA	*Complementary DNA*
cm	*Centimeter*
CO2	*Carbon dioxide*
DNA	*Deoxyribonucleic acid*
dNTP	*2'- Deoxyribonucleoside 5'-triphosphate*
EDTA	*Ethylene Di-amine Tetra Acetic acid*
g	*Gram*
IEG	*Immediate Early Gene*

IgG	*Immunoglobulin G*
IL1	*Interleukin 1*
kb	*Kilobase*
kDa	*Kilodalton*
kg	*Kilogram*
LTP	*Long Term Potentiation*
LTD	*Long Term Depression*
mg	*Milligram*
mGluR5	*Metabotropic Glutamate Receptor 5*
ml	*Milliliter*
mm	*Millimeter*
mM	*Millimolar*
mRNA	*Messenger ribonucleic acid*
ng	*Nanogram*
nm	*Nanometer*
NMDA	*N-methyl-D-Aspartate*
OD	*Optical Density*
rpm	*Revolutions per minute*
SDS	*Sodium Dodecyl Sulphate*
Taq	*DNA dependent DNA polymerase from*
TEMED	*N,N,N'N' - tetramethylethylethylenediamene*
TNF-α	*Tumor necrosis factor - alpha*

V	*Volts*
V/V	*Volume/volume ratio*

Introduction

Early growth response (EGR) genes are immediate early genes (IEGs) that encode transcription factors, proteins that bind to specific DNA sequences and control the transcription of their target genes. Moreover, IEGs are genes that are rapidly and transiently induced in various cell types independent of *de novo* protein synthesis (Curran & Morgan, 1987). Diverse intracellular messenger systems activate these genes linking membrane events to the nucleus. A subclass of immediate early genes in the nervous system encode inducible transcription factors that can act as third messengers coupling neurotransmission to a cascade of altered gene expression (Morgan & Curran, 1988). Members of the EGR family were first identified by several laboratories searching for genes whose expression was induced by growth factors. The family of EGRs is composed of four members: *Egr-1*, *Egr-2*, *Egr-3*, and *Egr-4* (Gashler & Sukhatme, 1995; Mages, Stamminger, Rilke, Bravo, & Kroczek, 1993).

1.1 Domain structure of Egr-2

All four EGR proteins have a highly conserved DNA-binding domain composed of three zinc-finger motifs. Zinc fingers, proposed to function in eukaryotic protein-nucleic acid interaction, are structural motifs found in several classes of proteins. These classes include the Cys4His5 group, the Cys2HisCys group and the Cys2His2 group (Beato, Chalepakis, Schauer, & Slater, 1989; Engelke, Ng, Shastry, & Roeder, 1980; Kim & Hudson, 1992; J. Miller, McLachlan, & Klug, 1985). The EGR proteins belong to the Cys2His2 class proteins. The Cys2His2 class zinc fingers are composed of tandemly repeated units of 28-30 amino acids containing two cysteine and two histidine residues that

fold around a tetrahedrally coordinated zinc ion and form a finger-like projection which then binds DNA in a sequence-specific manner (Lemaire, Revelant, Bravo, & Charnay, 1988). Cys2His2 zinc-finger motifs recognize a nine-base-pair consensus sequence (GCG (G/T) GGGCG), also known as GSG or EBS (Ets Binding Sites) motif, in the promoter regions of the target genes (X. M. Cao et al., 1990; Chavrier et al., 1988; B. Christy & Nathans, 1989; Crosby, Puetz, Simburger, Fahrner, & Milbrandt, 1991; Lemaire et al., 1990; Patwardhan et al., 1991).

The human *Egr-2* gene, also known as *Krox-20*, located on human chromosome 10q21.1 - q22.1, spans 4.3 kb and contains two coding exons. The human *Egr-2* and mouse *Krox-20* gene, share an overall homology of 75% at the nucleotide level: 87% in the coding region and 89% in the zinc-finger domain (Beckmann & Wilce, 1997; Joseph et al., 1988). The zinc fingers are also sufficient for nuclear localization of Egr-2 (Vesque & Charnay, 1992). Figure 1 shows a schematic structure of *Krox-20.*

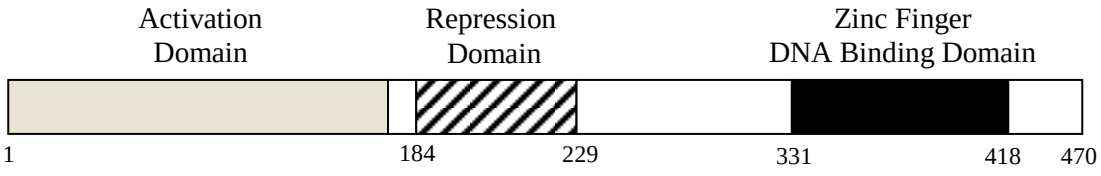

Figure 1: Schematic structure of murine *Krox-20*. Three functional domains have been defined: an N-terminal activation domain (residues 1-184), a repression domain (193-229), and a DNA-binding domain composed of three zinc fingers (331-418).

1.2 Function of Egr-2 protein

1.2.1 Knock out mouse experiments

To determine the role of *Egr-2* gene in normal physiological function, development of the animal and the phenotype caused by their deletion, *Krox-20* knock-out mutant mice (*Egr-2*$^{-/-}$) were established (S. L. Lee, Tourtellotte, Wesselschmidt, & Milbrandt, 1995; Schneider-Maunoury et al., 1993). *Egr-2*$^{-/-}$ mice showed a noticeable reduction or lack of rhombomeres 3 and 5 and abnormal segmentation of hindbrain during later stages of development (Schneider-Maunoury, et al., 1993). Although this phenotype was not lethal during embryonic development, about 50% of the *Egr-2* $^{-/-}$ mice died shortly after birth (48 h). Surviving *Egr-2* $^{-/-}$ mice showed a trembling phenotype, hypomyelination of the peripheral nervous system, and their Schwann cells were blocked at an early stage of differentiation. However, heterozygous *Egr-2* $^{+/-}$ mice, showed no neuropathologic abnormalities or functional impairment (Topilko et al., 1994).

1.2.2 Target genes

Potential EGR binding sites were identified in the upstream regions of synaptophysin and synaptobrevin II genes (Thiel, 1993), c-Ha-Ras, Int-2, human c-AN and histone H3.3, mouse metallothionein, JunB and JunD (B. Christy & Nathans, 1989). In particular, binding of EGR-2 transcription factor to the promoter regions of tumor necrosis factor α (TNFα) (Kramer, Meichle, Hensel, Charnay, & Kronke, 1994), insulin-like growth factor II, and the HoxA4 genes was reported (Chavrier et al., 1990; Holthuizen, Van Dijk,

Rodenburg, Koonen-Reemst, & Sussenbach, 1993). It is clear that EGR transcription factors are capable of regulating many genes encoding diverse proteins within different cell types. However, much more work is required to determine how EGR proteins regulate these genes *in vivo* and establish their function.

1.3 Physiological regulation of Egr-2 protein in Central Nervous System

The expression of immediate early genes (IEGs), including the EGR family, has been extensively studied in the mammalian central nervous system (CNS). Two major reasons for these studies are:

1- IEG activation can be used to map the activation of neuronal pathways (Sagar, Sharp, & Curran, 1988).

2- Understanding the induction of IEG-encoded transcription factors may provide a key for determining the gene expression program leading to the long-term effects of the neuronal stimulation.

Low basal expression levels of inducible transcription factors are detected in unstimulated neurons, revealing ongoing physiological processes in the animal. As a result, the expression of inducible transcription factors was used as a functional assay for the neuronal inputs and receptor systems involved in brain function. Also, it was thought that inducible transcription factors had a role in the development of the animal through control of cell growth and proliferation (X. M. Cao, et al., 1990; Sukhatme et al., 1988). The basal, developmental and inducible patterns of *Egr-2* expression are described below.

1.3.1 Basal expression

Northern blot experiments in rat brain showed the *Egr-2* mRNA as a 2.8kb transcript (K. J. Mack & Mack, 1992). To demonstrate the presence of *Egr-2* mRNA in the cortex and white matter tracts of the corpus callosum, *in situ* hybridisation was used and both nuclear and cytoplasmic localization for *Egr-2* was observed (K. Mack, Day, Milbrandt, & Gottlieb, 1990). Primary afferent neurons of sacral, lumbar, thoracic and cervical dorsal root ganglia showed some cytoplasmic staining for *Egr-2* (Herdegen, Kiessling et al., 1993). Cerebral cortex, hippocampus and diencephalic structures including the thalamus and hypothalamus show nuclear Egr-2 localization.

Egr-2 protein expression was observed in occasional brain stem nuclei, basal ganglia, granule cells of the cerebellum and cerebellar white matter (K. J. Mack & Mack, 1992). In addition, striatum, fornix, medial and lateral amygdala, olfactory bulb and hypothalamus (dorsomedial, medial tuberal and suprachiasmatic nuclei) contained *Egr-2* protein (Herdegen, Kiessling, et al., 1993). Distinct, basal, nuclear expression of *Egr-2* is prominent in layers II and III of the neocortex but sparse in layers IV and VI. In the hippocampus, pyramidal cells of the CA1-CA3 regions showed staining for *Egr-2* protein. However, only light staining was seen in the Dentate Gyrus (Herdegen, Brecht et al., 1993; K. Mack, et al., 1990; Williams et al., 1995).

1.3.2 Developmental expression

At embryonic day 9.5, *Egr-2* mRNA was expressed in rhombomeres 3 and 5 of the developing mouse hindbrain. Also, there was an evidence for *Egr-2* mRNA expression in

early neural crest cells and then neural-crest derived boundary caps as well as glial components of cranial and spinal ganglia (Wilkinson, Bhatt, Chavrier, Bravo, & Charnay, 1989). The Egr-2 protein, together with members of the Hox transcription factor family, is considered to have a role in the regulation of hindbrain segmentation and development (Beckmann & Wilce, 1997).

1.3.3 Induction of *Egr-2* expression in the Central Nervous System

The major excitatory amino acid neurotransmitter in the brain, glutamate, acts on both ionotropic and metabotropic subtypes of receptors. The metabotropic glutamate receptor is activated by quisqualate, ibotenate and aminocyclopentano-l,3-dicarboxylic acid (ACPD). The ionotropic receptors are divided into three classes by preferential agonist activation: 1- *N*-methyl-D-aspartate (NMDA) receptors that are permeable to calcium and monovalent cations have been extensively studied and are thought to play a critical role in the synaptic plasticity (change in the efficacy of the synapses) underlying learning, memory and neuronal development; 2- AMPA/quisqualate receptors that are coupled to a monovalent cation channel; 3- Kainate receptors which are same as AMPA receptors coupled to a monovalent cation channel (Beckmann & Wilce, 1997).

1.3.3.1. Egr-2 protein expression

Upon Kainic acid injection, Egr-2 protein expression was increased in the dentate granule cells and followed by increases in the CA1 and CA3 subfields of the hippocampal formation. Also, significant increased Egr-2 immunoreactivity was seen in the amyg-

dala, pyriform cortex, cingulate gyrus, olfactory tubercle and layers II, III and VI of the somatosensory cortex. Lower levels of Egr-2 protein expression were observed in the striatum and retrosplenial cortex (P. Gass, Herdegen, Bravo, & Kiessling, 1994).

Even though moderate hypoxia-ischemia did not alter Egr-2 protein expression in neurons, Egr-2 protein levels were increased in macrophage-like cells lying over the CA1 pyramidal cell layer ipsilateral to the stimulus; however, it should be noted that Egr-2 protein was not altered in other non-nerve cells (Dragunow et al., 1994). Also, Egr-2 immunoreactivity in neurons of the cortex and hippocampus was increased on the contralateral side of the brain after severe hypoxia-ischemia. However, only some pale non-nuclear Egr-2 staining was seen in non-nerve cells of the cortex and hippocampus on the ipsilateral side (Dragunow, et al., 1994).

Gamma-amino butyric acid (GABA) is the major inhibitory amino acid neurotransmitter in the central nervous system. The $GABA_A$ chloride ionophores have binding sites for neuromodulators such as the barbiturates and benzodiazepines and the channel blockers, pentylenetetrazole and picrotoxin. Bicuculline, which has similar effects as picrotoxin (Saffen et al., 1988; Worley, Cole, Saffen, & Baraban, 1990), is also a $GABA_A$ antagonist. Administration of bicuculline led to elevated Egr-2 protein expression in layers II, III and IV of the somatosensory cortex, the pyriform cingulate and retrosplenial cortices, and the fornix and amygdale. However, no bicuculline-induced Egr-2 expression was observed in the hippocampus (Herdegen, Kiessling, et al., 1993).

1.3.3.2. *Egr-2* mRNA expression

When apomorphine, a dopamine receptor agonist, was injected in reserpine-treated rats, *Egr-2* mRNA expression levels were increased in the caudate, putamen, and olfactory tubercle (Bhat, Worley, Cole, & Baraban, 1992). Middle cerebral artery occlusion did increase *Egr-2* mRNA expression in the penumbra surrounding the infarct, but did not affect expression in the core region of the infarct (Collaco-Moraes, Aspey, de Belleroche, & Harrison, 1994). Focal cerebral ischemia on the other hand, depending on the severity of the insult, increased *Egr-2* mRNA transcription in the cerebral cortex and hippocampus (An, Lin, Liu, & Hsu, 1992).

Some evidence showed that up-regulation of *Egr-2* (associated with down-regulation of *Egr-1*) in the striatum occurred when the adenosine 2A receptor was inactivated in mice (L. Yu et al., 2005) and that *Egr-2*, but not other EGR members, was down-regulated in the rat cortex when metabotropic glutamate receptor 5 (mGluR5) antagonists were used (J. T. Gass & Olive, 2008). Additionally, *Egr-2* was shown to be regulated by brain-derived neurotrophic factor (BDNF)/TrkB in a CCAAT/enhancer binding proteins (C/EBPα,-β) dependent manner (Calella et al., 2007).

Upon electroconvulsive shock, *Egr-2* mRNA expression was increased in dentate gyrus and pyriform cortex (Bhat, et al., 1992). The expression of *Egr-2* mRNA was also induced after a high frequency stimulus consisting of 50 trains (Worley et al., 1993; Yamagata et al., 1994). Giving 10 trains only induced long-term potentiation (LTP), a long-lasting increase in the magnitude of the synaptic response of a neuron, but did not increase expression of the *Egr-2* mRNA (Worley, et al., 1993). However, the 50 train pa-

radigm not only produce a longer-lasting synaptic enhancement (Jeffery, Abraham, Dragunow, & Mason, 1990), but also was coupled to a long-lasting elevation of Egr-2 protein expression (Williams, et al., 1995). The induction of Egr-2 protein expression was correlated with the persistence of LTP suggesting that the Egr-2 protein may also be involved in the stabilization of LTP (the stabilization of changes in synaptic strength) (Williams, et al., 1995).

1.4 Regulation of Egr-2 protein

Each protein's expression and function is regulated at several stages. Cis-acting elements often located in the 5' upstream promoter region of the genes, regulate the transcription of the genes. These elements can be up to several kb apart or even located within the introns of the genes (Beckmann & Wilce, 1997). The mRNAs from some genes are differentially spliced with unknown controlling mechanisms; therefore, mRNA can be differentially translated into proteins and proteins are regulated by post-translational modification by such processes as phosphorylation, glycosylation, etc. Interactions with other cellular components or proteins and allosteric mechanisms can modulate the final activity of the proteins (Beckmann & Wilce, 1997). The expression of the *Egr-2* gene is regulated at all of these levels as discussed in the next sections.

1.4.1 Cis-acting elements and the second messenger system

Cis-acting elements are regions of DNA that regulate the expression of genes located on that same strand. These elements may be located in the promoter region 5' to the

gene it controls, in an intron, or in the 3' untranslated region. Data concerning the upstream promoter elements of immediate early genes, such as the EGR family, can help explain and predict which EGR proteins will respond to any given stimulus in a specified brain region or cell type (Beckmann & Wilce, 1997).

Neuronal stimuli are coupled to various intracellular second messenger cascades, for example: NMDA receptors allow the influx of Ca^{2+} and Na^{+}; activation of adenylate cyclase cause production of cyclic AMP (cAMP) via D_1 dopaminergic and β_1 adrenergic receptors; and α_1 adrenergic receptors are positively coupled to the phospholipase C cascade. cAMP and calcium ions caused phosphorylation and consequently activation of the transcription factor calcium response element-binding protein (CREB), which activated transcription of IEGs through the calcium response element (CRE) (Sheng, McFadden, & Greenberg, 1990).

Phospholipase C activation products induced the expression of immediate early genes through the serum-response factor which bound to the serum response element (SRE) (Gilman, 1988). These second messenger pathways were also subject to cross-talk at multiple levels (reviewed in Edwards, 1994). Two SREs, two Spl motifs, three potential AP-1 sites, and two possible CREs were observed in the upstream region of *Egr-2* (Chavrier et al., 1989).

1.4.2 Differential transcription

A second, short intron (100 bp) was identified in the mouse and human *Egr-2* genes immediately at the 5' of the first AUG codon (Chavrier, et al., 1989; Rangnekar, Aplin, &

Sukhatme, 1990). Mouse *Egr-2* mRNA maturation resulted in two mRNAs which differed by the presence of a short intron at the 5' end as the non-excision of this intron extended the open reading frame of the murine *Egr*-2 (Chavrier, et al., 1989; Vesque & Charnay, 1992); however, based on the prediction of Rangnekar *et al.* (1990) there was only a single transcript from the human *Egr-2* gene. The unspliced RNA would encode a putative transcription factor with an additional 50 amino acids at its N-terminus.

The protein encoded by the short splice variant had lower transactivation ability than the longer protein encoded by the unspliced variant (Vesque & Charnay, 1992). Whether both splice variants of *Egr-2* are present in neuronal cells is yet to be investigated. The 3' end of all the EGR mRNAs contains an AT-rich region which are potential instability determinants and may contribute to the rapid and transient time course of their mRNA expression (B. A. Christy, Lau, & Nathans, 1988; Lemaire, et al., 1990; P. S. Miller, Reis, Calvert, DePeters, & Baldwin, 1991; Patwardhan, et al., 1991; Sukhatme, et al., 1988)

1.4.3 Differential translation

A further level of complexity was created by the possibility of differential translation of the *Egr-2* mRNA. In nuclear extracts of brain, two forms of Egr-2 (79 kDa and 55 kDa) were detected (K. J. Mack & Mack, 1992). However, the apparent molecular weight of *in vitro* translated Egr-2 had been shown to be approximately 60 kDa (X. M. Cao, et al., 1990). The relationship of these two forms of Egr-2 proteins is currently unclear. It should be noted that Egr-2 protein could exist in both cytoplasmic and nuclear forms

(Herdegen, Kiessling, et al., 1993; Herdegen, Sandkuhler et al., 1993). The implications of a cytoplasmic form of Egr-2 are discussed below.

1.4.4 Post-translational modification

Several levels of post-translational regulation were observed for transcription factors including EGR family. A site for N-linked glycosylation in the second zinc finger of all EGR proteins with a possible role in the regulation of DNA-binding activity of these proteins was identified (Muller, Baniahmad, Kaltschmidt, & Renkawitz, 1991). The zinc fingers of Egr-2 protein contained the nuclear localization signal (Matheny, Day, & Milbrandt, 1994). Interestingly, this nuclear localization sequence also contained one of the potential N-linked glycosylation sites that might be involved in nuclear localization of the Egr-2 proteins. Nuclear translocation of transcription factors seems to be a key control mechanism (Beckmann & Wilce, 1997).

Some regulatory mechanisms might have been conserved among all members of the EGR family. For instance, the loss or gain of the zinc ion from the finger changed the DNA-binding activity of all EGR proteins (Huang & Adamson, 1993). Other known regulatory mechanisms conserved between all EGR family members included: the interaction domain for NAB1 between Egr-1 and Egr-2, the N-linked glycosylation site in the DNA-binding domain, the redox potential and the nuclear localization signal. It should be mentioned that NAB proteins, NAB1 and NAB2, had initially been described as repressors of the transcriptional activity of EGR proteins (Russo, Sevetson, & Milbrandt, 1995; Svaren et al., 1996; Swirnoff et al., 1998).

1.5 Synaptic plasticity and memory

Knowing that even memory of the shortest incidents can last for a long time, it is not surprising that learning and memory requires long lasting modifications in neural circuits of brain. According to Donald Hebb's hypothesis (1949), a synapse linking two neurons is strengthened when the neurons are activated at the same time (Shapiro, 2001). He furthermore differentiated between short-term memory (STM), based on temporary electrical activity within the brain and long-term memory (LTM), based on development of longer lasting neurochemical changes (Baddeley, 2001). It is mostly believed that plasticity of synaptic connections, also known as activity-dependent modifications of synaptic strength or synaptic plasticity, plays a critical role in learning and memory formation (Rioult-Pedotti, Donoghue, & Dunaevsky, 2007).

There are a variety of hypotheses about activity-dependent synaptic plasticity and the different types of memory that we now know to exist (Kandel & Schwartz, 1982; Lynch & Baudry, 1984; McNaughton & Morris, 1987; Morris & Frey, 1997). However, all these distinct hypotheses share a common central idea, called the synaptic plasticity and memory (SPM) hypothesis. According to this hypothesis, during memory formation, activity-dependent synaptic plasticity that is induced at appropriate synapses is required for the information storage essential for the type of memory mediated by the brain area in which that plasticity is observed (S. J. Martin, Grimwood, & Morris, 2000).

1.6 Long Term Potentiation (LTP)

Bliss and Lomo were recording postsynaptic responses in the hippocampus of an anesthetized rabbit after delivering a single high frequency tetanization to the pre-synaptic axons of cortical afferents, when they noticed a pattern of enhanced neuronal responses that persisted for several hours. This long-lasting increase in the magnitude of the synaptic response, now defined as long-term potentiation (LTP), could provide a cellular substrate for information storage (Bliss & Lomo, 1973). LTP was thought by many to be a form of long-term synaptic plasticity (Bliss & Collingridge, 1993; Holscher, 1999; Izquierdo & Medina, 1997).

LTP has been most extensively studied in the hippocampus even though it occurs at many types of synapses throughout the brain. Also because many of the main components of the circuitry are conserved in hippocampal slices that are maintained *in vitro*, electrophysiological studies can be carried out easily in this structure (Adams & Dudek, 2005). It should be noted that, even within the hippocampus, LTP occurs at multiple locations and by means of different mechanisms, with the majority of LTP researchers' attention given to the excitatory synapses between CA3 and CA1 pyramidal neurons (CA3-CA1) due to the availability of a hippocampal slice preparation in which LTP can be relatively easily studied using extracellular field recordings (Blundon & Zakharenko, 2008).

By stimulation of Schaffer collateral fibers, using an extracellular electrode, axon collaterals from CA3 pyramidal neurons providing synapses that excite the apical dendrites of CA1 pyramidal neurons and excitatory postsynaptic potentials (EPSPs) are eli-

cited (Figure 2). EPSPs are recorded with a microelectrode positioned in the stratum radiatum of CA1 region.

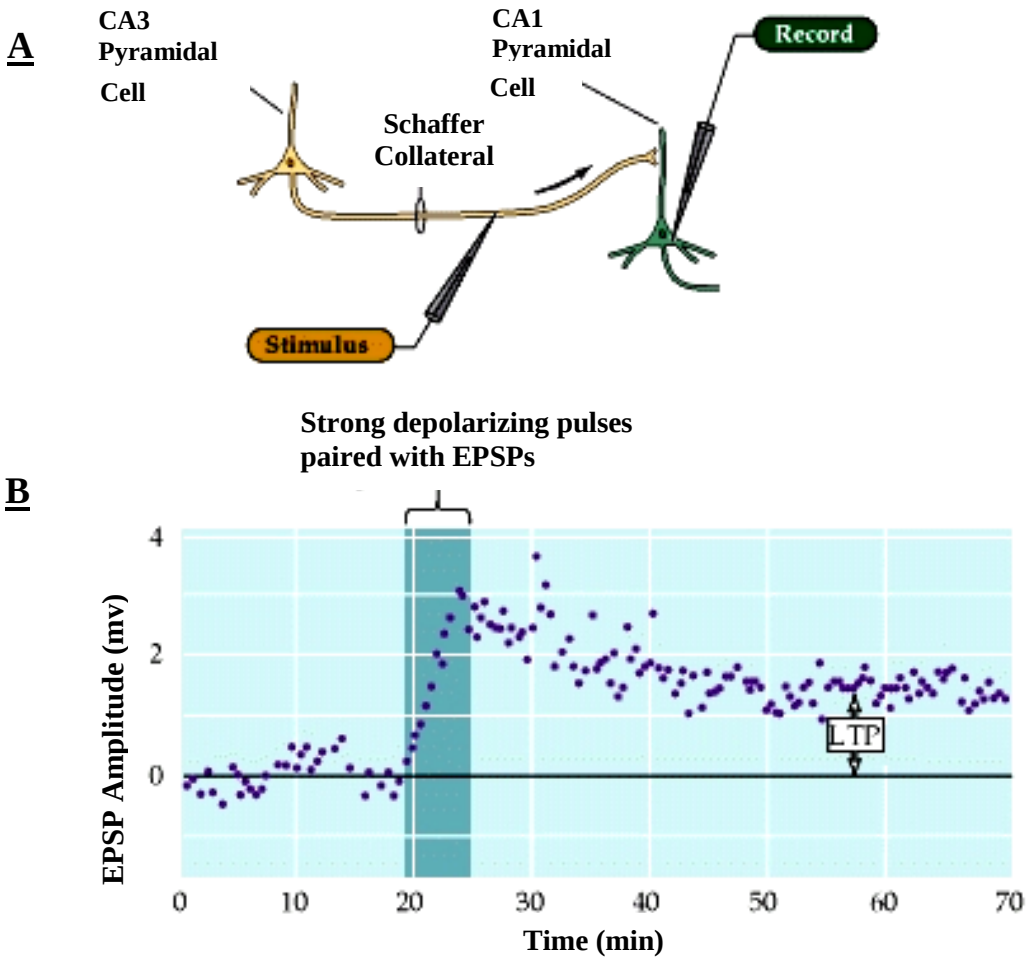

Figure 2: Pairing pre-synaptic and post-synaptic activity results in LTP. A) Positioning of stimulating and recording electrodes in hippocampal CA3-CA1 region. **B)** Single stimuli applied to a Schaffer collateral evokes EPSPs in the post-synaptic CA1 neuron and by applying stimulating pulses through the recording electrode a persistent increase in the EPSPs is observed. (Modified from http://www.ncbi.nlm.nih.gov/bookshelf/)

Stimulation of Schaffer collateral axons with strong depolarizing pulses causes a prolonged enhancement of the EPSPs and this potentiation of synaptic transmission persists for several hours (Figure 2B). These synapses are classical chemical synapses in the mammalian central nervous system and considerable information on synaptic transmission has been provided from studies conducted on these synapses (Nguyen, 2006).

A variety of stimulation protocols were studied in order to find the one that is similar to neuronal activity in the intact brain. Since the original stimulus protocol that ranged from 10 to 100 and used by Bliss and Lomo in the anesthetized rabbit preparation (Bliss & Lomo, 1973), an array of LTP induction protocols have emerged in the literature mostly composed of high-frequency trains of stimulation (tetanization) to be delivered to presynaptic axons, mostly with frequencies between 25 to 400 Hz (Blundon & Zakharenko, 2008). In 1986, Larson and others developed the theta-burst stimulation (TBS) protocol similar to tetanization but with a more complex pattern, to mimic hippocampal activity recorded in awake or sleeping animals and to produce LTP (Larson, Wong, & Lynch, 1986).

1.7 Molecular mechanisms underlying LTP

LTP at CA3-CA1 synapses regardless of the stimulation (induction) protocol depends on the activation of NMDA receptors (NMDARs). In a study carried out by Grover and Teyler in 1990, LTP induced by 25-Hz tetanization at CA3-CA1 synapses was completely blocked by NMDAR antagonists, whereas LTP elicited at the same synapses by 200- Hz tetanization was only partially blocked (Grover & Teyler, 1990). In the same

study, dialyzing the postsynaptic neuron with calcium chelators blocked the induction of LTP by either 200- or 25-Hz tetanizations suggesting that the induction of both forms of LTP was initiated by the postsynaptic neuron. However, they showed that two different mechanisms were used for calcium influx: NMDAR-independent LTP used L-type voltage-gated calcium channels (L-VGCCs) but NMDAR-dependent LTP used the NMDAR pathway (Grover & Teyler, 1990).

Moreover, pharmacological inhibition of some synaptic receptors, particularly NMDARs, blocked expression of many activity- dependent genes suggesting that biochemical signals, such as proteins, relay the information by transport from the potentiated synapse to the nucleus (Matsuo, Murayama, Saitoh, Sakaki, & Inokuchi, 2000; Steward & Worley, 2001; Worley, et al., 1993). For instance, kinases (Deisseroth, Mermelstein, Xia, & Tsien, 2003; K. C. Martin et al., 1997), the transcription factor nuclear factor kappa B (NF-κB) (Meffert, et al., 2003), and transport proteins such as importins could carry other signals as cargo (Thompson et al., 2004). Activation of constitutive transcription factors (TFs) as a proposed mechanism for communication between synaptic events and the nucleus represented a link between synaptic activity and gene expression (Kaltschmidt, Baeuerle, & Kaltschmidt, 1993; Kandel, 2001b; Meberg, Kinney, Valcourt, & Routtenberg, 1996).

1.7.1 Regulation of *Egr-2*

The earliest genomic response to synaptic activity is the induction of expression of immediate early genes (IEGs) such as *Fos*, *Jun*, or *Egr* family that might play an impor-

tant role in learning and memory processes (Bozon et al., 2003; Dragunow, 1996; Knapska & Kaczmarek, 2004). Activated IEGs, in response to neuronal activation, encoded many functionally different products such as cytoplasmic enzymes, secreted proteins and inducible transcription factors that could activate downstream target genes (Herdegen & Leah, 1998). Regulated expression of IEGs following neuronal activation is a key mechanism for the recruitment of specific target genes that might be involved in the stabilization of neural modifications required for the formation of long-term memories (LTM).

Many studies carried out on IEGs focused on the functional role of the EGR gene family as inducible transcriptional regulators in synaptic plasticity and memory processes (Abraham, Dragunow, & Tate, 1991; Bozon, Davis, & Laroche, 2002; Tischmeyer & Grimm, 1999). It was shown that the EGR genes are activated by stimuli that mediate mechanisms of synaptic plasticity (Beckmann & Wilce, 1997; O'Donovan, Tourtellotte, Millbrandt, & Baraban, 1999). *Egr-1, Egr-2* and *Egr-3* genes were all rapidly regulated following the induction of LTP in the dentate gyrus suggesting that they might induce the transcription of target genes required for the maintenance of certain forms of activity-dependent synaptic plasticity. The regulation of *Egr-2* and *Egr-3* appeared to require stronger LTP-inducing stimuli. However, the expression of the proteins lasted longer than that of *Egr-1* (Cole, Saffen, Baraban, & Worley, 1989; Williams, et al., 1995; Worley, et al., 1993; Yamagata, et al., 1994).

As in synaptic plasticity paradigms, the induction of *Egr-1* occurred rapidly and transiently after learning or exposure to learning-associated cues and may have had a role

in the transition from short- to long-term memory. Much less is known about the regulation of other EGR family members, including *Egr-2*, during learning (Poirier et al., 2008). Although, induction of *Egr-2* in single trial contextual fear conditioning was not clearly observed (Malkani & Rosen, 2000), a recent study by Desteno and Schmauss (2008) showed that expression of *Egr-2* was induced in the forebrain of mice performing an attention-set-shifting task (ASST), but not in a spatial working memory task. In the same study, induction of *Egr-1* and *Egr-3* was not detected in the same brain structures in the ASST, suggesting that EGR family members may be differentially induced during selective cognitive tasks (DeSteno & Schmauss, 2008). Further research is required to expand our knowledge of the specific conditions under which distinct EGR family members are activated in relation to specific types of learning.

1.7.2 Role of transcription factors

The primary search for the role of transcription factors (TFs) in memory consolidation was mainly focused on only one family of constitutive TFs, the cAMP response element binding protein (CREB) family. A series of studies in rodents, Aplysia and Drosophila suggested that CREB activation was an evolutionarily conserved mechanism involved in neural plasticity associated with memory formation (Kaang, Kandel, & Grant, 1993; Kinney & Routtenberg, 1993; Yin & Tully, 1996). However, regulation of gene expression was a process that required the involvement of multiple factors such as a defined set of TFs and co-activators (Marini et al., 2004). Recently, other transcription factors have been implicated in experience-based synaptic adaptations including members of

the NF-κB/Rel family (Kassed, et al., 2002; Mattson & Camandola, 2001; Meffert, et al., 2003; O'Neill & Kaltschmidt, 1997).

NF-κB/Rel factors were originally implicated as central regulators of the immune and inflammatory responses. Later, both basal expression and stimulus-coupled induction of NF-κB /Rel factors in neurons and glial cells were reported (Guerrini, Blasi, & Denis-Donini, 1995; C. Kaltschmidt, Kaltschmidt, & Baeuerle, 1993, 1995; Mattson & Camandola, 2001; Meberg, et al., 1996; Meffert, et al., 2003).

1.8 NF-κB in the nervous system

More than 20 years ago, in the laboratory of David Baltimore, the nuclear factor kappa B transcription factor (NF-κB) was discovered due to its ability to bind to the enhancer of the immunoglobulin (Ig) k light chain gene in B cells (Sen & Baltimore, 1986). In mammals, the NF-κB /Rel family of transcription factors comprised five members, p50, p52, p65 (Rel-A), c-Rel and Rel-B, which shared the Rel homology domain (RHD), a N-terminal 300 amino acid, allowing DNA binding, dimerization and nuclear localization (Baeuerle & Henkel, 1994).

The homo- or hetero-dimers formed by these proteins were present in the cytoplasm in an inactive form by interaction with inhibitory molecules, called IκBs, which masked the NF-κB nuclear localization and DNA binding domains (Ghosh & Karin, 2002; Malek, Chen, Huxford, & Ghosh, 2001; Whiteside & Israel, 1997). IκBs were composed of IκBα, IκBβ, IκBε, IκBγ, IκBζ, Bcl-3, and the precursors of p50 and p52, p105 and p100, respectively (Whiteside & Israel, 1997; Yamamoto et al., 2004;

Yamazaki, Muta, & Takeshige, 2001). Multiple stimuli including inflammation, infection, injury and stress could induce nuclear translocation of NF-κB (Pahl, 1999).

Upon stimulation, an IκB kinase (IKK) complex, composed of two catalytic subunits, IKK1/α and IKK2/β, and a regulatory subunit NF-κB essential modulator (NEMO)/IKKγ was activated. This complex caused phosphorylation of two N-terminal serines within the IkBs, leading to their ubiquitination and degradation through the proteasome pathway (Ghosh & Karin, 2002; May & Ghosh, 1998). Free and activated NF-κB dimers then translocated from the cytoplasm to the nucleus, bound to κB sites in the promoter or enhancer regions of target genes, and activated their transcription. The consensus sequence of κB site is GGGRNNYYCC (N = anybase, R = purine, and Y = pyrimidine) (Memet, 2006).

Genes encoding IκBα and IκBε were among the several genes regulated by NF-κB to provide a feedback mechanism such that newly synthesized IκBα and IκBε attached to DNA-bound dimers and exported them out of the nucleus to the cytoplasm (Arenzana-Seisdedos et al., 1995; Kearns, Basak, Werner, Huang, & Hoffmann, 2006; Pahl, 1999). An alternative pathway of NF-κB activation, that is currently thought to operate only in the immune system, involved phosphorylation of IKKα by NF-κB-inducing kinase (NIK), which then triggered inducible processing of p100, causing the release of p52-containing dimers (Bonizzi & Karin, 2004).

Although degradation and re-synthesis of the IκBs was a determining step in the control of NF-κB activation, many other regulatory mechanisms, including phosphorylation, acetylation, ubiquitination, or sumoylation of upstream or downstream effectors,

IKK subunits or NF-κB proteins themselves, provided a fine regulation of NF-κB signaling (for review see Memet, 2006).

1.8.1 Composition of NF-kB dimers

The overall aspects of the canonical NF-κB transactivation cascade within the nervous system are conserved. The p50/p65 hetero-dimer is the major transcriptionally active form of NF-kB in the nervous system, although other dimers are emerging as important alternate effectors (Memet, 2006). Dimers containing c-Rel were described in response to: hypoxia in the hippocampus (Qiu et al., 2001)], nerve growth factor (NGF) in sympathetic neurons (Maggirwar, Sarmiere, Dewhurst, & Freeman, 1998), interleukin (IL)-1β in primary cerebellar granular neurons (Pizzi, et al., 2002) and amyloid β (Aβ) peptide in primary cortical neurons (Pizzi et al., 2005).

The NF-kB transcriptional response is cell specific and the higher affinity of c-Rel homo-dimers for kB sites in comparison to p65 homo-dimers is likely one of the accounting factors (Pizzi, et al., 2002; Pizzi, et al., 2005). An additional factor may be that the kB site sequence itself determines the selectivity of the interaction with coactivators (Leung, Hoffmann, & Baltimore, 2004).

1.8.2 Activating stimuli

A wide variety of stimuli including cytokines (tumor necrosis factor α [TNFα] and Interleukin-1 [IL-1]), chemokines, lipopolysaccharide (LPS), injury or oxidative stress (caused by superoxide and nitric oxide) activates NF-κB in nervous system as well as the

immune system (Memet, 2006). However, some of these activators may exert additional functions in the nervous system; for instance, it had been reported that TNFα and nitric oxide can regulate synaptic plasticity (Albensi & Mattson, 2000; Beattie et al., 2002; Kantor et al., 1996; Stellwagen, Beattie, Seo, & Malenka, 2005).

In the nervous system, NF-κB is also activated by typical stimuli such as neurotrophins NGF and S100ββ (Alexanian & Bamburg, 1999; Carter et al., 1996; Wood, 1995), neurotransmitters such as glutamate (Guerrini, et al., 1995; C. Kaltschmidt, et al., 1995; Scholzke, Potrovita, Subramaniam, Prinz, & Schwaninger, 2003), mGlutR5 agonists (O'Riordan, et al., 2006), synaptic activity (Meberg, et al., 1996; Meffert, et al., 2003; Merlo, Freudenthal, Maldonado, & Romano, 2005; Yeh, Lin, Lee, & Gean, 2002), membrane depolarization (Lilienbaum & Israel, 2003), amyloid β (Aβ) peptide (Behl, Davis, Lesley, & Schubert, 1994), neural cell adhesion molecule (N-CAM) (Krushel, Cunningham, Edelman, & Crossin, 1999) or sleep deprivation (Brandt et al., 2004; Chen, Gardi, Kushikata, Fang, & Krueger, 1999).

In different reports, a relationship between cell type and stimulus was observed. For example, glutamate activated NF-kB p50/65 only in neurons (Guerrini, et al., 1995) and IL-1b activated NF-kB in astrocytes, but not in neurons (Srinivasan, Yen, Joseph, & Friedman, 2004). Even though all neurotrophins activated NF-kB in microglial cells (Nakajima et al., 1998) , in Schwann cells and oligodendrocytes, only neurotrophin NGF but neither (NT)-3 nor brain-derived neurotrophic factor (BDNF) induced NF-kB activity through the p75/neurotrophin receptor (NTR) (Carter, et al., 1996; Ladiwala et al.,

1998), but in neurons, tyrosine protein kinase receptor A (TrkA) was also required (Culmsee et al., 2002; Kaplan & Miller, 1997).

In neurons, several signaling cascades including p21 (Ras)/phosphatidylinositol-3-kinase (PI3K)/AKT, calcium/calmodulin dependent kinase II and protein kinase C trigger activation of NF-κB (Lilienbaum & Israel, 2003; Meffert, et al., 2003; Rojo, Salinas, Martin, Perona, & Cuadrado, 2004). Moreover, tyrosine phosphorylation of IκBα in primary hippocampal neurons or PC12 cells treated with NGF (Bui, Livolsi, Peyron, & Prehn, 2001) and Ca^{2+}-dependent protease calpain-degradation of IκBα upon glutamate treatment of cerebellar granule cells (Scholzke, et al., 2003) have been reported.

Interestingly, a novel mode of NF-κB activation by enzyme trans-glutaminase 2 which involves polymerization of IκBα by this enzyme has been described in microglia. This activation is independent of the classical IKK pathway and may account for a cell type specific signaling (J. Lee et al., 2004; Park, Choi, Yoon, Ahn, & Oh, 2004).

1.8.3 Inhibiting stimuli

In the nervous system, NF-κB activity can be negatively regulated by a number of molecules including transforming growth factor β (TGFβ), glycogen synthase kinase-3 (GSK-3β), Interleukin-10 (IL-10), Interleukin-4 (IL-4) and glucocorticoids (Kaltschmidt, Widera, & Kaltschmidt, 2005). For instance, in cortical neurons, the lipid peroxidation product 4-hydroxy-2-3-nonenal modified an upstream component of the NF-κB signaling pathway. In astrocytes, NF-κB activation was negatively regulated by GSK-3β and in cerebellar granule neurons, NF-κB activity was inhibited by TGFβ2. Under central nervous

system (CNS) neuro-inflammatory disease conditions, IL-4 inhibited NF-κB in activated glial cells via a peroxisome proliferator activated receptor (PPAR)-γ-mediated mechanism, allowing survival of differentiating oligodendrocyte precursors (Camandola, Poli, & Mattson, 2000; Kaltschmidt & Kaltschmidt, 2001; Paintlia, Paintlia, Singh, & Singh, 2006; Sanchez et al., 2003).

1.8.4 Target genes

Our knowledge of NF-κB target genes in the nervous system relies mostly on the genes identified in the immune system (Pahl, 1999). In more recent microarray studies, several new TNF-responsive genes were identified in a human glioblastoma cell line in comparison with 3T3 or HeLa cells (Schwamborn et al., 2003) as well as 17 genes regulated by p50 in mouse hippocampus upon treatment with trimethyltin (TMT), a neurotoxic chemical (Kassed, et al., 2004). In the CA1 region of the hippocampus, 38 genes were selectively modulated by contextual long-term memory consolidation and 12 of these genes contained c-Rel binding sites (Levenson, et al., 2004). Moreover, the protein kinase A (PKA) catalytic α subunit was reported to be a new NF-κB-responsive gene in a transcriptome analysis of hippocampus and cortex of mice in which NF-κB activity was selectively ablated in forebrain neurons (Kaltschmidt et al., 2006).

Other NF-κB target genes identified in the nervous system include amyloid β precursor protein (APP) (Grilli, Goffi, Memo, & Spano, 1996), calcium/calmodulin-dependent protein kinase II (CaMK II) δ (Kassed, et al., 2004), N-CAM (Simpson & Morris, 2000), inducible nitric oxide synthase (NOS II) (Madrigal et al., 2001), μ-opioid

receptors (Kraus, Borner, Giannini, & Hollt, 2003), β-secretase (BACE, the first and rate-limiting enzyme for APP cleavage) (Sambamurti, Kinsey, Maloney, Ge, & Lahiri, 2004), BDNF (Lipsky et al., 2001), and inducible cyclooxygenase-2 (COX-2) (Kaltschmidt, Linker, Deng, & Kaltschmidt, 2002).

1.9 NF-κB in long-lasting neuronal plasticity

In a study carried out by Meberg et al. (1996), NF-κB was linked with synaptic plasticity for the first time. In this study, hippocampal LTP was examined using ipsilateral tetanus of the rat perforant pathway *in vivo* (Meberg, et al., 1996). This study reported increased levels, relative to the contra-lateral side, of p50 and p65 mRNA in granule, CA3 and CA1 cell fields in both LTP experiments and in experiments using low-frequency stimulation controls at 60 minutes after treatment, but not at 30 or 120 minutes. This showed that the expression of both p50 and p65 genes was induced after neuronal activity. Also, when gene expression of the stimulation side was compared to the unstimulated side, p65 mRNA in granule cells was significantly greater in the LTP animals than in low-frequency controls. In addition, the increase of p50 mRNA after both high- and low-frequency treatments suggested NF-κB-dependent transcriptional activation because p50 gene expression was induced by NF-κB activation in a positive feedback system.

In another study by Albensi and Mattson (2000), stimulation of hippocampal slices in Schaffer collateral axons at low frequency (1 Hz) induced long-term depression (LTD), inverse of LTP; this was prevented by infusion of NF-κB decoy DNA. In addition

the same treatment significantly reduced the amplitude of LTP induced by high-frequency stimulation. The NF-κB decoy consisted of a double-strand DNA oligonucleotide containing a NF-κB consensus binding sequence that competed with endogenous NF-κB and thereby suppressed its function. Therefore, the decoy was administered to inhibit NF-κB function.

In 2002, a different study showed an increase in NF-κB and IκB kinase (IKK) activity after tetanization and that NF-κB decoy DNA infusion impaired LTP induction in amygdala slices (Yeh, et al., 2002). Together, these studies provided direct evidence of involvement of NF-κB in synaptic plasticity. Also, a growing body of experimental studies supported the participation of the Rel／NF-κB family of constitutive transcription factors in behavioural studies of memory as a transcription regulator as well as their roles as synapse-to-nucleus signalling molecules after activation at the synapse (Romano, Freudenthal, Merlo, & Routtenberg, 2006).

Furthermore, in a recent report (Freudenthal, Romano, & Routtenberg, 2004) two independent methods, electrophoretic mobility shift assays (EMSA) with nuclear extracts from total hippocampus and immunohistochemistry with an activated-specific p65 antibody that recognized the nuclear localization sequence region, were used to assess NF-κB activity in mouse hippocampus 15 min after tetanization of the perforant path as compared with control animals. The EMSA assay showed that NF-κB was specifically activated by LTP 15 min after its induction and immunohistochemistry revealed elevated expressions of activated p65 in most hippocampal subfields after tetanic stimulation.

1.10 NF-κB in mammalian memory models

Several studies, using pharmacological inhibitors of the NF-κB signaling pathway or different genetic mouse models, reported that NF-κB plays a role in memory formation, cognition and behavior (see Table 1). For the first time, in another animal model a link between NF-κB and long-term memory was proposed in crabs (Freudenthal & Romano, 2000). Later, reports of impaired memory reconsolidation after treatment of crabs with sulfasalazine, an inhibitor of IKKs, supported this hypothesis (Merlo, et al., 2005).

A growing body of studies supports the idea that NF-κB was an important molecular mechanism contributing to memory in mammals. The initial evidence to support this idea was acquired by a group studying fear conditioning in the rat (Yeh, et al., 2002). They found that NF-κB activation in the amygdala was required for memory storage in a fear conditioning paradigm. Another study in this model revealed that an association between NF-κB and the acetyl transferase CREB binding protein (CBP) was induced by fear conditioning and resulted in an increase in acetylated p65. The acetylation state of p65 in amygdala was prolonged after the administration of general deacetylase inhibitors and facilitated LTM (Yeh, Lin, & Gean, 2004).

Prior to these findings, Zhong et al. (1998) described the association of CBP with NF-κB, recruiting the acetyl transferase to promoters containing NF-κB sites. The ability of NF-κB to bind CBP depended on p65 phosphorylation by PKA, the cAMP dependent protein kinase which has been implicated in several models of memory (P. Q. Zhong, Zhi, Li, Xue, & Shu, 1998). An inhibitory avoidance task in mice provided the first evi-

Table 1: Knock-outs of NF-κB family members

Mice	Phenotype
$p50^{-/-}$	Increased neural damage after excitotoxic injury (Z. Yu, Zhou, Bruce-Keller, Kindy, & Mattson, 1999)
	Increased striatal neuronal apoptosis in an experimental model of Huntington's Disease (Z. Yu, Zhou, Cheng, & Mattson, 2000)
	Increased hippocampal neuronal apoptosis after chemical exposure (Kassed, Willing, Garbuzova-Davis, Sanberg, & Pennypacker, 2002)
	Increased susceptibility to noise- and age-induced hearing loss and neural degeneration (Lang et al., 2006)
$p65^{-/-}$	Impaired survival of E12 and E14 trigeminal and nodose sensory neurons to NGF and increased apoptosis in vivo (Hamanoue et al., 1999; Middleton et al., 2000)
	Myelin deficiency of DRG-derived Schwann cells in vitro (Nickols, Valentine, Kanwal, & Carter, 2003)
	Loss of Fas apoptosis inhibitory molecule (FAIM)-induced increase in neurite outgrowth of E15 cortical neurons (Sole et al., 2004)
$p65^{-/-}$,$TNFR^{-/-}$	Impaired formation of spatial memory in radial arm maze but normal excitatory behavior (Meffert, Chang, Wiltgen, Fanselow, & Baltimore, 2003)
$p65^{-/-}$,$TNF^{-/-}$	Increased Schwann cell apoptosis after axotomy (Boyle, Azari, Cheema, & Petratos, 2005)
$c\text{-}Rel^{-/-}$	Reduced hippocampal synaptic transmission, impaired late LTD, impaired formation of long-term passive avoidance memory (O'Riordan et al., 2006) and contextual fear memory (Kassed et al., 2004)
	Loss of Il-1β neuroprotective function against NMDA-mediated neurotoxicity (Pizzi et al., 2002)
	Hypoactive in open-field task, but normal nociception and anxiety behavior (Levenson et al., 2004)

(Memet, 2006)

dence that NF-κB activity was regulated in the hippocampus during memory consolidation, stressing the role of this transcription factor as part of the molecular mechanism for memory storage (Freudenthal et al., 2005).

1.11 Knock-out mice for NF-κB components: p50, p65

The use of transgenic mice shed further light on the study of NF-κB in learning and memory. Mice with a null mutation of p50 ($p50^{-/-}$) in an active avoidance task jumping to a platform showed acquisition deficits (Kassed, et al., 2002). This result demonstrated that p50 is required for normal learning. However, in a test for LTM performed 24 hours after training, no differences between knock-out and wild-type mice were reported, suggesting that p50 is not required for memory formation.

The $p50^{-/-}$ mice that lacked p50 and its precursor p100 were viable and fertile and did not have gross developmental and histological defects, but showed immune deficits. These animals had impaired learning in an active avoidance assay (Kassed, et al., 2002) and also displayed reduced anxiety-like behavior in exploratory drive and anxiety tests (Kassed & Herkenham, 2004).

Since deletion of the individual NF-κB gene occurs in every cell type of these knock-out mice, discrimination between the function of NF-κB in neurons versus glia was difficult. Recent data with cell-restricted ablation of NF-κB showed that loss of neuronal NF-κB impaired spatial long-term memory formation in the Morris water maze task whereas non-spatial working/episodic memory remained unchanged (Kaltschmidt, et al.,

2006). This strongly suggested that neuronal NF-κB had a prominent role in memory and cognition.

Although deletion of the p65 subunit was lethal during embryogenesis (Beg, Sha, Bronson, Ghosh, & Baltimore, 1995), concurrent deletion of type 1 TNF receptors (TNFR1) rescued the knock-out animals (Alcamo et al., 2001). Gross examination of p65/TNFR1 double knock-out mice brains appeared normal and no obvious behavioural abnormalities were described. In a study by Meffert et al. (2003), spatial memory performance of this double mutant was analyzed using the spatial and cued versions of the radial arm maze. The result suggested that p65 was necessary in the hippocampus for proper processing and storage of spatial information, whereas in other areas such as striatum that are involved in non-spatial learning, p65 was not required. Knock-outs of some NF-κB family members and their corresponding phenotypes are listed in Table 1 (Memet, 2006).

1.12 NF-κB - A synapse-to-nucleus signalling mechanism

The present view that signals leading to long-term neuronal changes are generated at the synapses and must reach the nucleus from axon or dendrites in order to regulate gene expression, is supported by a large body of experimental data such as the study carried out by Frey and Morris (1998). Some of the proposed synapse-to-nucleus mechanisms besides the NF-κB mechanism, include: cAMP dispersion from synapses to somatic cytoplasm, allowing PKA catalytic subunit translocation to the nucleus to eventually phosphorylate target transcription factors such as CREB (Kandel, 2001a), calmodulin

and⁄or CaMKIV translocation to the nucleus and MAPK translocation to activate nuclear kinases and transcription factors (Deisseroth, et al., 2003).

In a recent study, after inducing LTP chemically by activating NMDARs in hippocampal neurons of *Aplysia,* importin, a component of the retrograde transport machinery was found to translocate to the nucleus (Thompson, et al., 2004). This showed that the retrograde transport machinery was activated during stabilization of long-term synaptic changes.

Presence of inducible NF-κB in both pre- and postsynaptic sites (Guerrini, et al., 1995; C. Kaltschmidt, et al., 1993; Meberg, et al., 1996) suggested that NF-κB was locally activated by synaptic activity and then retrogradely transported to the nucleus to regulate gene expression. Therefore, NF-κB transcription factors were considered to function as both synaptic activity detectors and transcriptional regulators.

In a study carried out by Freudenthal & Romano (2000), local NF-κB activation was detected only after adequate synaptic stimulation required for long-term changes in neurons. Other evidence supporting this signalling hypothesis came from *in vitro* cellular studies by Kaltschmidt's group. Data from this study showed redistribution of p65 fused with enhanced green fluorescent protein (GFP) from neurites to nucleus after glutamate and either kainate stimulation or depolarization in hippocampal neuron cultures (Wellmann, Kaltschmidt, & Kaltschmidt, 2001).

Basal NF-κB DNA binding activity was detected in synaptosomal preparations from hippocampal neurons in culture (Meffert, et al., 2003). This basal activity was increased by glutamate stimulation and inhibited by blockers of NMDA receptors and L-

type Ca^{2+} channels. It should also be noted that in this study only the p65⁄p50 dimers were detected in synaptosomes and p65–GFP fusion proteins were detected in the cytoplasm and dendrites, including spine-like structures. Further studies with photo-bleaching showed that 30–45 min after stimulation, p65–GFP from distal processes reached the nucleus suggesting that recruitment of NF-κB from synapse to nucleus could be part of the molecular mechanism involved in long-lasting memory formation processes (Romano, et al., 2006).

1.13 Downstream effectors of NF-κB involved in memory

During the past decade, the involvement of NF-κB in the regulation of the expression of many genes was analyzed in different studies, many of which utilized cells other than neurons. Among those genes were: the protein kinases PKC δ and PKA catalytic α subunit; the immediate-early genes Erg-1 ⁄ Zif268, c-Fos, JunB and JunD; the neurotransmitters and neuropeptide receptors NMDA receptor 1 and 2A, δ and μ opioid receptors and insulin-like growth factor receptor; the neuropeptides angiotensinogen, proenkephalin and proopiomelanocortin; the proteasome component proteasome subunit LMP2; the cytokines TNF-α and -β and their receptors, and the interleukins; the enzyme inducible NO synthase (iNOS); and the cell adhesion molecule N-CAM (Kaltschmidt, et al., 2006; Levenson, et al., 2004).

A significant number of genes, regulated in part by NF-κB, produced proteins that had implications in neural plasticity and memory. In the context of memory formation however, the expression of only a few of these genes has been studied (Kaltschmidt, et

al., 2006; Levenson, et al., 2004). Therefore, to determine the specific role of NF-κB in the regulation of these genes during memory processes, more studies are required (Romano, et al., 2006).

1.14 Objectives and rationale of the study

A growing body of evidence supports an important role for the NF-κB transcription factor in the basic mechanisms of memory processing and storage. In previous experiments performed in this laboratory to investigate the role of NF-κB in neuronal activity dependent gene expression, the transcriptomes of LTP-induced hippocampal slices from NF-kB p50 knockout mice ($p50^{-/-}$) and its littermate ($p50^{+/+}$), were analyzed using Affymetrix Mouse DNA microarrays.

The results showed overexpression of many genes in wild-type animals vs. $p50^{-/-}$. *Egr-2*, whose expression is regulated during synaptic plasticity and memory formation (section 1.7.1) showed an approximate 3 fold increase in expression, and was therefore selected as a putative NF-κB target gene of interest in this study.

Based on this evidence, we hypothesized that *Egr-2* is induced by neuronal activity-dependent NF-κB activation. The overall objectives of this study were to verify the induction of *Egr-2* upon NF-κB activation through measurement of mRNA and protein expression of this gene and to determine whether NF-κB binds to the *Egr-2* promoter region and regulates expression of this gene at the transcriptional level.

Materials and Methods

2.1 Animals

Timed-pregnant CD-1 mice were purchased from Central Animal Care of University of Manitoba. Two to three month old C57BL/6 mice were purchased form Jackson Laboratory. Animal experiments were performed in accordance with the *Guide to the Care and Use of Experimental Animals*, published by the Canadian Council for Animal Care.

2.2 Mammalian cell culture

2.2.1 HeLa S3 cell line

2.2.1.1. Maintenance and passage of HeLa S3 cells

HeLa S3 cells (cervical carcinoma cell line) were chosen for this study. This cell line is insensitive to TNFα apoptotic activity (Sekine, Fujii, Abe, & Nishikawa, 2001), which makes it an ideal cell line for TNFα stimulation in order to evaluate the NF-κB dependent expression pattern of the *Egr-2* gene. Since it is easy to culture, manipulate and maintain compared to primary neurons, it was decided to begin the experiments using this cell line.

HeLa cells were grown on tissue culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin-streptomycin solution (Gibco). Cells were maintained as monolayer cultures at 37ºC in a humidified incubator with 5% CO_2 (Rosenstiel et al., 2003).

Cells were subcultured when they reached about 90 % confluence. Briefly, the conditioned medium was aspirated and 2-3 ml of trypsin (Gibco) was added to the cell monolayer and incubated at room temperature for 2-3 minutes. The trypsin was aspirated and the trypsinized cells were washed and suspended with 5 ml of conditioned medium. 1-2 ml of the suspended cells was added to a new flask (BD Falcon) containing 5-7 ml of Complete medium and incubated as described above.

2.2.1.2. Freezing and thawing of HeLa cells

For long-term storage, cells grown to ~ 90% of confluence in a tissue culture flask were detached with trypsin (section 2.2.1.1). The trypsinized cells were diluted into 8 ml of conditioned medium, and pelleted at 1200 rpm for 5 minutes. The conditioned medium was aspirated; the cells were re-suspended in 2 ml of cold Freezing Medium (Gibco/BRL), transferred to a cold pre-labeled cryovial, and stored at -80°C.

Cells stored in -80°C were thawed at 37°C. As soon as the vial contents thawed, 1 ml of conditioned medium was mixed with the cell suspension, and then transferred to a 15 ml conical tube containing 10 ml of conditioned medium. The cells were pelleted by centrifugation at 1200 rpm for 5 minutes. The conditioned medium was discarded and the cells were re-suspended in 10 ml of Complete Medium. The cell suspension was transferred to a tissue culture flask and incubated overnight at 37°C with 5 % CO_2.

2.2.2 Primary cortical neuron culture

Timed-pregnant CD-1 mice at gestational day 16-18 were used. Fetal brains were removed and cerebral cortices were dissociated by gentle pipetting. Dissociated tissue was filtered using a 40 µm cell strainer (BD Falcon), washed and suspended in Neurobasal medium (GIBCO) with B27 supplement (GIBCO), 5% FBS (Hyclone), 1.2 mM glutamine and 5 mM HEPES.

Cells were plated at 5 X 10^5 cells/cm^2 (total cells of ~10^6 per well) on poly-D-lysine-coated plates (NUNC, VWR). Twenty four hours after plating, 2mM Cytosine Arabinoside (Ara-c, Sigma) was added to inhibit non-neuronal cell growth. Subsequently, medium was completely replaced after another 24 hours with Neurobasal/B27. Experiments were performed on day 7–8 after plating. This method in particular was implemented in our laboratory by post doctoral fellow, Dr. Ning Ge.

2.3 RNA preparation

Two different methods were used for RNA isolation and the RNA concentrations were measured using a spectrophotometer (Beckman Coulter DU-640).

2.3.1 AllPrep DNA/RNA/Protein Mini Kit (QIAGEN) method

The AllPrep DNA/RNA/Protein Mini Kit (QIAGEN) was used following the manufacturer's instructions when RNA and protein extraction from the same sample was required. RNA and protein extraction from HeLa S3 cells was performed using this method.

2.3.2 TRIZOL (Invitrogen) method

The TRIZOL was used for RNA extraction only. Mostly RNAs of primary cortical neurons were extracted using TRIZOL following the manufacturer's instructions.

2.4 TNFα and SN50 treatment

To inhibit NF-κB with the cell-permeable synthetic peptide SN50 (Calbiochem), media of the HeLa cells plated in a 12-well plate (~ 0.5 $x10^6$ cells per well) and primary cortical neurons plated in a 6-well plate (~ 1.25 $x10^6$ cells per well) were replaced with 100 µg/ml of SN50 dissolved in conditioned media one hour before treating the cells with TNFα.

For HeLa cells, 10 ng/ml of TNFα was used and for primary cortical neurons 100 ng/ml of TNFα was used for each treatment (Dolga et al., 2008). After TNFα and SN50 treatment, mRNAs of the cells were extracted and their concentrations were measured as mentioned in the previous section.

2.5 Reverse Transcription PCR (RT-PCR)

For reverse transcription (RT) reaction, 1µg of total RNA isolated from HeLa cells and primary cortical neurons, was reverse transcribed to cDNA using the iScript™ cDNA Synthesis kit from Bio-Rad following the manufacturer's instructions.

2.6 Construction of plasmid DNA for quantitative real time PCR optimization

2.6.1 Primer design for human and mouse EGR-2 and GAPDH

The mRNA sequences of human and mouse EGR-2 and GAPDH were obtained from the Ensembl website (http://www.ensembl.org/). The identification (ID) of these genes and their transcripts are listed in Table 2.

Table 2: Human and mouse EGR-2 and GAPDH gene and transcript IDs

Species	Gene ID	Transcript ID
Mouse	*Egr-2* (ENSMUSG00000037868)	*Egr-2* (ENSMUST00000048289)
	Gapdh (ENSMUSG00000057666)	*Gapdh* (ENSMUST00000118875)
Human	*Egr-2* (ENSG00000122877)	*Egr-2* (ENST00000242480)
	*Gapdh (*ENSG00000111640)	*Gapdh* (ENST00000229239)

(Modified from http://www.ensembl.org/)

The FASTA format sequences of each gene's mRNA was used on the Primer 3 website (http://frodo.wi.mit.edu/) to design primers with melting temperatures (Tm) around 60°C and product size between 350 – 500 base pairs. Since these primer pairs were used for the endpoint PCR, they were called endpoint primers. Sequences of the endpoint primers are listed in Table 3.

Table 3: Endpoint primer pairs sequences

Human EGR-2	
Forward (5' - 3') :	TGGAGAGAAGAGGTCGTTGG
Reverse (5' - 3') :	TCTCTTCCCAATGATCCCAG
Human GAPDH	
Forward (5' - 3') :	CAGTCAGCCGCATCTTCTTT
Reverse (5' - 3') :	AAATGAGCCCCAGCCTTC
Mouse EGR-2	
Forward (5' - 3') :	TGGAGGGCAAAAGGAGATAC
Reverse (5' - 3') :	TGGGACATGGTACACACACC
Mouse GAPDH	
Forward (5' - 3') :	GCTGCCCAGAACATCATCC
Reverse (5' - 3') :	GGTCCAGGGTTTCTTACTCC

2.6.2 Polymerase Chain Reaction (PCR) amplification of DNA

The RNA extracted from a confluent flask of HeLa S3 cells and half of a C57B mouse hippocampus following the instructions mentioned in section 2.3, was reverse transcribed to cDNA (section 2.4). 2µl of each cDNA (0.2 to 0.5 µg) was mixed with 37 µl of double distilled water, 5µl of 10 X PCR Buffer (Invitrogen), 1µ1 of 10 mM dNTP (Invitrogen), 1µl of (10µM) sense and antisense primers of each gene (Table 3), 1.5µl of 50mM $MgCl_2$ and 1.5µl of 1unit Taq DNA Polymerase™ (Invitrogen).

The PCR amplification was performed in a BioRad Thermal Cycler. The reactions were incubated at 94°C for 2 minutes for the initial denaturation and the amplifications were carried out using 40 cycles with the following program setting: a denaturation step for 30 seconds at 94°C, an annealing step for 30 seconds at 60°C and an extension step

for 1 minute at 72°C. The 40 cycles of amplification were followed by the last extension for 7 minute at 72°C. 10µl of the PCR products were used to check the specificity of the amplification using agarose gel electrophoresis (section 2.5.3) and the rest of PCR products were purified using a GenElute™ PCR Clean-Up Kit (Sigma-Aldrich), following the manufacturer's instructions.

2.6.3 Agarose gel electrophoresis

DNA samples (10µl) were prepared for electrophoresis by adding 2µl of 6 X Sample Buffer (0.25% bromophenol blue [Sigma], 0.25% xylene cyanol [Sigma], 30% glycerol [Sigma]) to a final concentration of 1 X, and the mixture was separated by horizontal electrophoresis. The DNA fragments were analyzed by separation on 1% agarose gels prepared in 1 X TBE Buffer (40 mM Tris, 40 mM boric acid, 1 mM ethylene diamine tetra acetic acid [EDTA]) containing 0.5 µg/ml ethidium bromide (EtBr).

Electrophoresis was performed using 0.5X TBE Buffer as running buffer at 100 V for 35-40 minutes. The separated DNA fragments were visualized using an ultraviolet trans-illuminator and the sizes of the DNA fragments were determined by comparison with the 1 Kb DNA ladder (Fisher Scientific).

2.6.4 Bacterial medium and agar preparation

Luria Bertani (LB) broth and agar were prepared from pre-made mixtures (Invitrogen) as recommended by the manufacturer. Ampicillin (100 µg/ml) was added as a supplement when required.

2.6.5 Ligation of plasmid and insert DNA-transformation into competent *E. coli*

The purified PCR product was ligated into the plasmid or PCR 2.1 TOPO vector (Invitrogen -Figure 3) and then transformed into chemically competent *E.* coli using a classical TA Cloning Kit (Invitrogen) following the instructions of the manufacturer. Positive clones were selected by blue/white screening on LB agar plates containing 100 mg/l Ampicillin. White colonies represent the positive plasmid bearing *E. coli*.

2.6.6 Small scale plasmid DNA isolation (Miniprep)

A single white colony was inoculated into 3 ml of sterile LB Broth containing 100 µg/ml Ampicillin (LBA broth), and incubated overnight at 37°C with constant shaking at a maximum speed of 250 rpm. 1.5 ml of this culture was used for plasmid isolation and the remainder was retained to use as a starter culture for large-scale plasmid production.

Cells were pelleted from the 1.5 ml of culture by centrifugation at 16000 X g in an Eppendorf centrifuge for 10 seconds. The supernatant was discarded and the pellet was lysed in 100µl of GTE buffer (50 mM glucose, 25 mM Tris-Hcl pH 8.0, 10 mM EDTA pH 8.0). Then 200 µl of the buffer containing 0.2N NaOH and 1% sodium dodecyl sulphate (SDS) was added and mixed by inverting the tube untill the solution became clear. At this point 150 µl of potassium acetate solution (4 M potassium acetate, 2 M acetic acid) was added and mixed by vigorous shaking, then 400 µl phenol-Chloroform was added to the solution and centrifuged at 16000 X g for 3 minutes.

Three hundred and fifty µl of the aqueous top layer was transferred to a new tube and 400 µl 2-propanol added to it and mixed before centrifugation at 16000 X g for 3 minutes. The supernatant was completely discarded and 100 µl of 1 X TE (10 mM Tris-Hcl pH 7.5, 1 mM EDTA pH 8.0) and 1 µl of RNase (10 µg/µl) were added to the pellet and incubated at 37°C for 15 min.

After incubation, 120 µl of 2-propanol containing acetate (200 mM potassium acetate and 88% of 2-propanol) was added, vortexed and incubated at room temperature for 5 minutes. After incubation, it was centrifuged at 16000 X g for 10 minutes, the supernatant was discarded and 500 µl of 70% Ethanol was added to the pellet and centrifuged again at maximum speed for 2 more minutes. The supernatant was carefully aspirated and the pellet dried up using a Speed-Vac for 5 minutes. This final pellet containing the plasmid DNA was dissolved in 30 µl of double distilled water.

2.6.7 Plasmid digestion using restriction enzyme EcoRI

Less than half of the isolated plasmid DNA from 6 colonies for each gene were digested with the restriction enzyme *Eco*RI (Invitrogen) following the manufacturer's instructions. Since there are two *Eco*RI sites on the vector before and after each insert, if the insert was present, it would be cut out of the vector after digestion with *Eco*RI (Figure 3). Insert presence was confirmed by electrophoresis in 1% agarose gels.

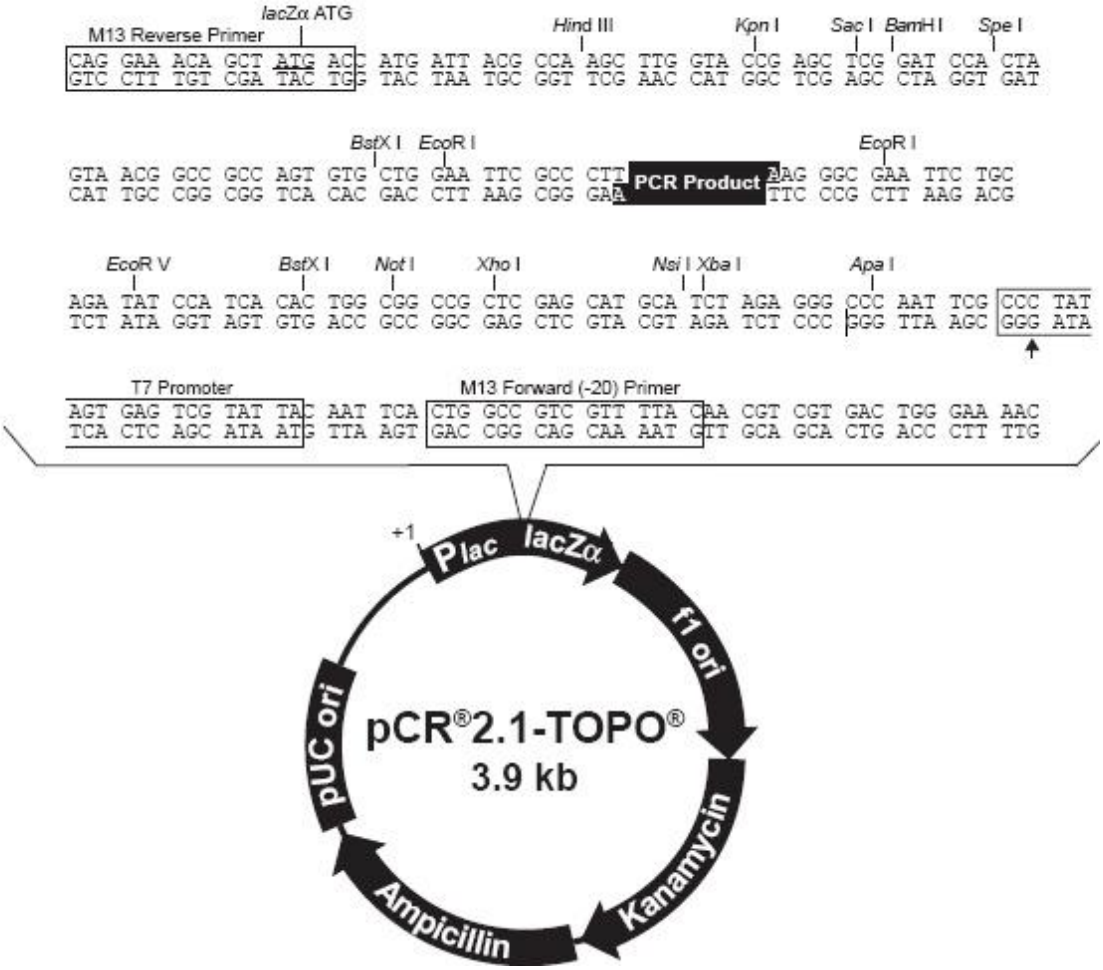

Figure 3: Map of the pCR 2.1 TOPO vector (Invitrogen) – (modified from http://tools.invitrogen.com/content/sfs/vectors/pcr2_1topo_map.pdf**)**

2.6.8 Determination of DNA concentration by spectrophotometric analysis

Later, the concentrations of the insert-containing plasmids were measured using a spectrophotometer (Beckman Coulter DU-640) in order to facilitate sending the requested concentration of these plasmids for DNA sequencing, thus confirming the sequence of the inserts.

2.6.9 Large scale plasmid DNA isolation

Two flasks, each containing 100 ml of LBA broth, were inoculated with 0.1 ml of starter culture with the correct insert (section 2.5.6) and incubated overnight at 37°C with constant shaking. The bacterial cells containing plasmid DNA were pelleted by centrifugation at 4500 X g for 15 minutes at 4°C.

The plasmid DNA was isolated from the bacterial cell pellet using a Plasmid Midi Kit from QIAGEN following the manufacturer's protocol. The concentration of isolated plasmid DNA was measured as in section 2.6.8.

2.6.10 Plasmid digestion using restriction enzyme EcoRV

The sequences of plasmid and inserts were analyzed for the restriction enzyme *Eco*RV sites. Only one *Eco*RV site outside of the insert was detected and since no site was found within the insert, this restriction enzyme was used to linearize the plasmid for the future use in serial dilutions required for quantitative real time PCR.

2.7 Gene expression quantification by quantitative real time PCR (qRT-PCR)

2.7.1 Optimization of the real time system

2.7.1.1. Serial dilutions for standard curve

Linearized plasmids (section 2.6.10) were purified using GeneElute PCR clean-Up kit (Sigma). The purified plasmid DNA concentrations were measured (section 2.6.8) and copy numbers were calculated using the following equation:

$$\text{Copies} = \frac{(6.02 \times 10^{23} \text{ copies}) \times (\text{plasmid concentration g/µl})}{(\text{Number of bases}) \times (660 \text{ Daltons/base})}$$

Tenfold serial dilutions of the plasmids (10^8 - 10^2 copies) were prepared and stored at -80°C to create standard curves for each gene. They were freshly prepared from the linearized plasmids if more than one month passed from their storage.

2.7.1.2. Primer concentration optimization

Primers for qRT-PCR were designed as mentioned in section 2.6.1. A list of these primers, called qPCR-primer pairs, is presented in Table 4. To obtain a standard curve with an efficiency approaching 100%, preliminary sets of qRT-PCR reactions were performed with the running protocol mentioned in section 2.7.2 and the annealing temperature set as average of melting temperatures of two primers. Primer concentrations of 500 nM, 750 nM, 1µM, 1.25 µM were tested. For each concentration of primers at least two of the plasmid serial dilutions (10^6, 10^7) were used as a DNA template.

QRT-PCR was performed using iQ SYBR Green Supermix and the iCycler iQ® real-time system, both from Bio-Rad. The cycle number at which sufficient amplified product accumulates to yield a detectable fluorescent signal is called the threshold cycle, or C_T. If perfect doubling occurs with each amplification cycle, the spacing of the fluorescence curves will be determined by the equation 2^n = dilution factor, where n is the number of cycles between curves at the fluorescence threshold (in other words, the difference between the C_T values of the curves or ΔC_T). For example, with a 10 fold serial

dilution of DNA, $2^n = 10$. Therefore, n = 3.32 and the C_T values should be separated by 3.32 cycles (Real-Time PCR Application Guide – Bio-Rad).

Table 4: qPCR primer pairs sequences

Human EGR-2	
Forward (5' - 3') :	TGGAGAGAAGAGGTCGTTGG
Reverse (5' - 3') :	ACCAGGGTACTGAGGGTCAA
Human GAPDH	
Forward (5' - 3') :	AGCGAGATCCCTCCAAAATC
Reverse (5' - 3') :	AAATGAGCCCCAGCCTTC
Mouse EGR-2	
Forward (5' - 3') :	ATGAACGGAGTGGCGGGAGATG
Reverse (5' - 3') :	TTCTAGGTGCAGAGATGGGAGCGAAG
Mouse GAPDH	
Forward (5' - 3') :	CGTGTTCCTACCCCCAATGTGTCC
Reverse (5' - 3') :	CTCAGATGCCTGCTTCACCACCTTC

The ΔC_T of paired reactions with similar primer concentrations and two serial dilutions were calculated and the concentration that generated the closest ΔC_T to 3.32 was considered as the optimal concentration for the primers of that gene. Table 5 shows the optimal primer concentrations for *Egr-2* and *Gapdh* genes.

Table 5: Optimized primer concentrations

Species	Gene	Primer Concentration
Mouse	m*Egr-2*	1 µM
	m*Gapdh*	750 nM
Human	h*Egr-2*	1 µM
	h*Gapdh*	1 µM

2.7.1.3. Annealing temperature optimization

Two plasmid serial dilutions (10^6, 10^7) were used as DNA templates for these experiments. The optimized concentration of primers for each gene (section 2.7.1.2) was applied and with all the other conditions being identical, a temperature gradient was programmed (65°C -72°C) for the annealing temperature in the running protocol (section 2.7.2) using the iCycler iQ™ Real-Time PCR Detection System Instruction Manual (Bio-Rad).

Eight sets of reactions for each DNA template were run, one for each time point and mentioned in section 2.7.1.2, the temperature that generated the closest ΔC_T to 3.32 was considered as the optimum annealing temperature for the primers of that gene. Table 6 shows the optimal annealing temperatures for the *Egr-2* and *Gapdh* genes.

Table 6: Optimized annealing temperatures

Species	Gene	Annealing Temperature
Mouse	m*Egr-2*	71.9 °C
	m*Gapdh*	69.4 °C
Human	h*Egr-2*	66 °C
	h*Gapdh*	65.7 °C

2.7.1.4. Creating a standard curve

To examine the efficiency of qRT-PCR reactions, a standard curve was created as follows: For each gene, eight plasmid serial dilutions (10^2 – 10^8) and one blank (double distilled water) sample using the optimized primer concentrations and annealing temperature of that gene and the corresponding running protocol (section 2.7.2) were run in triplicates to generate the standard curve. Later, the efficiencies (E) of these standard curves were used in calculations of gene expression fold changes (section 2.7.3.1).

2.7.2 QRT-PCR running protocol

One of the most important optimization factors of a qRT-PCR assay is determining the specificity of the primers chosen for amplification. A particular primer pair may induce primer-dimer formation and/or amplify other non-specific products. In turn, these non-specific products can greatly reduce amplification efficiency of a standard curve. Therefore, identification of all products amplified by a particular primer pair would be useful for optimization of qRT-PCR. Melt curve, a dynamic tool used to measure the

melting temperature (T_m) of double stranded DNA molecules, was used as a guide for improving real-time PCR assay development. A melt curve was used for identification of the peaks that represent the amplified products.

Considering all the optimized factors, the qRT-PCR running protocols were programmed on an iCycler iQ® real-time machine (Bio-Rad) following the instructions of the iCycler iQ™ Real-Time PCR Detection System Manual (Bio-Rad). Table 7 shows the running protocol used for Human Egr-2 and Gapdh qRT-PCR.

Table 7: QRT-PCR running protocol for human Egr-2 and Gapdh

Step	Temperature (°C)		Time	Cycle
First Denature	98		30 sec	X 1
Denature	95		1 sec	X 40
Anneal /Extend	*hEgr2*	*hGapdh*	15 sec	
	66	65.7		
Acquisition	83	80	10 sec	
Last Extend	72		1 min	X 1
Initial Denature for Melt Curve	95		1 min	X 1
Melt Curve	95 -> 25 (decreased by 0.5 °C)		10 sec	X 140
Hold	4		∞	

The qRT-PCR running protocol for Mouse *Egr-2* and *Gapdh* can be seen in Table 8. Other than higher annealing temperatures, the cycle number is also reduced in the second running protocol.

Table 8: QRT-PCR running protocol for mouse Egr-2 and Gapdh

Step	Temperature (°C)		Time	Cycle
First Denature	98		30 sec	X 1
Denature	95		1 sec	X 35
Anneal /Extend	*mEgr2*	*mGapdh*	15 sec	
	71.9	69.4		
Acquisition	80	72	10 sec	
Last Extend	72		1 min	X 1
Initial Denature for Melt Curve	95		1 min	X 1
Melt Curve	95 -> 25 (decreased by 0.5 °C)		10 sec	X 140
Hold	4		∞	

2.7.3 QRT-PCR data analysis

2.7.3.1. Relative quantification – The Pfaffl method

When comparing multiple samples using relative quantification, one of the samples is usually chosen as the calibrator, normally the untreated or control sample, and the expression of the target gene in all other samples (e.g. TNFα treated samples) is expressed as an increase or decrease relative to the calibrator. In this method, a gene such as Gapdh or β-Actin whose expression is not changed by the treatment under study (e.g. TNFα) is used as a reference gene.

To determine the relative expression of a target gene (e.g. *Egr-2*) in the test sample and calibrator sample using a reference gene (e.g. *Gapdh*) as the normalizer, the expression levels of both the target and the reference genes need to be determined using qRT-PCR. C_T values of target and reference genes in both test and calibrator samples are de-

termined from iCycler iQ Optical System Software and the Pfaffl method (Real-Time PCR Applications Guide from Bio-Rad) is used to calculate the expression ratio (fold change) of target gene between the sample and calibrator, using the following formula:

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta C_T,\text{target (calibrator – test)}}}{(E_{\text{ref}})^{\Delta C_T,\text{ref (calibrator – test)}}}$$

In the above equation, E_{target} and E_{ref} are the amplification efficiencies of the target and reference genes obtained from their standard curves. ΔC_T, target (calibrator – test) = C_T of the target gene in the calibrator minus the C_T of the target gene in the test sample, and ΔC_T, ref (calibrator – test) is the C_T of the reference gene in the calibrator minus the C_T of the reference gene in the test sample (*Real-Time PCR Applications Guide from Bio-Rad*).

2.7.3.2. Absolute quantification

In absolute quantification, the quantity (copy number) of the unknown sample is interpolated from a range of standards of known quantity. To construct a standard curve, the linear plasmid of each gene with known concentration was used. Dilution of this template was then performed (section 2.7.1.1) and these dilutions served as the standards. The unknown test samples were assayed with the standards in the same experimental run. The standard curve constructed from the diluted standard template was then used to determine the target quantity in the unknown sample by interpolation.

2.7.4 Gene expression quantification

QRT-PCR was performed using iQ SYBR Green Supermix and the iCycler iQ® real-time system both from Bio-Rad. HeLa S3 cells and primary cortical neurons were treated with 5-100 ng/ml of TNFα at different time points (see results). Total mRNA of HeLa S3 cells was extracted using an AllPrep kit (section2.3.1) and TRIZOL was used to extract total mRNA of the primary neurons (section 2.3.2). One µg of mRNA of each sample was reverse transcribed into cDNA as described in section 2.5.

Equal amounts of cDNAs were subjected to qRT-PCR using a proper running protocol (section 2.6.2) and a reaction without template was included as the negative control. QRT-PCRs were performed in triplicate and repeated three times for confirmation. Egr-2 expressions in HeLa S3 cells and cortical neurons were calculated after normalization with the Gapdh using the Pfaffl method (section 2.7.3.1).

2.8 Protein extraction and protein concentration determination

2.8.1 Whole cell protein extraction

2.8.1.1. AllPrep DNA/RNA/Protein Mini Kit (QIAGEN) method

Proteins of TNFα treated HeLa S3 cells were extracted using the All-prep/DNA/RNA Mini kit (QIAGEN) following the manufacturer's instructions.

2.8.1.2. Protein extraction using a lysis buffer

For the TNFα treated primary cortical neurons, after collecting the media, 60µl of the lysis buffer containing 50mM Tris-Hcl pH 6.8, 2% SDS and 1X Protease Inhibitor (PI) Cocktail (Roche) was added to each well. Using a cell scraper, neurons were transferred to a 1.5 ml tube on ice. To lyse the cells, all samples were frozen and thawed in liquid nitrogen four times. To remove unbroken cells and membrane, the samples were centrifuged in an Eppendorf 5810 R centrifuge at maximum speed for 15 minutes at 4°C. The supernatants from the cell lysate containing proteins were transferred to new tubes.

2.8.2 Nuclear and cytoplasmic protein extraction

To separate the nuclear extracts and cytoplasmic fractions and produce extracts enriched for proteins from these cellular compartments, the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) were used following the manufacturer's instructions.

2.8.3 Protein concentration measurement

The protein concentrations were measured using a Micro BCA protein assay kit (Pierce) following the manufacturer's protocol and the remainder of the samples were stored at -80°C.

2.9 Western blot analysis

2.9.1 Sample preparation and gel electrophoresis

The NuPAGE® Bis-Tris, 4-12% Mini Gels (1mm thickness) from Invitrogen were used for electrophoresis of proteins. Aliquots of proteins (10-80 µg) were prepared and separated on NuPAGE gels at constant voltage (100 V) for 120 min following the instructions from the NuPAGE® technical guide (Invitrogen).

2.9.2 Transfer to Immobilon-P Membrane (PVDF)

Proteins separated on a NuPAGE gel were transferred onto the PVDF membrane using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) following the manufacturer's instructions. Transfers were carried out at constant voltage (20 V) for 35 minutes at room temperature.

2.9.3 Detection of proteins using antibodies

After blocking the membrane with 5% skim-milk in TBS solution containing 0.2% Tween-Twenty (TBST) for 1 hour at room temperature or overnight at 4°C, the membrane was incubated with rabbit anti-Egr2 polyclonal antibody, diluted in TBST containing 1% skim milk, overnight with gentle shaking at 4°C. The membrane was washed 4 times in 30 minutes (15 minutes followed by 3 x 5 minutes) with TBST containing 1% skim milk.

After the last wash, the membrane was incubated with secondary antibody, goat anti-rabbit IgG conjugated with horseradish peroxidise (HRP) from Santa Cruz, diluted 1:6500 in TBST at room temperature with gentle shaking for 1 hour. For the last time, the membrane was washed 4 times in 30 minutes with TBST only. The chemiluminescence signal was visualized using the ECL-Plus detection system (GE Health Care Bio-Sciences) following the manufacturer's instructions.

2.9.4 Stripping the membrane

The Membrane was incubated in stripping buffer (100mM β-mercapto-ehtanol, 2% SDS, 62.5 mM Tris-HCl pH 6.8) with gentle shaking at 50°C for 30-40 min. After incubation, the membrane was washed 3 times in 45 minutes (3 x 15 minutes) with TBST. Actin (~43 kDa) was used as loading control for the cytoplasmic and whole cell proteins and HDAC1 (~62 kDa) was used as loading control for the nuclear proteins. Actin antibody (Sigma, 1:1000) and HDAC-1 antibody (Upstate Biotech, 1:500) were used to detect Actin and HDAC-1 respectively using the same protocol mentioned in the previous section.

2.10 Analysis of NF-κB-binding sites within the Egr-2 promoter

The mouse *Egr-2* upstream sequence information was obtained from the Ensemble website (http://www.ensembl.org/) and the gene ID listed in Table 2. Using the P-Match program (http://www.gene-regulation.com/), 3000 bp of the immediate upstream sequence of the Egr-2 gene promoter was searched for putative NF-κB binding sites. Prim-

ers targeting these regions (Figure 13) were designed using Primer 3 software (section 2.6.1) and used for chromatin immunoprecipitation (ChIP) analysis (section 2.11).

Moreover, the primer sequences were analysed using the BLAST-like alignment tool (http://genome.ucsc.edu/) which confirmed that these oligonucleotide sequences contained 100% homology to sequences within the *Egr-2* gene without homology to any other known sequences in the mouse genome.

2.11 Chromatin Immunoprecipitation (ChIP) Assay

The whole forebrains of CD-1 mice (E16 –E18) embryos were removed by dissection and kept in ice-cold PBS (0 – 5°C) solution. Using a Pasteur pipette, forebrains were dissociated to a single cell suspension and immediately incubated in 1% paraformaldehyde (PFA) in PBS at 37°C for 15 minutes. PFA cross-links the proteins to DNA or to the other proteins in their vicinity. To stop the cross-linking after 15 minutes, cells were incubated with 143 µl of 1 M Glycine per ml of solution at room temperature for 5 minutes and washed twice with an ice-cold PBS solution containing protease inhibitors (1 mM/L PMSF, 1 X protease inhibitor cocktail).

The cross-linked cells were homogenized in lysis buffer (1% SDS, 10 mM/L EDTA, and 50 mM/LTris-HCl pH 8.1, 1 mM/L PMSF, 1 X protease inhibitor cocktail) and incubated on ice for 10 minutes. Using a Branson Sonifier 350, the cell lysate was sonicated on ice for 10 – 15 times with 15 seconds pulses at 40% pulse strength and output control <4 and the lysate sat on ice for at least 1 minute between pulses. The genomic DNA fragment size was checked on an 1% agarose gel and sonication was repeated if

necessary until the fragment size was between 400–500 bp. At this time OD260 units of the sonicated lysate were measured.

Pierce Ultralink Protein A/G beads were washed twice with dilute buffer (0.01% SDS, 1.1% Triton X, 1.2 mM EDTA, 16.7 nM Tris-HCL pH 8.1 and 167mM NaCl) containing protease inhibitors (1mM/L PMSF, 1 X protease inhibitor cocktail). The samples were diluted in the above mentioned dilution buffer to have a 10 X OD260 (500 µg) DNA concentration in 400 µl of diluted sample. For each antibody two sets of samples were needed: one to add the antibody (Ab^+) and one without antibody (Ab^-) as a control. Sixty – hundred µl of the washed 50% slurry of beads was added to diluted samples and incubated at 4°C on a shaker overnight.

The samples were centrifuged at 2500 rpm for 2 minutes to precipitate the beads. The supernatants were collected and transferred to new tubes. BSA and tRNA, each to the final concentration of 500 µg/ml was added to each tube. Thirty µl of p50 antibody (goat polyclonal IgG from Santa Cruz - catalog number: sc-1190X) and 10 µl of p65 antibody (rabbit polyclonal IgG from Santa Cruz - catalog number: sc-109X) were added to the Ab^+ samples to immunoprecipitate DNA-p50 and DNA-p65 complexes. The Ab^+ and Ab^- samples were incubated on a shaker at 4°C overnight. Sixty µl of prewashed beads were added to the Ab^+ and Ab^- samples and incubated at 4°C on a shaker for 3 more hours.

After immunoprecipitations with beads were completed, the samples were centrifuged at 2500 rpm for 2 minutes, the supernatants were transferred to new tubes labelled as INPUT samples and the beads were washed as follows: 5 minutes with low salt buffer

(2 mM EDTA, 20 mM Tris-HCL pH 8.1, 150 mM NaCl, 0.1% SDS, 1% Triton X), 30 minutes with high salt buffer (2 mM EDTA, 20 mM Tris-HCL pH 8.1, 500 mM NaCl, 0.1% SDS, 1% Triton X), 30 minutes with LiCl buffer (1 mM EDTA, 10 mM Tris-HCL pH 8.1, 0.25 M LiCl, 1% NaDeoxycholate, 1% IGEPAL) and 5 minutes with TE buffer pH 8.0 (0.1 M Tris-HCl pH 7.5, 10 mM EDTA pH 8.0) twice.

To elute the specifically bound p50 and p65 complexes from the washed beads, 250 µl of freshly prepared elution buffer (1% SDS, 0.1 M NaHCO3) heated to 65°C was added to each sample and incubated at room temperature for 15 minutes on a shaker. The samples were centrifuged at 13000 rpm for 2 minutes and the eluted supernatants were transferred to new tubes. The elution step was repeated one more time and the two supernatants were combined.

Table 9: Sequences of primers used in ChIP assay

First and Second Binding Site *	
Forward (5' - 3') :	AGAGTTCTTGGAAGGCTCA
Reverse (5' - 3') :	GGGAGGATTGCAGCAG
Third Binding Site	
Forward (5' - 3') :	ACTTTCACGGTTGGCACGAGGAGAA
Reverse (5' - 3') :	TCCTGCCCTATTGGAGTACGGTGGTCA
Fourth Binding Site (Closest to TSS)	
Forward (5' - 3') :	TAGGGTCGGTGCTCGCGCCTTCTT
Reverse (5' - 3') :	AGGAACGATTTGCAGCCGGTGGAC

***** Positions of the binding sites on the promoter of *Egr-2* can be viewed in Figure 13.

To reverse cross-link the eluted DNA protein complexes and the INPUT samples, 25 µl of 5 M NaCl and 1 µl of RNase A (10 mg/ml) were added to 500 µl combined elu-

ates and INPUT samples and incubated at 68°C overnight. For protein digestion, 40 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCL pH 6.5 and 2 µl of proteinase K (20 mg/ml) were added to each sample and incubated for 2 hours at 55°C.

The DNA was extracted using the Qiaquick PCR purification kit (QIAGEN) following the manufacturer's instructions. Approximately 10% of the precipitated genomic DNA was used for PCR detection (section 2.6.2) using the primers targeting the four NF-κB binding sites on the promoter of *Egr-2* gene listed in Table 9, 10% of 5M Betaine to increase yield and specificity of PCR products, and the running protocol listed in Table 10.

Table 10: PCR protocol used for ChIP assay

Step	Temperature (°C)	Time	Cycle
First Denature	98	30 sec	X 1
Denature	92	1 sec	X 37
Anneal /Extend	70	15 sec	
Last Extend	72	1 min	X 1

2.12 Electrophoretic Mobility Shift Assays (EMSA)

2.12.1 Target probe design

Table 11 shows the sequences of the target probes that are DNA fragments containing the NF-κB (p50/p65) binding consensus sequence. These 20 bp sequences were designed so that they included the 10 bp NF-κB (p50/p65) consensus binding sequences

(underlined in both forward and reverse probes) detected on the fourth binding site (closest to TSS) upstream of the mouse *Egr-2* gene promoter (Figure 13).

Table 11: Sequences of target probes used for EMSA

Forward (5' - 3') :	CTCTGCAGTCCGGAGTCCCCGCTGCAGGCA
Reverse (5' - 3') :	GAGACGTCAGGCCTCAGGGGCGACGTCCGT

These probes were dissolved in TES buffer (1 M Tris-Hcl pH 8.0, 0.5 M EDTA, 0.15 NaCl). Using the annealing protocol listed in Table 12 and 40 µl of each primer (20 ng/µl in TES buffer), complementary oligonucleotides were annealed in a BioRad Thermal Cycler to form a duplex.

Table 12: Annealing protocol for target probes (EMSA)

Step	Temperature (°C)	Time	Cycle
1	95	5 min	X 1
2	95 – (1°C per Cycle)	1 min	X 70
3	4	∞	

2.12.2 Labelling and detection

The whole forebrains of CD-1 mice (E16 –E18) embryos were dissected and homogenized in lysis buffer (10 mM Tris pH 8, 60 mM KCl, 1 mM EDTA, 0.3 % NP40) with mortar and pestle, and kept on ice for 15 minutes to lyse cells. Samples were spun at high speed (10,000 rpm) to separate the cytoplasm. The pellet containing the nuclear

fraction was homogenized with another buffer (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA), mixed vigorously for 15 minutes at 4 °C, and spun at max speed for 5 minutes. Supernatant containing nuclear extract was combined with cytoplasmic extract and flash frozen.

Samples were thawed on ice, and a Bradford protein assay was performed (Bio-Rad). Tissue sample (10 µg) was incubated with ^{32}P-labeled oligonucleotide containing custom sequence to test for NF-κB consensus sequence (target probes - see section 2.12.1) or commercial NF-κB consensus sequence (Santa Cruz Biotechnology) for 30 minutes at room temperature. A 6 % native acrylamide gel was use to separate samples by vertical electrophoresis for 2 hours at 200 V. Gels were dried and placed on film overnight before exposing. For supershift assays, 2 µg of antibody specific for the p50 or p65 subunit of NF-κB (see section 2.11 for details) was added to samples and incubated for 1 hour prior to adding oligonucleotide.

This part of the study (section2.12.2) was conducted by Jason Schapansky in Dr. Gordon Glazner's laboratory.

2.13 Small Interfering RNA (siRNA)

2.13.1 Primary embryonic neocortical cell cultures

Timed-pregnant CD-1 mice were used to generate primary cultures. Neocortex was dissected and collected in Hank's Balanced Salt solution (HBSS). The tissue was incubated for 10 min at room temperature with 0.05 mg/ml trypsin (GIBCO). The cells were

pelleted by centrifugation (1200 rpm, 5 min), then resuspended and gently triturated to a single cell suspension in HBSS containing 100 μg/ml DNase I (Sigma). The cell suspension was transferred to a tube containing Neurobasal medium with B-27 (GIBCO) and penicillin-streptomycin-fungizone (100U/ml) (Life Technologies). Cells were counted and $3x10^5$ cells were plated per well (poly-D-lysine coated 24 well plate (Cel-lines Associates, Inc)) and cultured at 37^oC with 5% CO_2.

2.13.2 Transfection of Primary Cultures with siRNA

NF-κB p50 siRNA (m:sc-29408), NF-κB p65 siRNA (m:sc-29411) and control siRNA (sc-37007) were obtained from Santa Cruz Biotechnology. The siRNAs consist of pools of three to five specific 19-25 nucleotide siRNAs designed to knock-down NF-κB p50 or NF-κB p65 gene expression. Transient transfection using siRNA transfection reagents (sc-45064, Santa Cruz Biotech) was carried out on culture Day 2. 10 pmols (1μl) siRNA were added per well on 24 well plates following the Santa Cruz Biotech siRNA transfection protocol. 48 hr after transfection, cells were washed with cold PBS twice, and subjected to qRT-PCR.

2.13.3 RT-PCR

Total RNA, from siRNA transiently transfected cells, was prepared with RNA-Bee (TEL-TEST.INC). For reverse transcription (RT)-PCR, 1 μg of total RNA was used as a template to synthesize the first–strand cDNA. Pre-RT, RNA samples were treated by DNase I (Sigma) for 15 min at room temperature to digest the genomic DNA, then stop

solution was added to inactivate the DNase I at 70°C for 10 min. These sample tubes were immediately placed on ice.

The iScript™ cDNA synthesis kit (Bio-Rad) was used with 20 µl total reaction volumes. RT reactions were performed for 5 min at 25°C, 30 min at 42°C and 5 min at 85°C. The cDNA was used as the template for NF-κB p50, p65, Egr1, Egr2 and GAPDH amplification. PCR was performed in 25 µl reactions using 1µl cDNA and 5 pmol of each primer (listed in Table 13) and QIAGEN-HotstarTaq DNA polymerase (Cat#203205).

Table 13: Primers used for the siRNA experiment

Mouse GAPDH	
Forward (5' - 3') :	CACATTGGGGGGTAGGAACAC
Reverse (5' - 3') :	CTCATGACCACAGTCCATGC
Mouse EGR2	
Forward (5' - 3') :	ACCTCGAAAGTACCCTAACAG
Reverse (5' - 3') :	GTGAGTAGTAAGGTGGTCACT
Mouse EGR1	
Forward (5' - 3') :	GCTCACTCCACTATCCACTCT
Reverse (5' - 3') :	GTGGTCACTACGACTGAAGTTA
siRNA Primers	
NF-κB P50:	sc-29408-PR (NF-κB p50(m)-PR (Santa Cruz Biotech)
NF-κB P65:	sc-29411-PR (NF-κB p65(m)-PR (Santa Cruz Biotech)

This part of the study (section 2.12) was conducted by Shunzhen Zhang in Dr. David Eisenstat's laboratory.

2.14 Statistical Analysis

For Western blotting data, band intensities were quantified using ImageJ software (http://rsbweb.nih.gov/ij/). Statistical analysis was performed using the Students t-test with a 95% confidence interval followed by a one-way ANOVA post hoc Scheffe test using SPSS Software (SPSS Inc.). *$p < 0.05$ were considered to be significant.

For qRT-PCR data, plots of the *Egr-2* expression fold change in TNFα-treated samples were examined first. Extreme outliers were excluded for further comparative analysis. Comparisons of the *Egr-2* expression levels between different time points of TNFα stimulation were assessed using SPSS software as mentioned above.

Results

3.1 Analysis of *Egr-2* mRNA expression

3.1.1 TNFα treatment

To study the mRNA expression levels of the *Egr-2* gene upon NF-κB activation in both HeLa cells and primary mouse cortical neurons (section 1.8.2), cells were treated with mouse recombinant TNFα (R&D Systems Inc.). Since HeLa S3 cell line is insensitive to TNFα apoptotic activity, it is an ideal cell line for TNFα stimulation in order to evaluate the NF-kB dependent expression pattern of the *Egr-2* gene in human.

Previous LTP and Genechip experiments performed in this laboratory showed a 3-fold increase in *Egr-2* expression 3 hours after induction of LTP in hippocampal slices (section 1.14). However, since the time course of NF-κB activation in the cells upon stimulation with TNFα maybe different than following electrophysiological stimulation, different time points were considered for TNFα treatment to investigate the pattern of *Egr-2* expression.

3.1.2 *Egr-2* expression measurement in TNFα treated HeLa cells

To assess the time-dependent effect of TNFα treatment on the *Egr-2* mRNA expression, HeLa cells were exposed to mouse TNFα for 0, 1/2, 1, and 3 hours. After TNFα incubation, mRNA of the cells were extracted (section 2.3.1), their concentrations were measured (section 2.3), and transformed to cDNA (section 2.5).

Equal amounts of these cDNAs were used in previously optimized qRT- PCR reactions for both human *Egr-2* and *Gapdh* genes done in triplicate (2.7.2). The fold changes

of *Egr-2* in TNFα treated samples versus control were calculated using Pffafl method where *Egr-2* was considered our target gene and *Gapdh* our reference gene (section 2.7.3.1).

The whole experiment was repeated three more times and the final *Egr-2* expression fold change in different time points were statistically analyzed (methods described in section 2.14). The expression of *Egr-2* increased approximately 60 times 30 minutes after TNFα treatment, approximately 150 times 1 hour after TNFα treatment, and approximately three times 3 hours after TNFα treatment versus control. The increases in *Egr-2* expression 1 and 3 hours after TNFα treatment were statistically significant as demonstrated in Figure 4.

3.1.3 *Egr-2* expression measurement in TNFα and SN50 treated HeLa cells

To determine the effect of NF-κB inhibition on the expression of *Egr-2*, we decided to inhibit NF-κB with the cell-permeable synthetic peptide SN50 (Calbiochem). This compound contains the nuclear localization sequence (amino acids 360–369) of NF-κB p50 and inhibits nuclear translocation of the NF-κB active complex (section 2.4)

Based on the previous experiments, 1 hour stimulation with TNFα induces the maximum amount of *Egr-2* expression and therefore HeLa cells were incubated with TNFα for 1 hour before their mRNA were extracted and transformed to cDNA. Equal amounts of these cDNAs were used in previously optimized qRT- PCR reactions for both human *Egr-2* and *Gapdh* performed in triplicate (section 2.7.2).

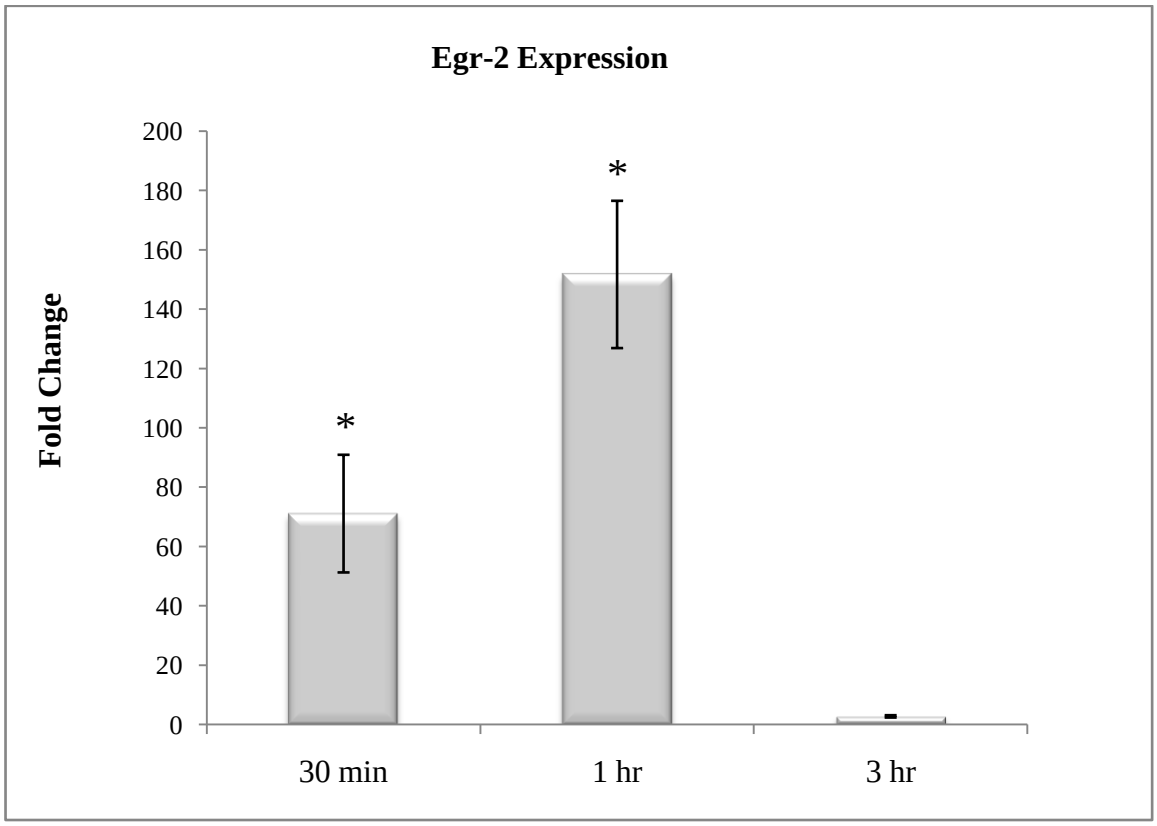

Figure 4. Egr-2 mRNA expression level in TNFα treated HeLa cells. HeLa cells were treated with mouse recombinant TNFα (5ng/ml) for the indicated periods of time. mRNA was extracted (section 2.3) and 1µg was reverse transcribed to cDNA (section 2.5). *Egr-2* mRNA expression levels in TNFα treated cells were quantified using qRT-PCR and normalized to *Gapdh* mRNA expression at each time point (section 2.7.3.1). Values represent the mean ± SEM (n = 4). Statistical analysis was performed as indicated in section 2.14. *$p < 0.05$ was considered to be significant.

The expression of *Egr-2* mRNA level in in TNFα treated cells in the presence and absence of SN50 was calculated using absolute quantification (section 2.7.3.2). The whole experiment was repeated 2 more times and the average of *Egr-2* expressions in HeLa cells upon TNFα treatment with or without SN50 inhibition were statistically analyzed (section 2.14).

Figure 5 shows that the expression of *Egr-2* mRNA, which is increased upon treatment with TNFα, is decreased in presence of the NF-κB inhibitor, SN50. The decreased transcript level of *Egr-2* in TNFα treated cells after treatment with SN50 supports the NF-κB dependency of Egr-2 induction.

3.1.4 *Egr-2* expression measurement in TNFα treated primary cortical neurons

To determine *Egr-2* mRNA expression in primary cortical neurons at different time points after treatment with TNFα, cortical neurons was treated with mouse recombinant TNFα (100 ng/mL) for 0, 1/2, 1, 2, and 4 hours. After TNFα incubation, mRNA of the neurons were extracted (section 2.3.2), their concentrations were measured, and transformed to cDNA (section 2.5).

Equal amounts of these cDNAs were used as templates in the previously optimized qRT- PCR reactions for both mouse *Egr-2* (target gene) and *Gapdh* (reference gene), performed in triplicate (2.7.2). The fold changes of *Egr-2* expression in TNFα treated samples versus control were calculated using Pffafl method (section 2.7.3.1).

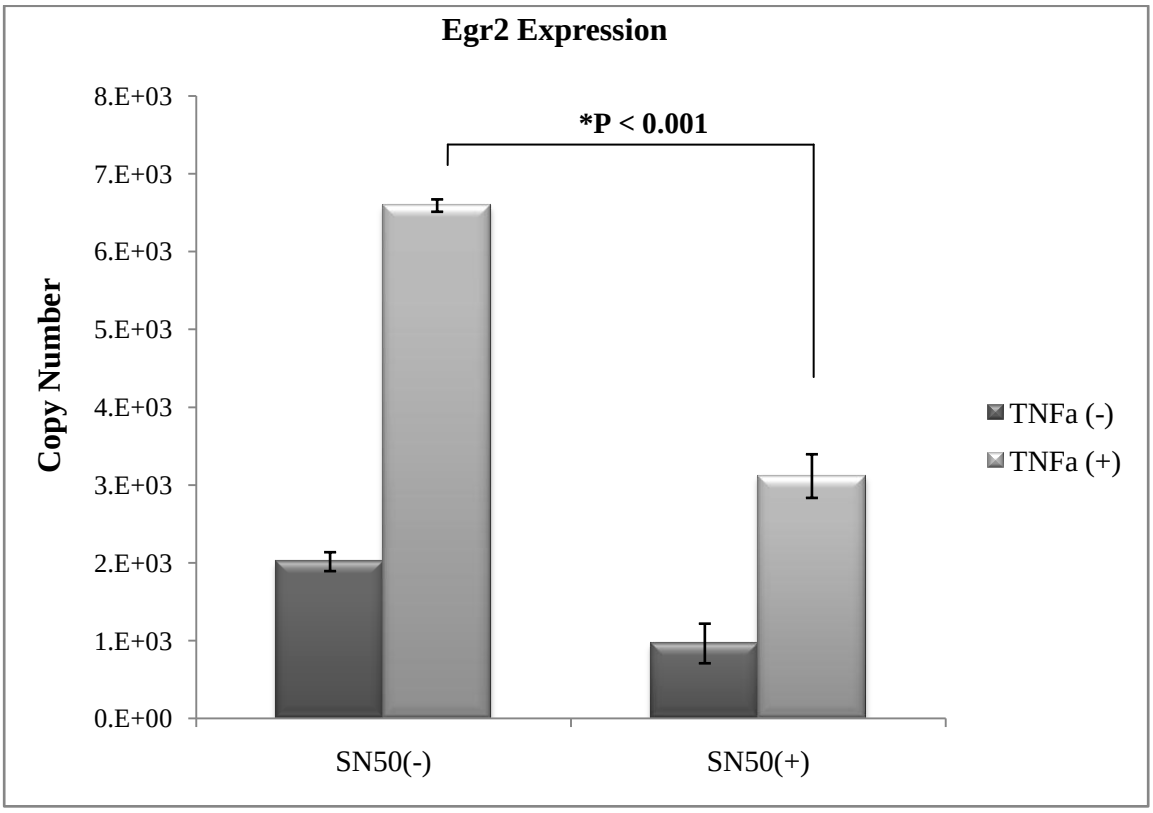

Figure 5: Egr-2 mRNA expression level in TNFα treated HeLa cells in the presence of SN50. HeLa cells were treated with SN50 (100 µg/ml, 1 hour) and subsequently treated with recombinant mouse TNFα (10 ng/ml) for the indicated periods of time (section 2.4). One µg of each mRNA sample was reverse transcribed to cDNA (section 2.5) and *Egr-2* mRNA expression (cDNA copy number) in the TNFα treated cells was quantified using qRT-PCR in the presence and absence of SN50 using absolute quantification (section 2.7.3.2). Values represent mean ± SEM (n = 3). Statistical analysis was performed as indicated in section 2.14. *$p < 0.05$ was considered to be significant.

This experiment was repeated twice more, the averages of *Egr-2* expression changes in different time points were measured, and the variations between the averages were statistically analyzed (section 2.14). Similar to HeLa cells, the expression of *Egr-2* significantly increased 30 minutes after TNFα treatment, reached to its maximum 1 hour after treatment and decreased 2 and 4 hours after treatment (Figure 6).

3.2 Analysis of Egr-2 protein expression

To determine whether enhanced levels of *Egr-2* mRNA transcript in TNFα treated cells (HeLa cells and primary cortical neurons) would lead to higher levels of protein, Western blotting was performed.

3.2.1 Egr-2 protein level in TNFα treated HeLa cells

3.2.1.1. Western blotting of whole cell proteins

Protein from the same HeLa cells whose mRNA was used for qRT-PCR, were extracted using an Allprep Kit (QIAGEN), their. After measuring the protein concentrations as described in section 2.8.3, 5-10 µg of each protein sample was used in Western blotting using the Egr-2 antibody from Santa Cruz (section 2.9) and Actin antibody (Sigma) was used as a loading control.

In Figure 7, expression of Egr-2 protein (~ 48 kDa) in HeLa cells did not change after treatment with mouse recombinant TNFα for indicated periods of time and nor did it change in the protein samples extracted from TNFα treated HeLa cells in presence or absence of SN50 for the same periods of time.

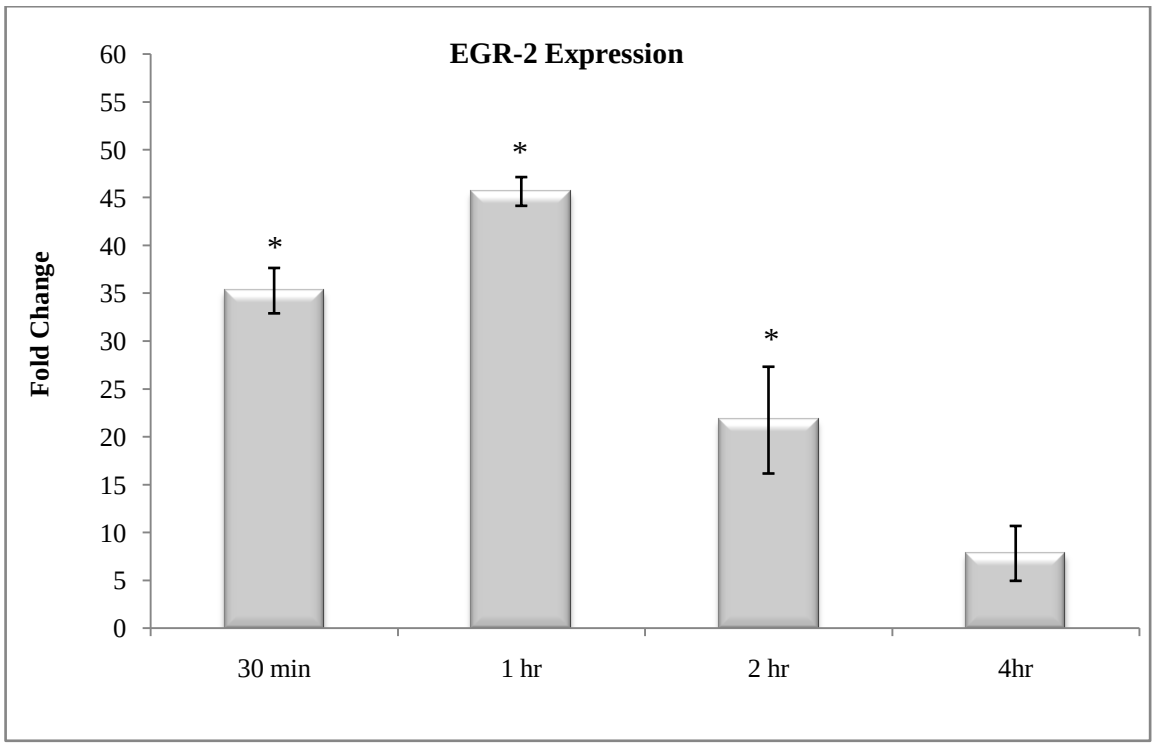

Figure 6: Egr-2 mRNA expression level in TNFα treated primary cortical neurons. Cortical neurons were treated with mouse recombinant TNFα (100 ng/ml) for the indicated periods of time and their mRNA were extracted (section 2.3). One µg of each mRNA sample was reverse transcribed to cDNA (section 2.5). *Egr-2* mRNA expression levels in TNFα treated cells were quantified using qRT-PCR and normalized to *Gapdh* mRNA expression at each time point (section 2.7.3.1). Values represent mean ± SEM (n = 3). Statistical analysis was performed as indicated in section 2.14. *$p < 0.05$ was considered to be significant.

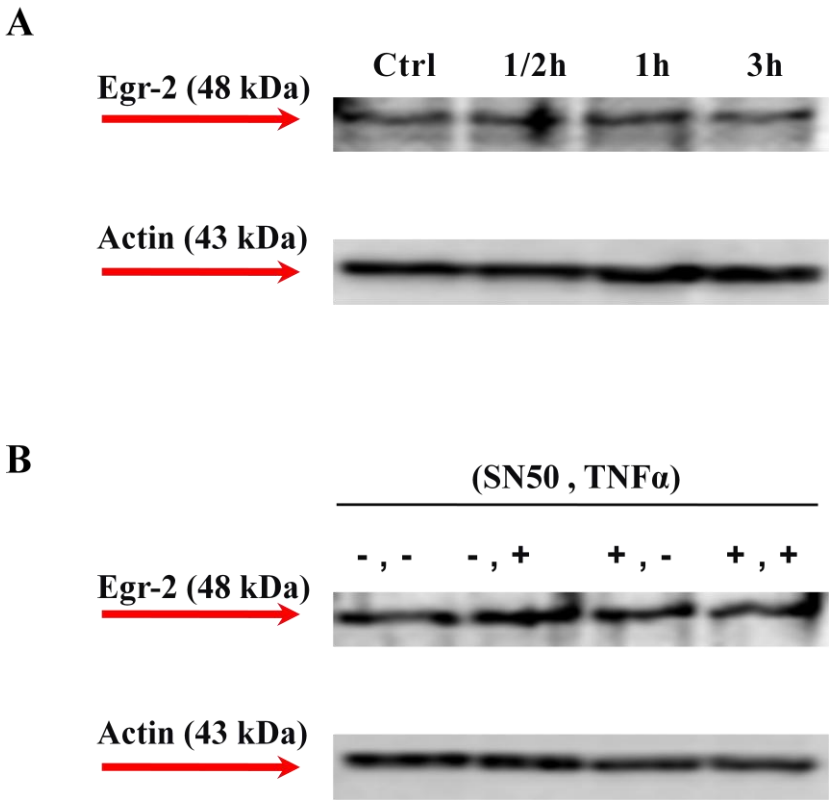

Figure 7: Egr-2 protein expression in TNFα treated HeLa cells. Expression levels of Egr-2 protein (~ 48 kDa) in HeLa cells did not change after treatment with mouse recombinant TNFα (5 ng/ml) for the indicated periods of time. (A) Proteins were extracted from 0, 1/2, 1, and 3 hours TNFα treated HeLa cells as mentioned in section 2.8.1 and 10 µg of total protein was used for Western blotting (section 2.9). (B) Proteins were extracted from TNFα treated HeLa cells in the presence or absence of SN50 (e.g. (-,-) indicates neither SN50 nor TNFα treatment) (section 2.4) and 5 µg of total protein was used for Western blotting (section 2.9). Egr-2 antibody from Santa Cruz (1:200) was used for both experiments.

These Western blots were repeated for all the protein samples extracted from HeLa cells and the results were the same for all the Western blots and no change of Egr-2 protein levels was observed (data not shown).

3.2.1.2. Western blotting of cytoplasmic and nuclear proteins

The nuclear and cytoplasmic proteins were extracted from HeLa cells as described in section 2.8.2 and immunoprecipitated as described in section 2.9 using Egr-2 antibody to detect the Egr-2 protein levels, Actin antibody as a loading control for cytoplasmic proteins, and HDAC1 antibody as a loading control for nuclear proteins.

Figure 8 shows the cytoplasmic proteins blot (A) and the nuclear proteins blot (B). As it can be seen in this figure, there was no change in the Egr-2 protein levels in the cytoplasm but increase in the nucleus upon treatment of HeLa cells with TNFα at 1 and 3 hours. This experiment was repeated 3 more times using higher concentrations of proteins and another Egr-2 antibody from Covance (Data not shown).

3.2.2 Egr-2 protein level in TNFα treated primary cortical neurons

The low basal levels of Egr-2 protein in HeLa cells could be one of the reasons why it was difficult to detect differences in Egr-2 protein levels upon treatment with TNFα. To determine if that was the case, a Western blot was performed using proteins extracted from forebrain tissue of a 2-3 month old mouse and it showed a considerably higher basal level of Egr-2 protein (data not shown). Therefore, we decided to measure Egr-2 protein levels in primary cortical neurons instead of HeLa cells upon treatment with TNFα.

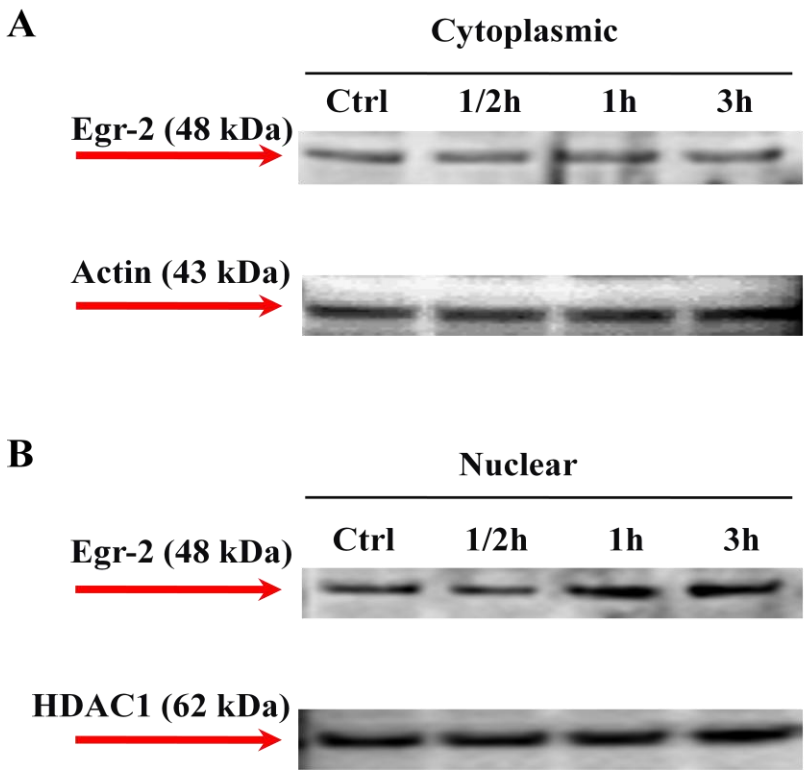

Figure 8: Egr-2 protein expression in cytoplasmic and nuclear fractions of TNFα treated HeLa cells. Expression of EGR-2 protein in the cytoplasmic fraction of HeLa cells did not show any change after treatment with mouse recombinant TNFα (5 ng/ml) for the indicated periods of time, whereas there was an increase of Egr-2 in the nucleus at 1 and 3 hours. (A) Cytoplasmic proteins were extracted from 0, 1/2, 1, and 3 hours TNFα treated HeLa cells (section 2.8.2) and 30 µg protein was used for Western blotting (section 2.9). (B) Fifteen µg of the nuclear proteins extracted from the same samples used for cytoplasmic proteins, was used for Western blotting. Egr-2 antibody from Santa Cruz (1:200) was used for both experiments.

Proteins extracted from primary cortical neurons treated with mouse recombinant TNFα at different time points were used in Western blotting as described in section 2.9. Figure 9 shows two different blots made of these samples one of which (A) was probed with the Egr-2 antibody from Lifespan Biosciences and the other one (B) probed with the Egr-2 antibody from Covance.

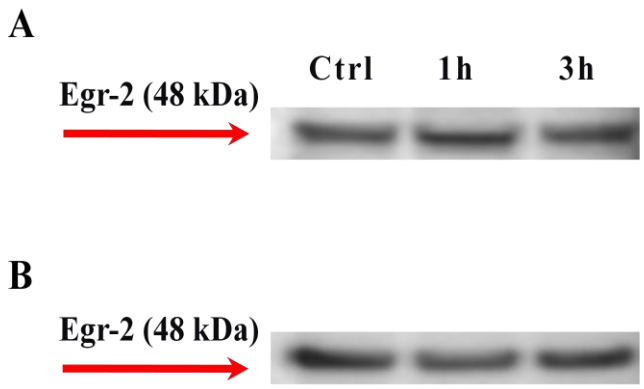

Figure 9: Egr-2 protein expression in TNFα treated cortical neurons. Expression of EGR-2 protein in cortical neurons did not change after treatment with mouse recombinant TNFα (100ng/ml) for 0, 1, and 3 hours (n=1). Fifteen µg of each protein was used for Western blotting (section 2.9). (A) Egr-2 antibody (Lifespan Biosciences, 1:500) was used to detect Egr-2 protein. (B) Egr-2 antibody (Covance, 1:500) was used to detect Egr-2 protein.

No difference in the Egr-2 protein levels can be seen in any of the blots. This experiment was also repeated two more times, once using higher concentrations of loaded proteins, and another time using proteins extracted from the mixture of cortical neurons and glial cells treated with TNFα for the same indicated times. For both Western blots, Egr-2 protein levels (Lifespan Biosciences antibody) showed no change (data not shown).

To study the effect of other TNFα treatment time points on Egr-2 protein expression, cortical neurons were treated with TNFα for 0, 6, 12, and 24 hours. The proteins extracted from these neurons using the method mentioned at section 2.8.1.1 were used for Western blotting using the Egr-2 antibody (Lifespan Biosciences) as the primary antibody (section 2.9).

The result of this experiment demonstrated a modest increase in the Egr-2 protein level 12 hours after treatment with TNFα. To confirm the results this experiment was repeated two more times and the Egr-2 band intensities were quantified using ImageJ software and presented as optical density ratios of Egr-2 (~48 kDa) to Actin (~ 43 kDa) for control. However, the bar graph of Figure 10 which represents the mean ± SEM (n = 3) from three different blots does not show any statically significant difference in the Egr-2 protein expression 12 hours after treatment with TNFα.

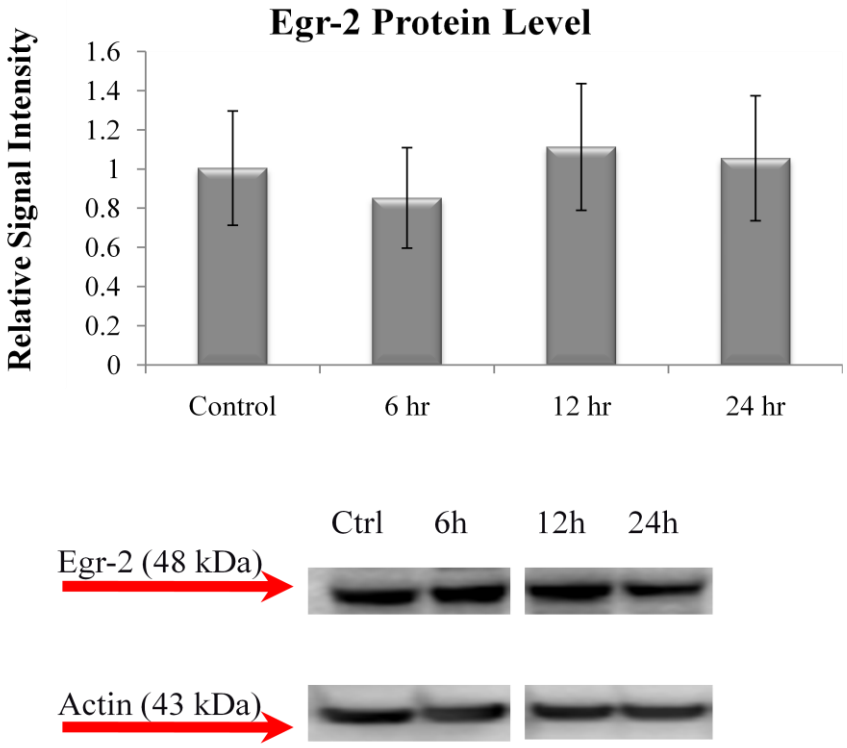

Figure 10: Egr-2 protein expression in TNFα treated cortical neurons. Cortical neurons were treated with mouse recombinant TNFα (100 ng/mL) for the indicated periods of time before their proteins were extracted (section 2.8.1.1). 40-50 µg of each protein was used in Western blotting (section 2.9) using the Egr-2 antibody (Lifespan Bioscience). The blot at the bottom shows a modest increase in Egr-2 expression in cortical neurons 12 hours after exposure to TNFα. The bar graph shows the quantification of the 48 kDa Egr-2 bands. The results shown represent the mean ± SEM (n = 3) from three different blots. Statistical analysis was performed as mentioned in section 2.14. The bar graph does not show any statistically significant ($p>0.05$) change in the Egr-2 protein expression.

3.2.3 Egr-2 protein level in TNFα treated primary cortical neurons in absence and presence of SN50

Since the first set of Western blots in cortical neurons showed a difference in Egr-2 protein level 12 hours after TNFα treatment, an additional set of Western blots were performed to assess the expression of Egr-2 twelve hours after treatment with TNFα with or without SN50. The proteins of primary cortical neurons were extracted twelve hours after treatment and used in Western blotting with the Egr-2 antibody from Lifespan Biosciences as the primary antibody as indicated in section 2.9. This experiment was repeated two more times.

Figure 11 shows one of the blots and the bar graph obtained from the quantification of the 48 kDa Egr-2 bands (n = 2). The bar graph does not show any difference in the Egr-2 protein expression 12 hours after TNFα stimulation with or without SN50, although Egr-2 levels were increased at 12 hours in the absence of SN50.

While repeating this experiment for the 3rd time, all the parameters were kept the same, but the protein extraction method was modified. The lysis buffer method was used as mentioned in section 2.8.1.2 and 50µg of the proteins were used for Western blotting. Surprisingly, the pattern of protein bands (specific and non-specific) on the blot was changed. Proteins extracted using this method produced a cleaner blot, with a fewer non-specific bands and stronger specific (Egr-2) bands. However, in this experiment no differences in the Egr-2 level were observed (data not shown).

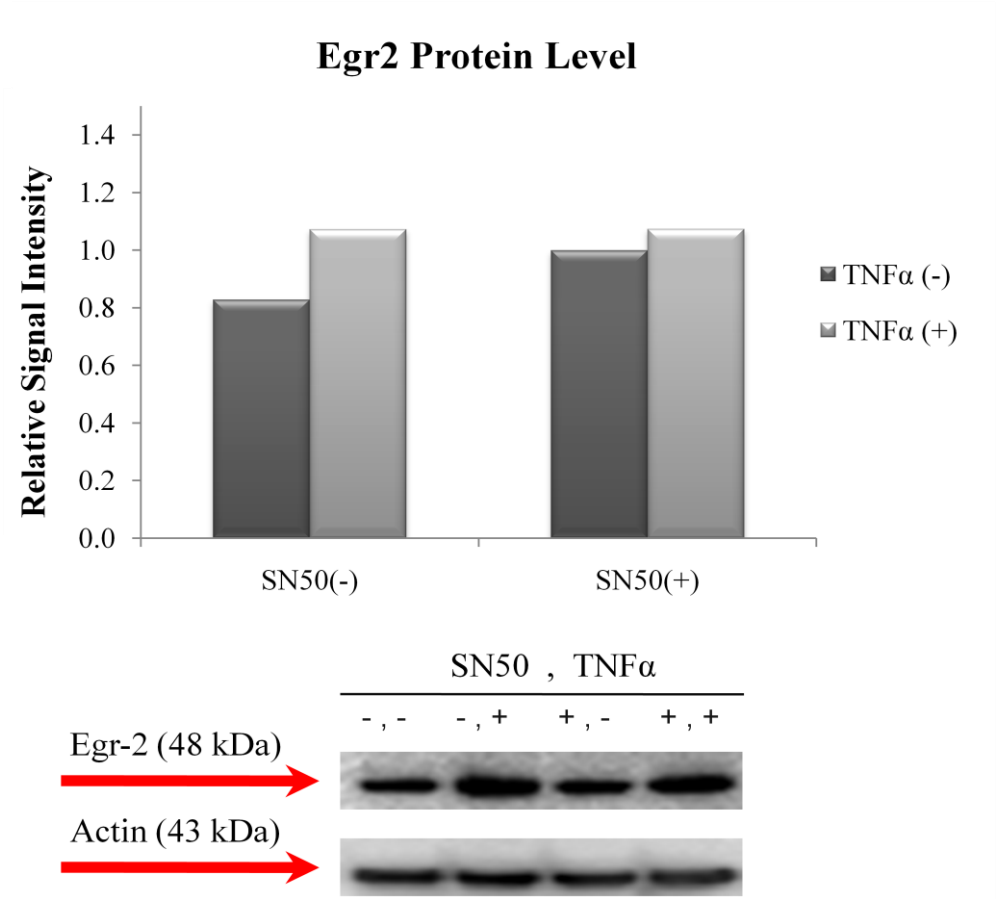

Figure 11: Egr-2 protein expression in TNFα and SN50 treated cortical neurons. Egr-2 expression in cortical neurons increases 12 hours after exposure to mouse recombinant TNFα (100 ng/mL) compared to control sample. However adding SN50 (100 µg/ml) to the neurons did not decrease the expression of Egr-2 upon TNFα treatment. Treatment is designated by a (+) sign and no treatment by a (-) sign (e.g. (-,-) indicates neither SN50 nor TNFα treatment). The bar graph shows the quantification of the 48 kDa Egr-2 bands (n=2).

3.2.4 Egr-2 protein level in TNFα treated primary cortical neurons - additional studies

Since our Western blots results did not show any differences in Egr-2 protein levels upon TNFα stimulation, neither in HeLa cells nor in primary neuronal culture, contrary to what we were speculating and was expected, we sought to repeat these studies under different conditions. The Western blot performed using the lysis buffer protein extraction method, described in section 2.8.1.2, looked promising in order to repeat our Western blot experiments. Therefore at this point, we decided to repeat the whole protein Western blot experiments using this new method of protein extraction, primary cortical neurons, Egr-2 antibody from Covance, and TNFα treatment time points of 0, 1/2, 1, 2, and 4 hours.

Mouse recombinant TNFα (100 ng/ml) was added to cortical neurons for the above mentioned time points. Proteins were extracted as mentioned in section 2.8.1.2 and their concentrations were measured (section 2.8.3). Ten to twenty five micrograms of each protein was used in Western blotting with the Egr-2 antibody from Covance as indicated in section 2.9. This experiment was performed twice and a representative result can be seen in Figure 12. Band intensities were quantified using ImageJ software and presented as relative signal density of Egr-2 to Actin as control.

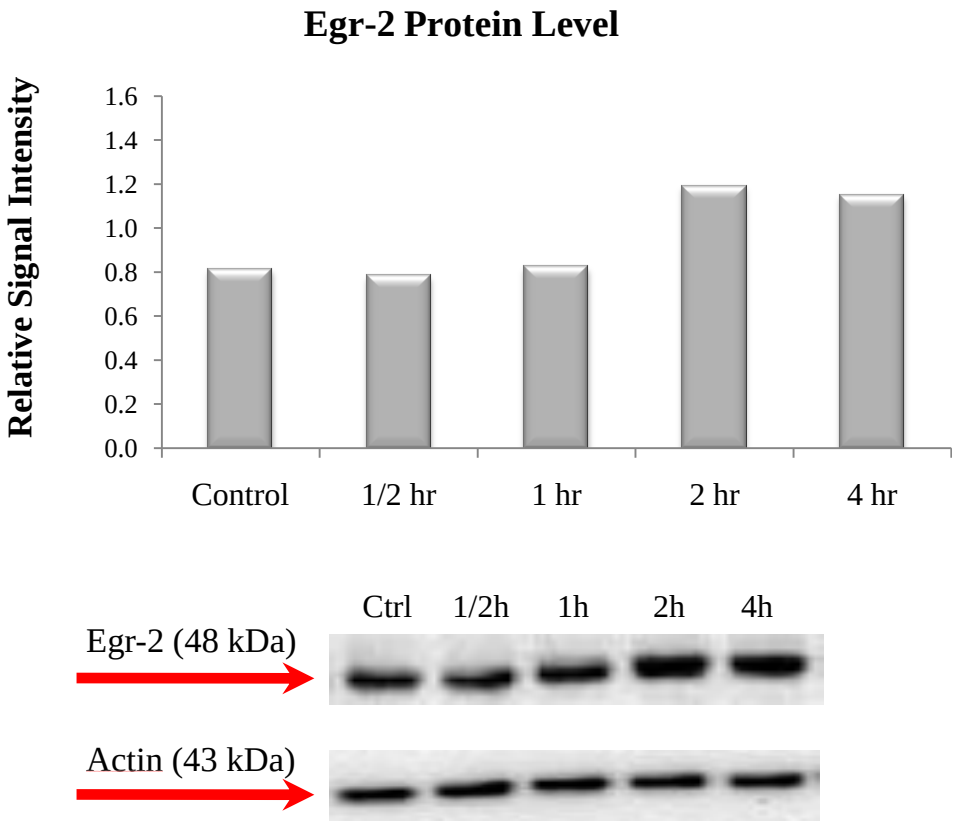

Figure 12: Egr-2 protein expression in cortical neurons. Expression of Egr-2 protein increases 2 and 4 hours after exposure to mouse recombinant TNFα (100 ng/mL) compared to controls. The bar graph represents (n = 2). Consistent with the qRT-PCR analyses and considering the delay between mRNA transcription and translation of its protein, the levels of Egr-2 were enhanced in 2 and 4 hours after treatment with TNFα compared to controls.

3.3 Analysis of NF-κB binding sites within the *Egr-2* promoter region

3.3.1 Bioinformatics approach

Using the method mentioned in section 2.10, four candidate κB binding sites with >80% homology to the NF-κB consensus sequence were identified within the promoter region of the *Egr-2* gene. Figure 13 shows the positions of these binding sites compared to the transcription start site (TSS).

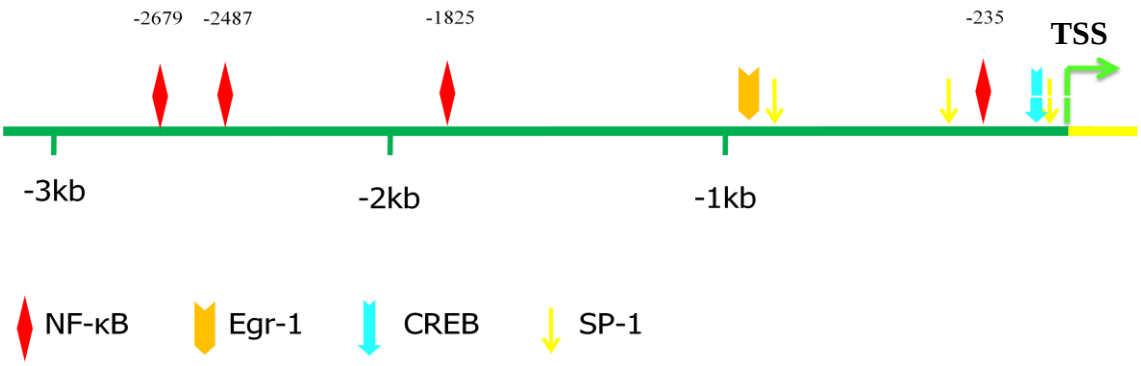

Figure 13: Schematic representation of the transcription factor binding sites in the Egr-2 promoter. 3kb upstream sequence of the *Egr-2* gene promoter was searched for putative κB binding sites. Transcription Start Site (TSS) represents the first nucleotide (+1) of the *Egr-2* gene.

3.3.2 ChIP assay

To further evaluate *Egr-2* transcriptional regulation by NF-κB, NF-κB interaction with the *Egr-2* promoter was investigated *in vivo*, using the ChIP assay with antibodies against specific NF-κB subunits (p50 and p65).

Forebrains of CD-1 mice (E16 - E18) were dissected, minced, and treated with 1% paraformaldehyde. Sonication was performed to mechanically shear the DNA to 300 - 400 bp sized fragments as illustrated in Figure 14 (A). Chromatin immunoprecipitation was performed with antibodies (both from Santa Cruz) against the p50 and p65 subunits of NF-κB. PCR was performed on the resultant products using primer sets listed in Table 9 that specifically amplify the candidate NF-κB binding sites 3kb within the upstream promoter of the *Egr-2* gene (Figure 13) as in section 2.11.

Results shown in Figure 14 (B) indicate that both p50 and p65 bind to the NF-κB binding region most proximal to the transcription start site (see Figure 13 for the locations of the four putative κB binding sites). This assay was repeated 2 more times and the results were consistent in each experiment confirming the interaction of NF-κB (p50/p65) with the promoter of the *Egr-2* gene and supporting its role in the induction of the *Egr-2* gene.

3.3.3 Electrophoretic Mobility Shift Assay (EMSA)

Nuclear NF-κB (p50/p65) DNA binding activity was measured by the electrophoretic mobility shift assay (EMSA) as described in section 2.12. As shown in Figure 15, the transcriptionally active NF-κB specifically binds to DNA in the mice forebrain. Re-

sults confirm a significant NF-κB/DNA binding activity in mice forebrain tissue and since this DNA sequence is a part of the region upstream of the *Egr-2* gene promoter, this experiment confirms specific NF-κB binding to the promoter region of the *Egr-2* gene.

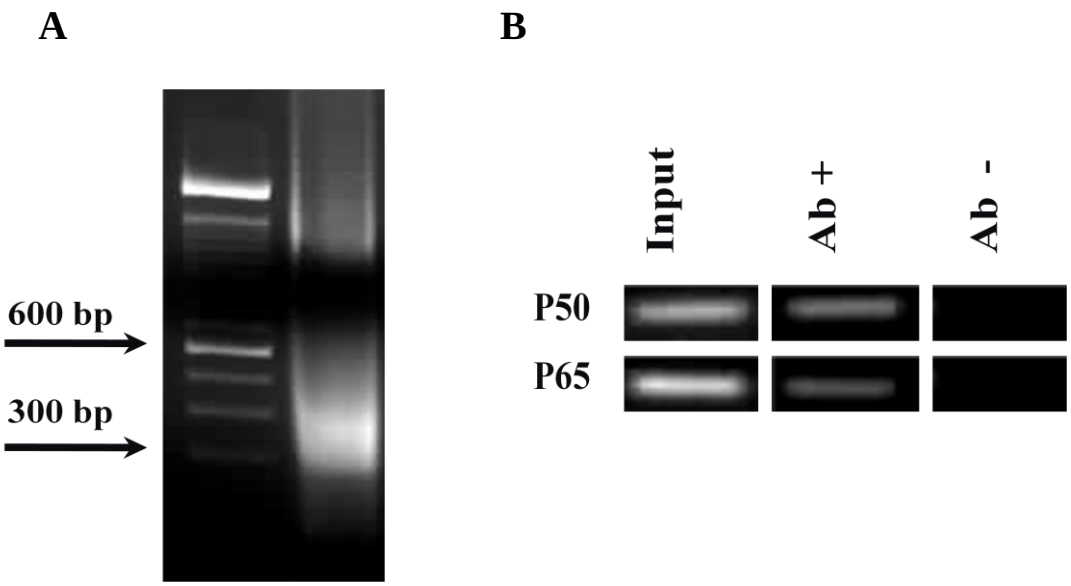

Figure 14: ChIP assay. (A) Chromatin was sonicated to fragments of 300 - 400 bp in size and run on a 1% agarose gel. Ethidium bromide staining of a representative gel is shown. (B) DNA representing the most proximal NF-κB binding site to the transcription start site of *Egr-2* promoter was amplified by PCR from genomic DNA fragments that were immunoprecipitated by a p50 or p65 antibody. Input shows the non-immunoprecipitated genomic DNA.

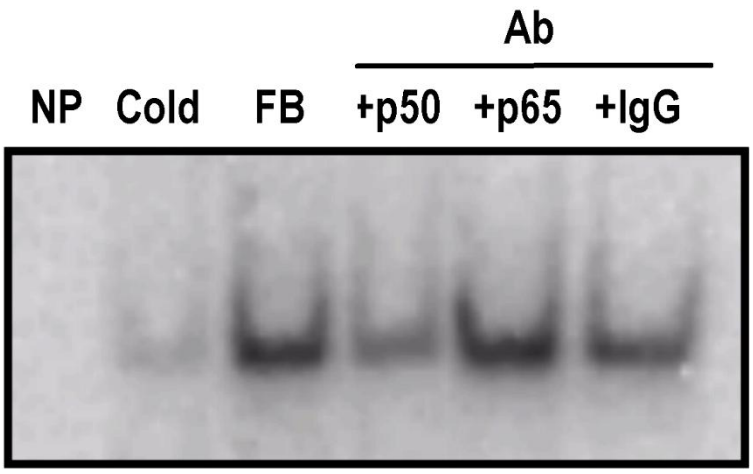

Figure 15: Detection of specific NF-κB DNA binding activity using EMSA. NF-κB DNA binding activities of the nuclear protein extracts prepared from CD-1 mice embryonic forebrain tissues (E16-E18) were determined by EMSA using ^{32}P-labeled oligonucleotide containing custom sequence to test for NF-κB consensus sequence binding (target probes - see section 2.12.1). For supershift assays, 2 µg of antibody specific for the p50 or p65 subunit of NF-κB (Santa Cruz Biotechnology) was added to nuclear extract samples and incubated for 1 hour prior to adding oligonucleotide. The darker band seen in the FB lane reflects NF-κB/DNA binding activity and the specificity of the bands were assessed by using a competition experiment with unlabeled probe (Cold lane) using the same extracts. A negative control was added with no probe (NP lane). The lighter band in p50 lane shows that DNA binds directly to the p50 subunit of NF-κB but not to the p65 subunit (p65 lane). IgG antibody was used as a control for the supershift assay and, as expected, the band in IgG lane shows no shift.

3.4 Small Interfering RNA (siRNA)

The siRNAs consist of pools of three to five specific 19-25 nucleotide siRNAs designed to knock-down NF-κB P50 or NF-κB p65 gene expression. Transient transfection was carried out on primary cortical neuronal cultures, Day 2, as described in section 2.13. 48 hours after transfection, cells were washed with cold PBS twice and subjected to qRT-PCR (section 2.13.3).

Briefly, total RNA, from siRNA transiently transfected cells, was prepared, reverse transcribed to cDNA and the cDNA was used as the template for NF-κB p50, p65, Egr2 and GAPDH amplification. Figure 16 shows the qRT-PCR results. Reduced p50 subunit expression by small interfering RNA (siRNA) knockdown resulted in the loss of *Egr-2* expression, suggesting that this transcription factor is required for the *Egr-2* gene expression.

The p50 subunit of NF-κB was found by ChIP assay (section 3.3.2) and EMSA (section 3.3.3 - conducted by Dr. Glazner's laboratory) to be associated with the upstream region of the *Egr-2* promoter. This siRNA experiment (conducted by Dr. Eisenstat's laboratory) therefore provides yet more evidence supporting the specific role of NF-κB in the regulation and induction of *Egr-2* gene expression.

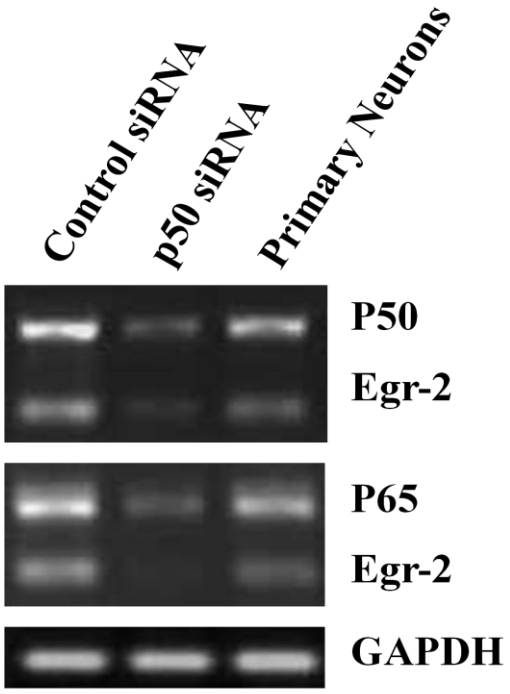

Figure 16: NF-κB p50 subunit knock-down using siRNA. siRNAs to NF-κB p50 subunit were transfected into primary embryonic cortical neurons. Neurons were incubated for 2 days and their RNAs were extracted, reverse transcribed to cDNA and subjected to qRT-PCR to measure the expression of *Egr-2* after NF-κB p50 subunit knock-down. Results show a decrease in *Egr-2* expression levels in neurons transfected with p50 siRNA supporting the role of NF-κB in induction of *Egr-2* gene.

Discussion

The research presented here was begun to explore target genes of NF-κB during long term potentiation (LTP) in mouse hippocampal slices. These studies were initiated in our laboratory by a postdoctoral fellow, Dr. Kensuke Oikawa. Dr. Oikawa induced LTP in the hippocampal slices from NF-kB p50 knockout mice ($p50^{-/-}$) and its littermate ($p50^{+/+}$) using the theta-burst stimulation (TBS) protocol consisted of five bursts of four pulses at 100Hz separated by 200ms repeated six times with 10sec intervals between each train. In order to investigate the role of NF-κB in neuronal activity dependent gene expression 3 hours after stimulation, the transcriptome of stimulated and unstimulated hippocampal slices of both p50 knockout ($p50^{-/-}$) and its wild-type littermate ($p50^{+/+}$) were analyzed using Affymetrix Mouse DNA microarrays.

The results showed overexpression of several genes in $p50^{+/+}$ animals versus $p50^{-/-}$ suggesting the possible involvement of NF-κB in the induction of their expression. Among these genes *Egr-2*, whose expression is regulated during synaptic plasticity and memory formation (Cole, et al., 1989; Williams, et al., 1995; Worley, et al., 1993; Yamagata, et al., 1994), showed around a 3 fold increase and was therefore selected as a putative NF-κB target gene of interest in this study. Moreover, Nikols et al. (2003) suggested that the *Egr-2* transcription factor might be one of the NF-κB target genes that coordinates the myelination process in Schwann cells (Nickols, et al., 2003) and *Egr-2* expression, upregulated early in the myelination process, may be a critical regulator of genes encoding the proteins that mediate myelination (Jessen & Mirsky, 2002).

Although the protein-protein interactions between NF-κB p50 and p65 subunits and CREB binding protein have been reported (S. Cao, Zhang, Edwards, & Mosser, 2006;

Yang, Ma, Lin, Orr, & Wathelet, 2004; H. Zhong, May, Jimi, & Ghosh, 2002) as well as the interaction between CREB binding protein and Egr-2 protein (Yoo & Lee, 2004), no study to date has shown any interplay between NF-κB (p50/p65) and the *Egr-2* gene. Furthermore, a recent study conducted by Lemberger et al. (Lemberger, Parkitna, Chai, Schutz, & Engblom, 2008) showed that in mouse forebrain neurons, CREB1 (cAMP responsive element binding protein 1) and CREM (cAMP responsive element modulator) proteins are necessary for expression of the *Egr-2* gene. However, there is no evidence in the literature that suggests a direct role of NF-κB (p50/p65) in the regulation of *Egr-2* gene expression.

To study the possibility of NF-κB binding to the *Egr-2* promoter region, the 3kb immediate upstream sequence of the *Egr-2* gene promoter was searched using P-Match software (section 2.10) for possible κB binding sites. As demonstrated in Figure 13, four binding sites with >80% homology to the NF-κB consensus sequence were identified within the promoter region of the *Egr-2* gene suggesting that NF-κB might be regulating this gene at the transcriptional level.

To confirm the overexpression of *Egr-2* gene upon NF-κB activation, we measured *Egr-2* mRNA levels in the HeLa S3 cell line as well as in primary neuronal cultures upon TNFα treatment. The HeLa S3 cell line is a human cervical carcinoma cell line and since it is insensitive to TNFα apoptotic activity (Sekine, et al., 2001), it is an ideal cell line for TNFα stimulation studies (section 1.8.2). TNFα, a 17 kDa protein, can bind to TNF receptors (TNFRs) expressed on glia, neurons and neural stem cells and activate NF-κB within the central nervous system (Bruce et al., 1996; Klassen et al., 2003).

Therefore, to measure *Egr-2* mRNA levels in HeLa cells and primary cortical neurons before and after NF-κB activation, quantitative real time PCR (qRT-PCR) was performed before and after TNFα treatment. To obtain the most accurate results from qRT-PCR, its parameters such as primer concentrations and annealing temperatures were optimized using samples made of plasmids containing human and mouse *Egr-2* and *Gapdh* cDNA. For each gene, a standard curve was created using the optimized parameters for that gene which was later used in calculation of the changes in the expression of *Egr-2* at different time points upon TNFα treatment.

mRNAs were isolated from TNFα treated HeLa cells and subjected to qRT-PCR. The fold changes of human *Egr-2* expression in TNFα treated samples versus control were calculated. The results that can be observed in Figure 4 show an increase in the expression of this gene 30 min after TNFα treatment that reaches its maximum at 1 hour after TNFα treatment and decreases to around 3 fold at 3 hours after TNFα treatment. Later, mRNA isolated from TNFα treated primary cortical neurons was also used in qRT-PCR experiments to measure the mouse *Egr-2* expression level changes in TNFα treated samples versus control. Results shown in Figure 6 confirm that, as in HeLa cells, *Egr-2* expression increases 30 min after TNFα treatment and reaches its maximum at 1 hour after TNFα treatment and approaches basal levels at 4 hours after TNFα treatment. These results are all consistent with the fact that *Egr-2* is an IEG and has a rapid and transient induction pattern (Curran & Morgan, 1987).

Based on the data obtained so far, TNFα stimulation induces *Egr-2* expressions in both HeLa cells and primary neurons and it is known that TNFα activates NF-κB

(Memet, 2006). However, to confirm the direct role of NF-κB in this induction, we inhibited NF-κB with SN50, a cell-permeable synthetic peptide that inhibits nuclear translocation of the NF-κB active complex, in HeLa cells and measured the expression of *Egr-2* upon stimulation with TNFα. To fulfill this, the mRNA extracted from HeLa cells in the presence and absence of SN50 before and after TNFα treatment was subjected to qRT-PCR and *Egr-2* mRNA levels were calculated using absolute quantification (section 3.1.3). The results demonstrated in Figure 5 show that the expression of *Egr-2* mRNA, which is increased upon treatment with TNFα, is decreased in presence of the NF-κB inhibitor, SN50. The decreased transcript level of *Egr-2* upon TNFα stimulation after treatment with SN50 supports the direct involvement of NF-κB on the induction of *Egr-2* expression. This experiment could be repeated using primary cortical neurons to confirm the role of NF-κB on the *Egr-2* induction in a more relevant model system.

To determine whether enhanced levels of *Egr-2* mRNA transcript in TNFα treated cells (HeLa cells and primary cortical neurons) would lead to higher levels of protein expression, whole cell proteins of the same HeLa cells used for qRT-PCR in the previous sets of experiments (section 3.1.2) were extracted and used in Western blotting. These proteins were extracted using the method described in section 2.8.1.1 that was also used for simultaneous mRNA extraction. We expected to observe an increase in Egr-2 protein levels at the same time point that showed the maximum increase in *Egr-2* mRNA levels upon TNFα stimulation, i.e. one hour after TNFα treatment. However, no change was identified between the Egr-2 protein levels at the different time points of TNFα stimulation (0, ½, 1, 3 hours). This experiment was repeated but all experiments showed the

same results as demonstrated in Figure 7. Therefore, we repeated these Western blots using another Egr-2 antibody, the Egr-2 antibody from Santa Cruz was replaced by an Egr-2 antibody from Covance. However, even with the new Egr-2 antibody, no change was observed at the Egr-2 protein levels at the indicated time points of TNFα stimulation.

Egr-2 protein can exist in both the cytoplasm and nucleus (Herdegen, Kiessling, et al., 1993; Herdegen, Sandkuhler, et al., 1993); however, the zinc fingers of the Egr-2 protein contain the nuclear localization sequence (Matheny, et al., 1994) and this sequence might be involved in nuclear localization of the Egr-2 proteins (Beckmann & Wilce, 1997). Considering the fact that Egr-2 is a DNA binding transcription factor and nuclear translocation of transcription factors is a key control mechanism, possibly higher concentrations of Egr-2 exists in the nucleus rather than cytoplasm. For that reason we repeated the Western blot experiments using the nuclear and cytoplasmic fractions of HeLa cell proteins after treatment with TNFα. These experiments were performed as mentioned in section 3.2.1.2 using both Egr-2 antibodies (Santa Cruz and Covance) and higher concentrations of proteins but showed similar results (Figure 8) as the previous Western blots. No difference in Egr-2 protein levels was observed in the cytoplasmic protein fractions extracted from HeLa cells upon TNFα stimulation. However, levels in the nucleus appeared to increase at 1 and 3 hours after TNFα stimulation.

Whole cell protein extracted from forebrain tissue of a two month old mouse (C57B) in the same gel next to HeLa cell proteins showed a higher level of Egr-2 protein suggesting a higher basal level of Egr-2 protein in mouse brain tissue compared to HeLa cells (data not shown). Also, since the difference in the cytoplasmic and nuclear levels of

Egr-2 protein was not significant, we extracted the whole cell proteins of primary cortical neurons treated with TNFα for the next series of Western blots.

Using the same Egr-2 antibody from Covance that was used for HeLa cells and also another Egr-2 antibody from Lifespan Biosciences (Figure 9) did not show any difference between the Egr-2 protein levels at different time points upon TNFα treatment. Even higher concentrations of loaded proteins did not show any change at the Egr-2 protein level (data not shown).

Since our first Genechip experiments were performed on the mRNA extracted from hippocampal tissue and not sole neurons, to determine whether glial cells might be the cells responsible for the observed enhancement in *Egr-2* mRNA transcription level after induction of LTP, a Western blot was performed on the proteins extracted from a mixture of neurons and glia after they were treated with TNFα using the same method of extraction used for HeLa cells and neuronal culture (section 2.8.1.1). It should be mentioned that this experiment was performed before the qRT-PCR experiment was performed on primary cortical neurons that showed a difference in the *Egr-2* mRNA levels in the primary neurons. This Western blot result also did not show any difference at the Egr-2 protein level after treatment with TNFα (data not shown).

In a study published by Dolga et al. (Dolga, et al., 2008), the survival rate of cortical neurons when challenged with glutamate increased when they were pre-incubated with TNFα via a NF-κB dependent pathway. Pre-incubation of neurons with TNFα for up to 6 hours had no significant protective effect against glutamate-induced excitotoxicity, but the number of surviving neurons gradually increased when the TNFα pre-treatment lasted

for more than 6 hours up to 24 hours. It is possible that NF-κB activity in cortical neurons is enhanced 6 h to 24 h after treatment with TNFα. At this time, we examined the level of Egr-2 protein upon TNFα stimulation for longer periods of time (e.g. 6, 12, and 24 hours). Therefore, the primary cortical neurons were exposed to TNFα for the indicated times and their proteins were extracted using the same extraction method (section 2.8.1.1).

Western blotting was performed using the Egr-2 antibody from Lifespan Biosciences and since the first blot showed an increase at the Egr-2 protein level 12 hours after TNFα stimulation (Figure 10), this experiment was repeated two more times. However, after quantifying the Egr-2 protein band intensities, pooling the data obtained from all three experiments and performing statistical analysis (section 2.14), no significant change was observed in the Egr-2 protein levels after TNFα treatment (Bar graph in Figure 10).

When Egr-2 protein levels appeared to be increased 12 hours after TNFα stimulation, another Western blot was performed to test the inhibitory effects of SN50 on the Egr-2 protein level at 12 hours. For this experiment, the primary cortical neurons were treated with TNFα in the presence and absence of SN50 for 12 hours (section 3.2.3) (Figure 11).

Although it appears that SN50 might have an inhibitory effect on Egr-2 protein levels, statistical analysis did not show any significant difference at the Egr-2 protein level upon TNFα stimulation with or without SN50. However, when this experiment was repeated for the 3rd time, another protein extraction method was used (lysis buffer method mentioned in section 2.8.1.2) and surprisingly, the resulting blot was different from the

other two in terms of Egr-2 band specificity, clarity of the bands , and fewer non-specific bands. This blot also did not show any difference for Egr-2 protein levels, even after 12 hours stimulation with TNFα in the absence of SN50 (data not shown). This observation was concurrent with the results of the previous sets of Western blots where 12 hour stimulation of primary cortical neurons with TNFα did not confirm an increase at the Egr-2 protein level (Figure 10).

Based on all the above results and the importance of the protein extraction method on determining Western blotting outcome, we repeated the whole set of Western blot experiments using the lysis buffer method for protein extraction (section 2.8.1.2) from primary cortical neurons, the Egr-2 antibody from Covance and the primary TNFα treatment time points of 0, 1/2, 1, 2,and 4 hours which approximate the time points showing an increase at the mRNA level of *Egr-2* expression and are also more consistent with the expected timing for Egr-2 protein level enhancement as an IEG.

Considering these new parameters, additional Western blots were performed and as can be seen in the blot presented in Figure 12, the Egr-2 protein level increased 2 and 4 hours after TNFα stimulation. This result is consistent with the qRT-PCR analyses and seems plausible considering the delay between mRNA transcription of a gene and translation of its protein. To obtain even more statistically accurate results, we will repeat this Western blot at least two more times in the future.

On another note, to evaluate *Egr-2* transcriptional regulation by NF-κB, the ChIP assay was performed using antibodies against NF-κB subunits (p50 and p65) to examine the interaction of NF-κB with the *Egr-2* promoter *in vivo*. This assay was performed on

mouse embryonic (E16-E18) forebrain tissues (section 3.3.2). Results presented in Figure 14 (B) indicate that both p50 and p65 bind to the NF-κB binding region most proximal to the transcription start site (Figure 13). This assay was performed 3 times overall with the consistent results supporting a direct role of NF-κB in the induction of *Egr-2* gene at the transcription level. However, NF-κB may or may not be bound directly to the Egr-2 promoter.

Therefore, to detect specific NF-κB (p50/p65) DNA binding activity, electrophoretic mobility shift assays (EMSA) were performed by Dr. Glazner's laboratory. In these experiments, the nuclear protein extracts prepared from CD-1 mice embryonic forebrain tissues (E16-E18) were tested by EMSA for NF-κB consensus binding sequence (target probes) within the upstream region of the Egr-2 promoter that was identified as bound to p50/p65 in our ChIP assay (section 2.12.1). NF-κB/DNA binding in the forebrain nuclear extracts and the specificity of the binding was assessed using a cold competition experiment with unlabeled probe with the same extracts. Also, when no probe was added to the negative control there was not any binding (Figure 15).

When 2 µg of antibody specific for the p50 or p65 subunits of NF-κB was added to nuclear extract samples and incubated for 1 hour prior to adding oligonucleotide, a supershift was observed with the p50 antibody but not with the p65 antibody. IgG antibody was used as a control for the supershift assay and, as expected, the band in the IgG lane showed no shift (Figure 15). This supports direct binding of the NF-κB p50 subunit to Egr-2 promoter region DNA. However, we cannot rule out the possibility of direct binding of the p65 subunit since different p65 antibodies might show different results. Results

obtained from EMSA confirm specific NF-κB p50 subunit/DNA binding in mouse forebrain tissue and since this DNA is a part of the upstream region of the *Egr-2* gene promoter, this experiment confirms specific NF-κB binding to the promoter region of the *Egr-2* gene.

To confirm the role of NF-κB in the regulation of *Egr-2* expression, we knocked down p50 and p65 gene expression in primary cortical neurons using small interfering RNA (siRNA) and assess the expression of Egr-2 using qRT-PCR. In this experiment, conducted by Dr. Eisenstat's laboratory, the siRNAs were designed to knock-down NF-κB p50 or p65 gene expression. Transient transfection was carried out on primary cortical neurons culture, day 2, as described in section 2.13.

The qRT-PCR results showed that the reduction of p50 expression by siRNA knock-down resulted in decreased *Egr-2* expression, suggesting that this transcription factor is required for the induction of *Egr-2* gene expression (Figure 16). Even though association of NF-κB with the promoter region of *Egr-2* has been confirmed using EMSA and ChIP assay, the siRNA experiment provides us with strong evidence regarding the direct involvement of this transcription factor in regulation of *Egr-2* induction.

Taken together, our data suggest that NF-κB binds to the promoter region of the *Egr-2* gene as shown by EMSA and ChIP assays and induces *Egr-2* mRNA expression in both HeLa cells and primary cortical neurons as revealed by qRT-PCR and the SN50 experiments and confirmed by siRNA experiments. Since NF-κB itself is also activated upon neuronal activity (1.9), and *Egr-2* mRNA enhancement has been shown in LTP by the Genechip experiments previously performed in our laboratory (3.1.1), it can be con-

cluded that the *Egr-2* gene is directly induced by neuronal activity-dependent NF-κB activation that happens in LTP as well as in learning and memory.

Future Directions

Our data suggest that Egr-2 is directly induced by neuronal activity-dependent NF-κB activation. However, more studies need to be done to determine the exact role of NF-κB in the transcription of the *Egr-2* gene especially in terms of interaction of this transcription factor with the other transcription factors or co-factors necessary for the induction or repression of this gene's expression.

Egr-2 protein levels in cortical neurons after TNFα treatment are increased only 1.5 times more than the basal level. This is considerably less than the enhancement of *Egr-2* mRNA expression which shows around a 45-fold change at its peak, one hour after TNFα stimulation. Whether the majority of the induced mRNA gets degraded before leaving the nucleus to be translated to the proteins or there are other involved regulatory mechanisms, needs to be investigated.

To investigate the role of Egr-2 transcription factor in the induction of other genes involved in LTP and therefore learning and memory, an Egr-2 knock out model of mice can be utilized for the same sets of experiments performed in this study. Also, the same experiments can be carried out starting with a series of behavioral experiments to train the animals instead of the LTP experiments in slice preparations before hippocampal mRNA extraction.

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