THESIS

USE OF FLUORESCENT IMMUNOHISTOCHEMISTRY TO INVESTIGATE NF-κB INVOLVEMENT IN BRAIN PATHOLOGIES

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2013

Master's Committee

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ABSTRACT

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Nuclear Factor Kappa Beta (NF-κB) is a transcription factor ubiquitously expressed in mammalian cells and involved in a broad spectrum of physiological responses. In the central nervous system (CNS), NF-κB is responsible for the regulation of several brain-specific processes, ranging from synaptic plasticity to neuroinflammation (Mattson and Camandola, 2001). The effects of NF-κB activation are highly variable and the transcription factor appears to play a dichotomous role in brain pathologies.

Parkinson's Disease (PD) is the second most prevalent neurodegenerative disease in the world and is characterized by the progressive, irreversible loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Neuroinflammation, a CNS-specific immune response facilitated by glial cells, is now know to be an important contributing factor to PD pathology. Many neuroinflammatory responses have been linked to glial NF-κB activation, although the mechanisms have not yet been established.

Drug-induced seizures are a serious adverse drug reaction (ADR) associated with both CNS- and non-CNS-targeting drugs. The current methods of drug safety evaluation rely solely on behavioral analysis and therefore often fail to identify potentially seizurogenic activity. Studies have suggested that neuronal NF-κB activation may be an early stress response and serve

a neuroprotective function. The molecular mechanisms involved in seizurogenesis are also largely unknown.

To study the role of NF-κB in these models of CNS injury, I employed fluorescent immunohistochemical (IHC) staining, a molecular technique that utilizes antibody-antigen binding to identify and visualize specific proteins in tissue. IHC has a wide range of applications and is often used for both laboratory research and clinical diagnostics. The following studies examined the role of NF-κB in CNS injury by using fluorescent IHC staining to characterize a transgenic mouse containing a NF-κB-driven enhanced green fluorescent protein (*cis*-NF-κB^{eGFP}) construct in order to detect cell-specific changes in NF-κB activity. Using this method in the MPTP neurotoxin-induced model of Parkinson's Disease, I found that NF-κB-mediated glial activation accompanied loss of dopaminergic neurons and that treatment with novel pharmacological inhibitors of NF-κB attenuated this response. In the kainic acid (KA) model of drug-induced seizures, this method showed that neuronal activation of NF-κB occurs at sub-seizurogenic doses and may be an early, neuroprotective stress response.

Fluorescent IHC staining in models of neuropathologies is useful for mechanistic research, but may also be an effective tool in drug-development. Use of fluorescent IHC with the NF-κB transgenic mouse allows for characterization of NF-κB signaling, as it relates to other proteins *in vivo.* Further optimization of this method could be extremely advantageous to NF-κB research.

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Chapter 1. Literature Review

Chronic Neuroinflammation in CNS Injury

Due to the presence of the blood-brain barrier (BBB), a cellular barrier which separates extracellular fluid in the brain from circulating peripheral blood, the brain was long considered an “immune privileged” organ (Tansey et al., 2007). However, research has since proven that the brain is fully capable of eliciting an inflammatory response. Glial cells, particularly microglia, are the key mediators of CNS-specific immune responses. In the event of stress or trauma, glial cells undergo phenotypic changes which result in the upregulation of a wide array of signaling molecules. During this process, both neurotrophic and neurotoxic, pro-inflammatory factors are released. Activation of this response is generally acute and neuroprotective, subsiding upon resolution of the stress. However, if the stimulus persists, the response is chronically activated and creates a self-propelling cycle in which pro-inflammatory signaling is sustained (Taylor et al., 2013). Chronic inflammation in the CNS causes an increase in oxidative stress and accumulation of neurotoxic signaling factors which ultimately results in neuronal death (Hirsch and Hunot, 2009). This discovery has been critical to neuroscience research and has led to a tremendous amount of understanding on the molecular mechanisms behind CNS disease. Neuroinflammation is now considered to be an important contributing factor in many neuropathologies, ranging from neurodegenerative diseases to neuronal injury following excitotoxicity. However, the molecular signaling involved is extremely complex and the precise mechanisms behind neuroinflammation-induced pathologies remain enigmatic.

Microglial Activation

Microglia are considered the resident macrophage cells of the CNS and are the primary cells involved in the initiation of CNS-specific immune responses. Under normal physiological conditions, microglia are involved in maintaining homeostasis in the CNS by actively surveying the microenvironment. However, in the event of injury or trauma, microglia transition to an "activated" phenotype where they exhibit immune defense mechanisms (Kettenmann et al., 2011). Activated microglia upregulate major histocompatibility (MHC) factors to recruit other immune cells and become phagocytic in nature (Kim and Joh, 2006). During this process, microglia also release an array of other immune response molecules. Some factors are neurotrophic, such as Brain-Derived Neurotrophic Factor (BDNF) and Insulin-like Growth Factor-1 (IGF-1), whereas other factors are anti-inflammatory, such as IL-10 and Transforming Growth Factor-β (TGF-β) (Taylor et al., 2013). However, majority of the molecules that are upregulated by activated microglia are proinflammatory, including cytokines IL-1β and TNF-α, as well as free radicals such as NO (nitric oxide) (Block et al., 2007). These factors amplify the inflammatory response by activating other glial cells and recruiting immune cells to the site of injury. In circumstances of acute trauma, activated microglia return to their state of surveillance and inflammatory signaling ceases upon resolution of the stressful stimulus. In some situations, however, the stimulus persists and causes chronic activation of this inflammatory response, referred to as chronic neuroinflammation (Taylor et al., 2013). While acute microglial activation serves to be neuroprotective, chronic neuroinflammation results in an overproduction of pro-inflammatory cytokines and free radicals. Accumulation of these cytotoxic molecules causes

neuronal death that eventually results in a feed-forward signaling loop, causing further inflammation and neurodegeneration.

Reactive Astrogliosis

Astrocytes make up the largest population of glial cells in the brain, yet their physiological functions remain largely unknown. In recent years, research has shown that astrocytes have fundamental and dynamic roles in brain development and homeostasis, despite the early beliefs that they were primarily an inert structural component of the brain (Sofroniew and Vinters, 2009). It is now known that astrocytes are intimately involved in maintenance of the CNS microenvironment by providing metabolic and trophic support to neurons (Ambrosini and Aloisi, 2004). Their close proximity to both blood vessels and other brain cells allows astrocytes to mediate a number of homeostatic functions, including blood-brain barrier permeability, regulation of blood flow and immune response mechanisms. Furthermore, emerging evidence shows that disruption of normal astrocyte function may be a critical element of many inflammation-related brain pathologies. Current research suggests that astrocytes are involved in both innate and adaptive immune responses in the CNS (Farina et al., 2007).

Despite that they lack the antigen-presenting capabilities that characterize microglia as the resident immune cells of the CNS, astrocytes are also highly responsive to changes in the brain microenvironment. Although microglia are often considered to be the first-line immune response cells of the CNS, many of the factors secreted by activated microglia cause phenotypic changes in astrocytes, which further amplify the local innate immune response. In turn, astrocytes release an array of pro-inflammatory signaling molecules, many of which are also

released by microglia, including IL-1β and TNF-α (Ambrosini and Aloisi, 2004). Additionally, astrocyte activation results in the upregulation of mediators involved in neuronal survival, such as neurotrophic factors, anti-inflammatory cytokines and chemokines. As with microglial activation, if the insult is not resolved, inflammatory signaling persists and interaction between the two types of glial cells results in a self-propelling state of chronic information.

Similarly to microgliosis, the dichotomous effect of astrocyte activation makes the resulting molecular signaling network extremely complex. One hypothesis that explains the paradoxical role of astrocyte activation in the pathologic brain is that activation results in either pro-inflammatory or anti-inflammatory signaling depending on a number of variables. A number of factors, such as physical proximity or timing of the insult, may determine astrocyte phenotype after pathogen recognition (Sofroniew and Vinters, 2009). While it has become clear that astrocytes are functionally critical to the maintenance of a healthy CNS environment, as well as key mediators of local immune responses, more research is needed to elucidate the precise mechanisms involved.

NF-κB in the CNS

One of the most widely studied inflammatory pathways of neuroinflammation is that of Nuclear Factor Kappa B (NF-κB). NF-κB is a transcription factor that is ubiquitously expressed in nearly all animal cell types and is involved in a multitude of cellular processes, particularly in immune responses. Activation of NF-κB results in the transcriptional regulation of an array of immunomodulatory genes. In mammalian cells, there are five proteins in the NF-κB family, p65 (RelA), RelB, c-Rel, p50 (p105), and p52 (p100). In the absence of stimuli, these proteins exist

in the cell as inactive dimers bound to inhibitor proteins of the IκB family. A variety of extracellular ligands are able to activate NF-κB by binding to membrane receptors. The best characterized pathway of NF-κB activation is the "classical pathway," (Fig.1.1) in which stimulation causes activation of the enzyme IκB Kinase (IκK), which in turn phosphorylates the inhibitory protein IκB and signals it for degradation via the ubiquitin proteosome system (UPS) (Camandola and Mattson, 2007). Once IκB is dissociated from the complex, the activated NF-κB dimer is able to translocate from the cytoplasm into the nucleus, where it binds to specific regulatory regions on the DNA (response elements) and initiates transcription of a variety of pro-inflammatory genes (Hayden, 2004). Many of the target genes mediated by NF-κB signaling are

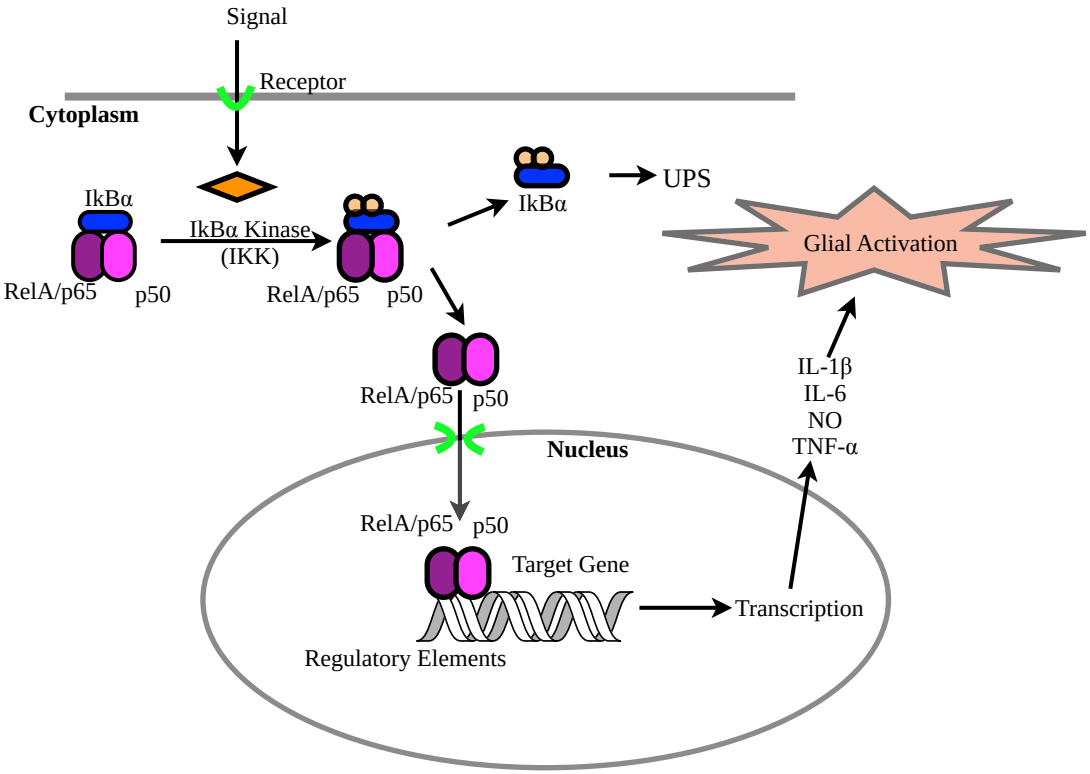

Figure 1.1. Canonical NF-κB signaling pathway. Inactive NF-κB is a cytosolic protein complex that exists as a heterodimer bound to the inhibitory protein IkBα. Receptor mediated cell-signals in response to harmful cellular stimuli cause activation of IkBα Kinase (IKK) which phosphorylates IkBα, signaling the inhibitory protein for proteosomal degradation. Removal of IkBα allows for the RelA/p65-p50 heterodimer to translocate from the cytosol to the nucleus and act as a transcription factor. RelA/p65-p50 binds to regulatory elements on the target gene and upregulates transcription of a variety of proinflammatory molecules, such as IL-1β and NO. Consequently, these proinflammatory signals activate glial cells (microglia and astrocytes), the immune-response cells of the brain. Although acute glial activation is neuroprotective, chronic glial activation results in a positive feedback loop which sustains upregulation of the proinflammatory molecules, many of which are cytotoxic.

seen in glial activation-mediated cytotoxicity, making it an important factor in neuroinflammation research.

NF-κB in the CNS is involved in diverse functions and has a multitude of consequences. Emerging evidence shows that NF-κB signaling in the CNS may have unique functions that are divergent from that of the peripheral tissues (O'Neill and Kaltschmidt, 1997). It is now known that basal NF-κB expression in the brain is highly variable, depending on the region and cell type.

The role of NF-κB in neurons is extremely complex and it is debated whether activation of NF-κB is neuroprotective or a pathological response to stress. Interestingly, neurons are known to express both constitutive and inducible forms of NF-κB (Kaltschmidt and Kaltschmidt, 2000). Constitutive NF-κB expression is thought to be involved in synaptic plasticity and cognitive function (Fridmacher et al., 2003; Mattson and Meffert, 2006). NF-κB is also inducible in neurons and can be activated by both inflammatory factors as well as neuron-specific stimuli, such as nerve growth factors and neurotrophic factors (Camandola and Mattson, 2007). While many studies have shown that NF-κB activity in neurons is neuroprotective, others have indicated that dysregulated NF-κB activity contributes to a number of neuropathologies (Fridmacher et al., 2003; Mattson and Meffert, 2006; Nakai et al., 2000). Neuronal NF-κB activation induces transcription of a number of anti-apoptotic factors, including BCL-2 and Superoxide Dismutase (SOD).

Although NF-κB activation in neurons is generally thought to be neuroprotective, induction in glial cells, particularly microglia, has shown to have opposing effects. In recent years, an extensive amount of research indicates that the pro-inflammatory signaling from

chronic NF-κB activation in glial cells is neurotoxic rather than neuroprotective (O'Neill and Kaltschmidt, 1997). In support of this hypothesis, the transcription of many neurotoxic and neuroinflammatory factors secreted by activated glial cells are known to be regulated by NF-κB induction. Pro-inflammatory cytokines such as TNF-α and IL-1β, as well as NO and other free radicals are known target genes of NF-κB (Mattson and Camandola, 2001).

Perhaps the most widely accepted theory behind the paradoxical role of NF-κB is that activation in neurons serves as a pro-survival mechanism, whereas NF-κB induction in glial cells yields a neurotoxic response, and in chronic neuroinflammation this response overcomes that of the neurons (Camandola and Mattson, 2007). It is important to note that research on NF-κB activation in the CNS that is done *in vivo* or in mixed neuron-glia cultures may be biased towards the effects of glial activation, due to the large population of glial cells (Mattson and Meffert, 2006).

Establishing a better understanding of NF-κB signaling may provide important insights into a number of CNS disorders, however, a better understanding of the implications of NF-κB activation must be established in order for it to be a useful therapeutic target. Since NF-κB in the brain has important functions in cognition and neuronal processes, generalized inhibition of NF-κB may be deleterious. Instead, neuron-specific or glia-specific targeting may be a more effective approach in the development of NF-κB-modulating therapeutics.

Neuroinflammation in Neurodegenerative Diseases

Alzheimer's and Parkinson's Disease

Alzheimer's Disease (AD) and Parkinson's Disease (PD) are the two most common age-related neurodegenerative diseases in the general population (Tansey et al., 2007). Although the clinical manifestations of these diseases are very different, AD being primarily associated with cognitive dysfunction and PD being mainly motor dysfunction, there are many common pathological features. Both diseases are classified as neurodegenerative diseases and as proteinopathies, due to the loss of neuronal mass and presence of protein aggregates. While hereditary genetic mutations have been identified in familial forms of both AD and PD, the vast majority of cases are idiopathic and the etiologies remain largely unknown. Multiple underlying factors are thought to contribute to neurodegeneration, including environmental exposures and protein misfolding, but current research suggests that chronic neuroinflammation may be a point of convergence between these factors (Tansey et al., 2007). A great amount of information supporting this hypothesis has been generated in recent years by both *in vitro* and *in vivo* studies, as well as by post-mortem pathological findings (Niranjan, 2013).

Evidence for Neuroinflammation in Alzheimer's and Parkinson's Disease

In 1988, reactive microglia expressing an antigen-presenting cell surface receptor was identified in the brains of Alzheimer's and Parkinson's Disease patients (McGeer et al., 1988). Along with the canonical pathological features of AD and PD, neuroinflammation is now considered an important feature of both diseases. Clinical evidence from post-mortem analysis has shown that a number of factors secreted by activated glial cells, particularly those regulated

by NF-κB transcription, are upregulated in PD and AD patients compared to levels in control brains. Inflammatory cytokines, such as TNF-α, have been identified in the cerebrospinal fluid and the substantia nigra (SN) in the brains of Parkinson's Disease patients (Mogi et al., 1994). Studies have also repeatedly shown increased levels of the same inflammatory markers in the serum, cerebrospinal fluid, and postmortem tissues of AD patients (Akiyama et al., 2000; Glass et al., 2010). Interestingly, research has determined that both Aβ cleavage products from APP (Amyloid Precursor Protein), which are found in the senile plaques in AD patients, is able to activate glial cells and elicit a neurotoxic inflammatory response (Kitazawa et al., 2004). Similarly, modified forms of alpha-synuclein, one of the primary proteins found in Lewy bodies in Parkinson's Disease, have been shown to induce microglial activation and inflammation (Reynolds et al., 2008). Mechanistic studies of factors such as TNF-α, IL-1β, and IFN-γ, have determined that these glial signaling molecules are potent inducers of iNOS (NOS2; inducible nitric oxide synthase), a key mediator in the formation of reactive nitrogen species, which have also been implicated in neuroinflammation-induced toxicity in PD brains (Hunot and Hirsch, 2003; Kennedy, 2002). Therefore, inflammation may be a convergence point between the neurodegeneration and protein misfolding that occurs in AD and PD. While further investigation into the mechanisms is needed, evidence supporting this link continues to emerge.

Neuroinflammation in Epilepsy and Drug-Induced Seizures

Drug-Induced Seizures

Due to their lethality and prevalence, drug-induced seizures are an adverse drug reaction (ADR) of serious concern in the development of new pharmacological agents. Although the

majority of compounds with this ADR target the CNS, many therapeutic agents targeting non-CNS areas have also been associated with seizure liability, including compounds targeting the cardiovascular and respiratory systems (Easter et al., 2009). Despite the severity of such an ADR, the current methods used to assess the seizurogenic potential of new compounds are performed in late-stage pre-clinical trials and are strictly behavioral, eliciting a "positive" response only when overt motor effects are observed. This system is highly insensitive and often fails to detect subtle behavioral changes. In addition to the low-sensitivity of this method, behavioral assessment is also extremely labor-intensive and requires requires constant observation by highly trained personnel with acute observational skills. As a result, the current method of safety assessment for new pharmacological agents is insufficient and new protocols are needed to decrease the risk of seizure-liable compounds that make it to the market.

NF-κB Activation in Neuronal Excitotoxicity

Convulsions occur as a result of sudden, transient, increases in neuronal firing. Such changes in neuronal excitability are caused by complex changes in molecular signaling and respective alterations in gene expression. In order to develop new drug-screening techniques for seizurogenic compounds, the cellular mechanisms preceding these events must be further elucidated. The Nuclear Factor-Kappa B (NF-κB) pathway is involved in the regulation of a number of cellular processes, ranging from inflammation to synaptic plasticity. Furthermore, NF-κB plays a key role in the regulation of early response genes, and therefore may be an important indicator of proconvulsant phenotypes in the CNS.

Although the mechanisms remain largely unknown, there is substantial evidence supporting NF-κB involvement in seizures. Post-mortem analysis has shown that NF-κB is overexpressed in the hippocampi of epileptic patients in comparison to that of non-epileptic hippocampi (Crespel et al., 2002). Mechanistic *in vitro* studies of hippocampal slices have shown that signal transduction pathways activate NF-κB in response to kainate, a widely used chemical-inducer of status epilepticus (continuous seizures) (Lubin et al., 2005). Furthermore, the formation of reactive oxygen species (ROS), which are known to induce NF-κB activity, is thought to be an important feature of excitotoxicity (Mattson and Camandola, 2001; Rong and Baudry, 1996). Further studies must be conducted to investigate the transcriptional changes induced by activation of NF-κB during seizurogenesis, but current information implies that NF-κB plays a neuroprotective role early on in the process (Lubin et al., 2007). Understanding the regulatory implications of NF-κB in excitotoxic phenotypes may provide valuable information for early detection of drug-induced seizures. Such modifications of the drug-screening process would eliminate seizure-liable compounds early on in the drug-development process.

Chapter 2. Experimental Methods

Transgenic NF-κB Reporter Mouse

In order to investigate the role of NF-κB-mediated neuroinflammation *in vivo,* we utilized a transgenic mouse strain expressing an NF-κB-driven enhanced green fluorescent protein (eGFP) reporter construct (cis-NF-κB^{eGFP})(Magness et al., 2004). In comparison to other *in vivo* models, this strain of mouse allowed us to evaluate real-time NF-κB activation without providing exogenous substrate. Use of this tissue samples from this mouse with immunohistochemical staining also enables us to visualize NF-κB expression as it relates to other proteins and to identify the NF-κB expressing cells.

Fluorescent Immunohistochemistry

The primary goal of this document is to explore the use of fluorescent immunohistochemical (IHC) staining to investigate the mechanisms of NF-κB activation neuroinflammatory disorders. Immunohistochemistry is an extensively used molecular tool with applications in both clinical diagnostics and laboratory research (Xiao Chen, 2010). The theory behind IHC is that it combines histology, immunology, and chemistry to detect antigens by the use of specific antibodies (Ramos-Vara, 2005). Although the methodology of IHC has evolved over the years to increase specificity and sensitivity, the basic concept is rather simple. Specific antibodies are used to bind an antigen of interest and then the antibody-antigen reactions are visualized by a marker. There are different types are markers available, but majority are either fluorescent, enzymatic, or radioactive. In the case of indirect fluorescent immunohistochemistry, the marker is a fluorophore conjugated to the secondary antibody.

There are multiple methods of IHC used for antigen detection, most commonly direct and indirect. The direct method of IHC is less tedious and requires the use of only one antibody. The Ab targeted against the antigen of interest is labeled with a marker and therefore the antibody-antigen reactive products are directly visualized. In the indirect method of IHC, two antibodies are used. A primary, unlabeled antibody directed against the specific protein of interest is used first to bind the antigen. Then a second antibody, which has been labeled with a visual marker, is used to bind to the antibody-antigen complex. This secondary antibody has been produced in a different species than the first and usually targets the IgG of the primary antibody host species. In this method, several secondary antibodies are able to bind to a single primary Ab. This allows for amplification of visual signal and therefore, indirect IHC is more sensitive than the direct method. Due to increased sensitivity, all studies in this document utilize the indirect method of IHC.

The steps to indirect immunofluorescent staining are as follows: tissue preparation, sectioning, blocking, antigen retrieval, incubation with primary antibody, incubation with secondary antibody, and preparation for visualization (Xiao Chen, 2010). While the precise methods used are highly variable, the protocols used in this document have been optimized specifically for the investigation of proteins involved in NF-κB-mediated neuroinflammation in the particular murine model used in our lab. Since the reagents and antibodies used vary slightly between studies, protocol specifics will be provided in the methods section of each study. However, because this document focuses on the methodology behind these studies, it is important to fully understand the concepts behind the protocols used.

Tissue preparation is important for the preservation of antigens and cellular architecture of the tissue. A wide variety of fixative method are used during this process, depending on the tissue used and experimental needs. The most commonly used reagents are acetone, paraformaldehyde, and paraffin. Proper fixation with any of these reagents allows for the tissue to be stored while retaining antigenicity (Xiao Chen, 2010).

After the sample has been fixed, it must be sectioned in order to obtain tissue slices from the area of interest. Again, the protocol used during this step are highly variable. In our neuroinflammation research, the brain is frozen in liquid nitrogen and the frozen sections are cut using a cryostat microtome. The sectioned tissue is then preserved in a cryoprotectant solution and ready for IHC staining.

The tissue sections are removed from the cryoprotectant and rinsed (usually with Tris-buffered saline) before they are placed on the slides. Some antigens, such as tyrosine hydroxylase on dopamine neurons (Fig. 1) require an additional step before blocking, a process called antigen retrieval. Antigen retrieval reverses any protein cross-linking that occurred during fixation and makes it easier for the antibody to bind to the antigen of interest. There are also various ways to perform antigen retrieval, based on the type of fixation. The most common methods use heat, enzymes, or chemicals. We typically use heated sodium citrate in our research, as the higher pH provides for more vigorous antigen retrieval. However, one limitation of using the eGFP reporter mouse is that antigen retrieval decreases GFP signal in formaldehyde fixed, frozen tissue. When identifying GFP co-localization (NF-κB activation) with other antigens, antigen retrieval is not advised. Before the primary antibody can be applied, the tissue must incubate in a blocking buffer to prevent false-positive staining. Various endogenous proteins can

interfere with antibody-antigen binding and increase background signal in the stain. Fc receptors, which bind the Fc region of the antibody, are the primary concern due to their high level of expression on immune-related cells (Xiao Chen, 2010). However, a simple blocking buffer containing serum from the host animal usually is effective at preventing unspecific binding.

Since IHC uses antibody-antigen binding to detect the presence of a specific antigen, the efficacy of this method is highly dependent on the primary antibody (Ab) used. Polyclonal antibodies are produced by immunizing multiple animal species with a purified form of the particular antigen. Due to the presence of several antibodies produced to target the antigen,

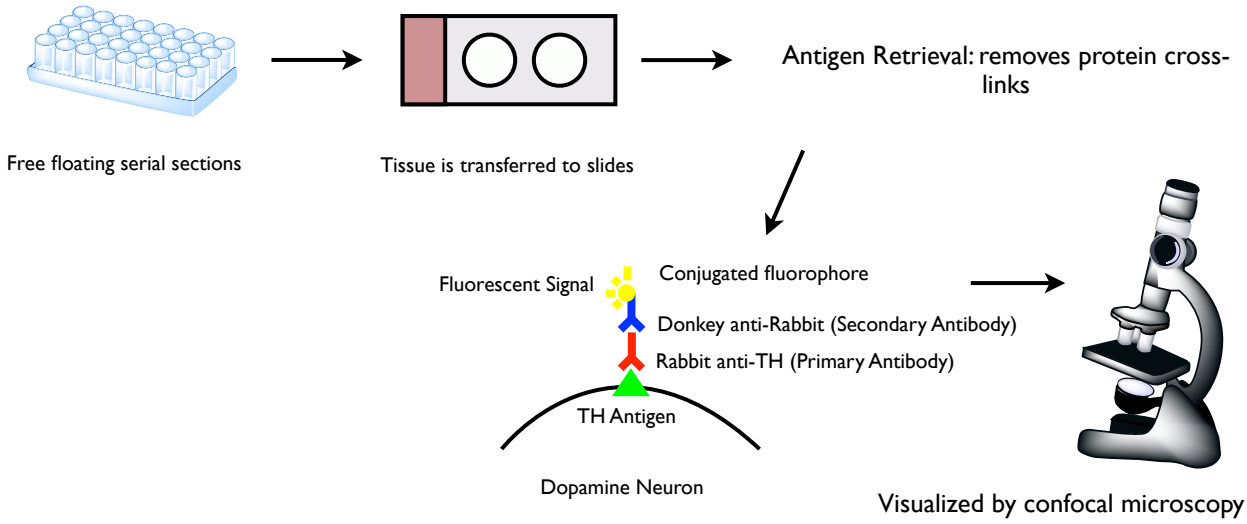

Figure 2.1. Schematic of indirect fluorescent immunohistochemical staining for tyrosine hydroxylase in dopamine neurons. Serial sections are removed from the cryoprotectant solution and rinsed repeatedly with TBS before being transferred to slides. In the case of tyrosine hydroxylase (TH) staining, antigen retrieval must be used to reverse protein cross-links and increase antigen availability. This is done by immersing the tissue in microwave-heated sodium citrate for 20 minutes. Afterwards, the tissue is blocked with serum from the host-animal for an hour to prevent unspecific binding. The tissue is then incubated overnight at 4^{o} C with the primary antibody, anti-TH produced in rabbit. The next day, following several washes with TBS, the tissue is incubated with the secondary antibody for three hours at room temperature. When staining for TH, the secondary antibody used is an anti-rabbit antibody produced in donkey. This antibody is conjugated to a fluorophore that emits a fluorescent signal when stimulated at a particular wavelength. Once the final incubation is complete, the tissue is again washed with TBS and covered by protective coverslips using a mounting medium containing DAPI, a marker of cell nuclei. The coverslips are sealed with clear nail polish for long-term storage and the tissue can then be visualized using a confocal microscope.

polyclonal Abs are more likely to detect multiple epitopes and have both a higher affinity and broader spectrum of reactivity than monoclonal Abs (Ramos-Vara, 2005). These traits also increase the likelihood of cross-reactivity between the polyclonal Ab and other antigens, so there are advantages and disadvantages to both.

The incubation solution containing the primary antibody is usually some concentration of antibody in a 1% blocking buffer (TBS plus host-species serum) containing a non-ionic detergent, such as Triton X-100. The detergent in this step assists in antibody-antigen binding of non-membranous proteins by disrupting the cell membrane. Following application of the primary antibody, the tissue is incubated overnight at 4˚ C.

Once the primary antibody is removed and rinsed with TBS, the secondary antibody can be applied. The secondary antibody is targeted to the IgG of the primary antibody host-species and is conjugated with a fluorophore that emits a fluorescent signal when stimulated by the appropriate wavelength of light. The solution used in this step does not contain detergent or serum, as neither are necessary to promote reactivity. The tissue is incubated with the secondary antibody for three hours at room temperature.

Once incubation is finished, the tissue is washed with TBS and coverslips are placed on top using mounting media. The mounting media helps the coverslip adhere to the tissue and preserves the tissue. The mounting media used in our lab also contains a counterstain, DAPI (4', 6-diamidino-2-phenylindole), an intercalating fluorophore that allows for visualization of cell nuclei. Finally, the slides are visualized using a laser scanning confocal microscope.

IHC Controls

Both positive and negative controls are necessary to effectively optimize an IHC protocol. The three main types of controls are primary antibody controls, secondary antibody controls, and label controls. IHC controls are conducted to confirm specificity of the reaction. Since all of the antibodies used in this document have been used by our lab in previous studies, controls were performed prior to my involvement in this research. However, it is important to understand the use of controls, as they are critical to the development of an IHC protocol.

Primary antibody controls demonstrate specificity of the primary antibody to the antigen of interest, along with the effects of tissue processing (Burry, 2011). There are many primary antibody control methods available, both genetic and non-genetic, and use of multiple controls is recommended. One of the most effective methods of primary antibody control for *in vivo* studies is to use tissue from an animal in which the gene encoding the antigen has been genetically knocked-out (KO). Since the antigen of interest is not expressed in this tissue, the primary antibody should not bind. This is the preferred method of many researchers because the KO tissue can be processed and stored using the same procedures as the original tissue, therefore minimizing any discrepancies in tissue condition. However, KO mice may not always be available or attainable for a particular protein. Furthermore, KO mice may contain non-functional genes or mutated versions of the gene, in which the primary antibody may still bind. Non-genetic methods of primary antibody control are also used, most frequently western blots and absorption assays. Western blots are a commonly used analytical technique which use antibodies to detect specific proteins in tissue or cell homogenate. One limitation of this method of antibody control is that many of the protein conditions vary between IHC and western blots.

Instead of detecting fixed antigens in tissue slices, western blots detect proteins in which the secondary and tertiary structures have been denatured. Absorption controls are another commonly used non-genetic method in which the primary antibody is incubated with purified antigen prior to use on the tissue. If the antibody specifically binds the antigen, then the antibody-antigen complexes will already be formed in the test tube and the antibody will not bind to the antigens in the tissue (Burry, 2000). This method, however, can produce false-negatives. If the antibody is capable of binding to multiple proteins, but is saturated with the antigen of interest during this assay, the antibody will appear to be specific to the antigen of interest. It is recommended that other methods are used in conjunction with absorption controls to confirm antibody specificity (Burry, 2011).

In the case of indirect fluorescent IHC, it is important to use controls to test the specificity of the secondary antibody in order to confirm that the fluorescence is from the antibody-antigen complex. The preferred method of secondary control is to incubate the secondary antibody with tissue in the absence of the primary antibody. If the antibody does specifically target the antigen, no staining should occur.

Although the likelihood of unwanted endogenous fluorescence is unlikely, labeling controls are also needed to determine that the fluorescence is intentional and corresponds to the antibody-antigen complex. This can be done rather simply by incubating a tissue sample in all of the same reagents and buffers used in the procedure, with the exception of any antibodies (Burry, 2011).

Experimental Models of Parkinson's Disease

There are currently multiple neurotoxin-based models of Parkinson's Disease, most commonly 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Use of these chemicals in rodent models have hugely impacted our understanding of the disease and have been provided valuable information for therapeutic targets in drug development.

The chemical structure of 6-hydroxydopamine (6-OHDA) (Fig. 2.2) closely resembles that of dopamine, which gives it a high affinity for catecholamine transporters and allows it to enter the neuron via the dopamine transporter. One limitation of 6-OHDA is that the compound cannot readily cross the blood-brain barrier and therefore must be directly injected into the brain in order to cause neurodegeneration. The site of stereotaxic injection affects the resulting pathology and there are two primary sites of injection for the 6-OHDA experimental model, the medial forebrain bundle (MFB) and the striatum. Stereotaxic injection into the medial forebrain bundle (MFB) results in a more rapid degeneration of the neurons, producing a nearly complete lesion only 5 weeks post-injection. Injection into the striatum produces a slower, dose-dependent loss in neurons, which allows for simulation of the disease at various stages (Bové and Perier, 2012).

The exact mechanisms by which 6-OHDA causes neuronal death are poorly understood, but it is thought to be through oxidative damage from the production of reactive oxygen species (ROS). It is currently believed that neuronal death caused by injection into the MFB is caused by necrosis, but studies have shown that striatal injection may be apoptotic, and the topic is highly debated (Bové and Perier, 2012; Ries et al., 2008).

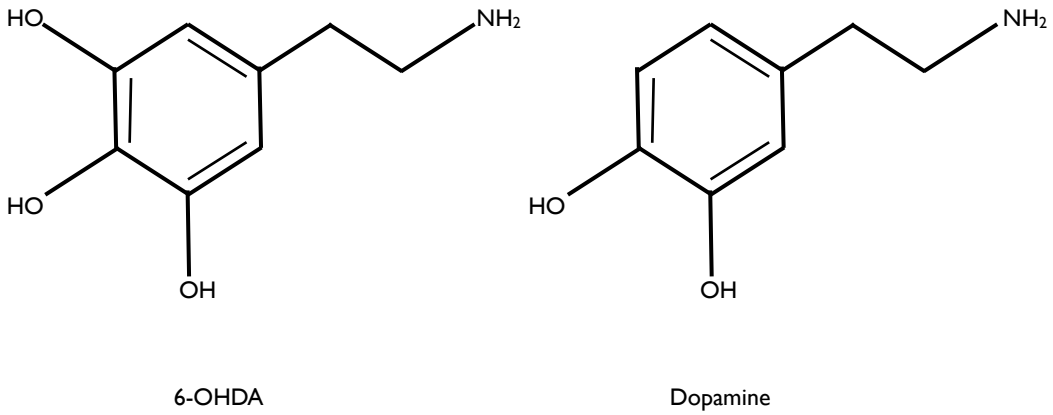

Figure 2.2. Chemical structures of 6-hydroxydopamine (6-OHDA) and dopamine

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is another neurotoxin used in rodent models of Parkinson's Disease. The toxin was accidentally created by a student as a synthetic, heroin-like recreational drug. The neurotoxic effects of MPTP were discovered when all of the users that had injected the drug developed Parkinsonian-like symptoms that were very similar to those of idiopathic PD (Hisahara and Shimohama, 2011). Analysis of the users' brain revealed a Parkinson's-like pathology with severe dopaminergic loss in the SNpc, although the presence of Lewy bodies was absent. MPTP has since become a widely used neurotoxin for experimental models of PD in mice. It is important to note that rats are extremely resistant to MPTP toxicity and therefore are not used in this model (Bové and Perier, 2012).

MPTP is a protoxin that is converted to 1-methyl-4-phenylpyridinium (MPP+), which is responsible for the pathological effects (Fig. 2.3). MPTP is highly lipophilic and rapidly crosses the BBB when administered systemically (Bové and Perier, 2012). The molecule is first oxidized by monoamine oxidase B (MAO-B) to produce the unstable intermediate molecule 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+) (Chiba et al., 1984). MPDP+ dismutation

results in the production of MPTP and MPP+, the latter of which is then taken up by dopamine transporter (DAT) on the plasma membrane of dopamine neurons (Bové and Perier, 2012).

MPP+ toxicity results in a permanent, progressive loss of dopaminergic neurons and elicits similar symptoms, both motor and non-motor. The severity of the disease model is variable depending on the route of administration and the intoxication regimen. There are several intoxication regimens used, but the most common involved systemic injections of MPTP over a period of time ranging from days to months. In general, acute models involve injections of lower doses of MPTP at shortened intervals and these regimens are used to study neuroinflammation. Alternatively, sub-acute models using injections of slightly higher doses of MPTP at longer intervals are more commonly used for studies of cell-death mechanisms.

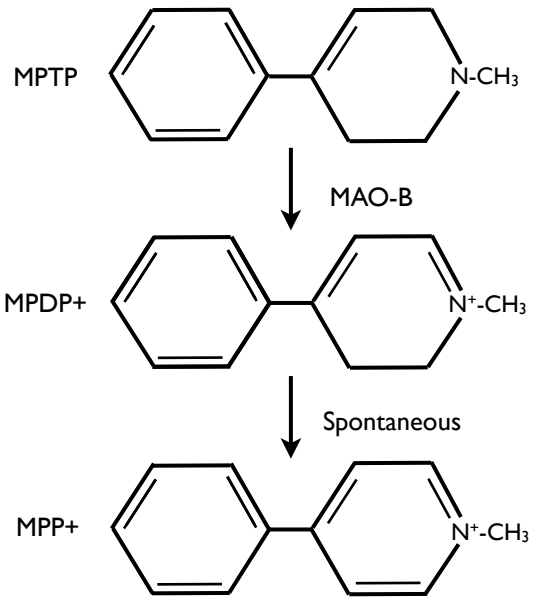

Figure 2.3. Production of the MPP+ neurotoxin from protoxin MPTP. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is metabolized by the enzyme monoamine oxidase B (MAO-B) in the glia to form the unstable metabolite, 1-methyl-4-phenyl-2,3-dihydropyridinium (MPDP+). The unstable intermediate is spontaneously converted to 1-methyl-4-phenylpyridinium (MPP+) which enters dopaminergic neurons via dopamine transporters (DAT) on the membrane.

Chronic models of the disease create a more progressive disease state and result in only a partial loss of dopaminergic neurons. In these models, it is common for only the older mice to exhibit motor symptoms, therefore allowing for assessment of the disease at earlier stages. Chronic models are important in the development of neuroprotective therapeutics (Bové and Perier, 2012).

Although the mechanism of MPP+ toxicity has not been fully elucidated, the widely accepted belief is that it interferes with the mitochondrial electron transport chain by inhibiting enzyme complex I, this prevents proper mitochondrial respiration (Bové and Perier, 2012). Altered mitochondrial respiration results in the formation of reactive oxygen species, which is thought to play a large role in MPTP neurotoxicity. MPTP is also known to activate microglia, indicating that inflammation-induced cytotoxicity may contribute to the formation of ROS and cytokines that stimulate neuronal death.

Experimental Models for Seizure Assessment

Due to the broad spectrum of excitotoxic phenotypes, there is a multitude of animal models that can be used for seizure research and a wide variety of syndromes can be simulated. In our studies, we utilized the prototypic seizurogenic compound, kainic acid (KA), to induce a proconvulsant phenotype in the transgenic NF-κB reporter mouse. Several studies have shown that NF-κB activation may be involved in epilepsy and evidence suggests that NF-κB may be an early indicator of excitotoxicity-induced stress response (Crespel et al., 2002; Lubin et al., 2005; 2007) . Additional supporting research has shown that NF-κB activation can occur via stimulation of the kainate receptors (Cruise et al., 2000; Nakai et al., 2000).

Kainic acid is glutamate analogue isolated from red algae that exerts its excitotoxic effects by specifically binding to kainate receptors, a subtype of ionotropic glutamate receptors(Wang et al., 2005). KA mimics the effects of glutamate, the primary excitatory neurotransmitter in the brain. The mechanisms involved in glutamate excitotoxicity are complex, but accumulation of intracellular calcium is thought to play a large role. The influx of calcium signals for a cascade of molecular events, including mitochondrial dysfunction and ROS generation, which eventually lead to cell death (Zhang and Zhu, 2011). Although the precise molecular events involved in kainic acid toxicity have not been fully elucidated, KA is a well established hippocampal neurotoxin. Systemic injection of kainic acid selectively targets the CA1 and CA3 regions of the hippocampus. The reason for this increased sensitivity is unknown, but it is likely due to the high levels of kainate receptors located in these regions (Wang et al., 2005).

KA is a useful model in the research of drug-induced seizures because it causes extensive neuronal death in the hippocampus, which causes an epileptic phenotype which is capable of producing reoccurring seizures. Treatment with convulsant doses of kainic acid causes excessive Ca^{+} influx, ROS, and endoplasmic reticulum stress, resulting in mitochondrial dysfunction and glial activation of inflammatory responses (Zheng et al., 2011). The complex signaling network caused by KA-toxicity has provided valuable information about the mechanisms of seizure development, lending to better understanding of drug-induced seizures. KA has also been used in studies of neuroinflammation, as neuronal death induced by KA-toxicity has been shown to cause a marked glial response (Wang et al., 2004).

Discussion

While there are a multitude of techniques available for neuroinflammation, implementing those that are most effective and efficient will help expedite scientific progress. We have been striving to optimize the protocol of fluorescent IHC staining of tissue from the transgenic *cis*-NF-κB^{eGFP}-containing reporter mouse for use in neuroinflammatory research. This method has enabled us to uncover many molecular mechanisms of neuroinflammation. Continuing to optimize these models will undoubtedly lead to better understanding of NF-κB involvement in neurodegeneration and excitotoxicity.

Chapter 3. Neuroinflammation in the MPTP Model of Parkinson's Disease

Introduction to Parkinson's Disease

The substantia nigra pars compacta (SNpc) is the brain region within the basal ganglia that most severely affected in Parkinsonian disease. Neurodegeneration in this brain region is characterized by severe loss of dopaminergic neurons, as well as protein aggregates, termed Lewy bodies or Lewy neurites, in the remaining neurons (Tansey and Goldberg, 2010). The most prominent symptoms of PD are associated with the loss of neurons that occurs in the basal ganglia, resulting in decreased motor function such as bradykinesia and resting tremors. PD is categorized into two forms, familial and sporadic. Familial PD tends to follow patterns of Mendelian inheritance and is associated with an earlier age of onset than idiopathic Parkinson's Disease. A number of causative genetic mutations have been identified in familial forms of PD which have provided valuable insight into the molecular alterations in the Parkinsonian brain. However, the heritable genetic mutations involved in the development of familial Parkinson's Disease are not associated with idiopathic development of the disease and therefore only explain up to 15 percent of overall cases (Dawson and Dawson, 2003).

Age is the single largest risk factor for developing sporadic Parkinson's Disease, with the average age of onset being 70 years. The age-association of idiopathic PD implies that cumulative CNS damage may play a large role in pathogenesis of the disease. In more recent years, research has accumulated in favor of this hypothesis by establishing that neuroinflammation is an important pathological feature of Parkinson's Disease. Emerging evidence for the role of inflammatory processes in Parkinson's Disease supports the idea that

oxidative stress and cytokine-derived toxicity from chronic neuroinflammation are likely contributors to PD neurodegeneration. Despite the increasing evidence that neuroinflammatory responses are involved in idiopathic PD etiology, the exact pathological mechanisms have yet to be elucidated. Currently, the only available therapeutics for PD aim to alleviate symptoms, no drug has made it to the market that has been able to slow neuronal loss thus far. Understanding the neuroinflammatory processes involved on a molecular level may unveil a number of new therapeutic targets that may slow or possibly prevent further neurodegeneration in Parkinson's Disease.

Neuroinflammation in Parkinson's Disease

There is substantial evidence supporting the involvement of chronic neuroinflammatory responses in Parkinson's Disease pathology, including the presence of activated microglia, oxidative damage, and cytokine accumulation in the brains and cerebrospinal fluid (CSF) of PD patients (McGeer et al., 1988; Mogi et al., 1994; Tansey et al., 2007). While the exact mechanisms by which neuroinflammation contributes to neurodegeneration have not yet been established, it is likely that a combination of factors are involved. Chronic activation of glia-mediated immune responses cause an overproduction of cytokines and reactive oxygen species, which cause neuronal death in large quantities. In comparison to neurons in other regions of the brain, midbrain dopaminergic neurons are more susceptible to oxidative damage and cytokine-induced toxicity (Floyd, 1999; McGuire et al., 2001; Taylor et al., 2013). Accumulation of these toxic insults is thought to create a feedback mechanism responsible for propagation of the disease. Unfortunately, the customary diagnostic procedures rely largely on the assessment of

motor symptoms, which do not arise until neurodegeneration in the SNpc has already begun. Furthermore, the only PD drugs on the market are palliative and do not affect progression of the disease. Understanding the mechanisms by which inflammation contributes to Parkinson's Disease pathology may provide novel therapeutic targets capable of slowing or stopping neuronal loss (Taylor et al., 2013).

The Involvement of NF-κB in PD

Transcription factor NF-κB is known to be involved in the regulation of a large number of physiological processes, which has made it a popular target for drug-development research in recent years (Camandola and Mattson, 2007). Although the molecular mechanisms remain unknown, there are multiple lines of evidence connecting NF-κB activation with PD pathophysiology. Under normal physiological conditions, NF-κB resides as an inactive complex consisting of two subunits, p50 and p65, and is bound to inhibitor protein IkB. Upon stimulation, IkB is phosphorylated by the enzyme IkB Kinase (IKK) and dissociates from the complex. The NF-κB heterodimer is then able to translocate to the nucleus and bind to regulatory elements on the DNA, regulating transcription of certain target genes (Camandola and Mattson, 2007; Stephane Hunot, 1997). Nuclear translocation of NF-κB, as well as the upregulation of NF-κB mediated inflammatory genes, have been observed in both post-mortem brains and in experimental models of PD (Stephane Hunot, 1997). Concordant with these findings, tumor necrosis factor-α (TNF-α) has been detected in activated microglia and is a known activator of NF-κB (Boka et al., 1994; Mogi et al., 1994; Stephane Hunot, 1997). Similar studies have confirmed that dopaminergic neurons are particularly sensitive to TNF-α-induced

toxicity, which would explain the selective neuronal loss seen in PD neurodegeneration (McGuire et al., 2001). Further understanding these mechanisms is critical for drug development and may reveal a multitude of new therapeutic targets capable of modifying progression of Parkinson's Disease (Camandola and Mattson, 2007).

Fluorescent IHC Markers Used in PD Research

The use of indirect fluorescent immunohistochemistry and the NF-κB reporter mouse has generated valuable information about NF-κB activation in Parkinson's Disease pathology. In our research, we used fluorescent IHC to identify the NF-κB-mediated mechanisms of MPTP-toxicity in dopaminergic neurons of the substantia nigra pars compacta (SNpc).

Tyrosine Hydroxylase

Parkinson's Disease neurodegeneration is characterized by the progressive loss of nigrostriatal dopaminergic neurons. Dopamine (DA) is produced in the neuronal bodies located in the SNpc and then projected to the basal ganglia and released in the striatum (the putamen and caudate nucleus). Therefore, loss of dopaminergic neurons in the SNpc results in decreased synaptic dopamine levels in the striatum (Dauer and Przedborski, 2003). Tyrosine hydroxylase (TH) is the rate-limiting enzyme involved in catecholamine synthesis and is responsible for the conversion of amino acid L-tyrosine into L-3,4-dihydroxyphenylalanine (L-DOPA), which is then converted into dopamine. DA synthesizing neurons in the SNpc can therefore be identified by using fluorescent immunohistochemical staining with antibodies generated against the tyrosine hydroxylase antigen (Haavik and Toska, 1998).

MPTP Intoxication Protocol

In order to evaluate loss of TH+ cells in the SNpc, we used a sub-acute MPTP treatment routine to create a progressive lesion. 12 week-old transgenic mice (As previously described, (Magness et al., 2004)) containing the cis-NF-κB^{eGFP} reporter construct were injected subcutaneously every other day with MPTP (25 mg/kg) and intraperitoneally (i.p.) with probenecid (250 mg/kg) for 7 days. Probenecid is an adjuvant which decreases MPP+ clearance when co-administered with MPTP in sub-acute MPTP models (Petroske et al., 2001). Control-treated mice were given i.p. injections on the same schedule using only saline and probenecid (250 mg/kg). Mice were terminated either after directly after the 7 day treatment or after 14 days (Further characterization of this treatment strategy is described by (De Miranda et al., 2013).

Stereological Assessment of Dopaminergic Neurons and Nerve Terminals

Tyrosine hydroxylase staining of every third free floating serial section was used for stereological assessment of dopamine neurons in the SNpc and dopamine nerve terminals in the striatum. This selection protocol was developed in order to assess TH expression throughout the entire SNpc. The selected sections were rinsed in 0.05 M Tris-buffered saline (TBS, pH 7.6) and then placed directly on slides. Heat-induced antigen retrieval was then performed by submerging the sections in heated 10% sodium citrate for 20 minutes, followed by incubation with 1% donkey serum and 1% goat serum in TBS for one hour. The sections were then incubated with a rabbit anti-tyrosine hydroxylase primary antibody (1:500 in blocking buffer containing 0.5% Triton X-100; Chemicon, Temecula, CA) overnight at 4˚ C. The following day, sections were rinsed in TBS and incubated with an Alexafluor conjugated anti-rabbit secondary antibody

(1:500 in blocking buffer; Invitrogen, Carlsbad, CA) for three hours at room temperature. The tissue sections were again rinsed in TBS and mounted in media containing 4',6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei. The finished slides were stored at 4˚ C until stereological counting was performed using Slidebook software (De Miranda et al., 2013).

IHC Staining for Tyrosine Hydroxylase

As previously described in the Methods chapter of this document, free-floating serial sections were used for IHC staining of tyrosine hydroxylase. TH is an extremely effective marker to assess loss of dopaminergic neurons and can easily stained using IHC. Stereology is a method used to collect unbiased quantitative data from three-dimensional material. In our research, we utilize TH staining in the SNpc for stereological assessment of TH+ neurons (Fig. 3.1).

The primary antibody used in IHC is one the most critical factors in determining the efficacy of staining. In our studies, we tried several anti-TH antibodies and had variable success. The most affective antibody for TH staining in the SNpc in our mouse model was a rabbit anti-tyrosine hydroxylase (Chemicon) at a concentration of 1:500.

Problems did arise, however, in double staining for TH+ and MAP2+ neurons in the SNpc. In order to confirm that the TH+ cells of the SNpc are neuronal, we had hoped to co-localize the TH staining with MAP2, a structural filament protein expressed by neurons. However, we found that staining with mouse anti-MAP2 was unsuccessful and created a diffuse, non-specific stain. The most effective MAP2 antibody in mice was a rabbit polyclonal anti-MAP2 (AbCam, 1:250 in blocking buffer containing 0.5% Triton X-100), which presented

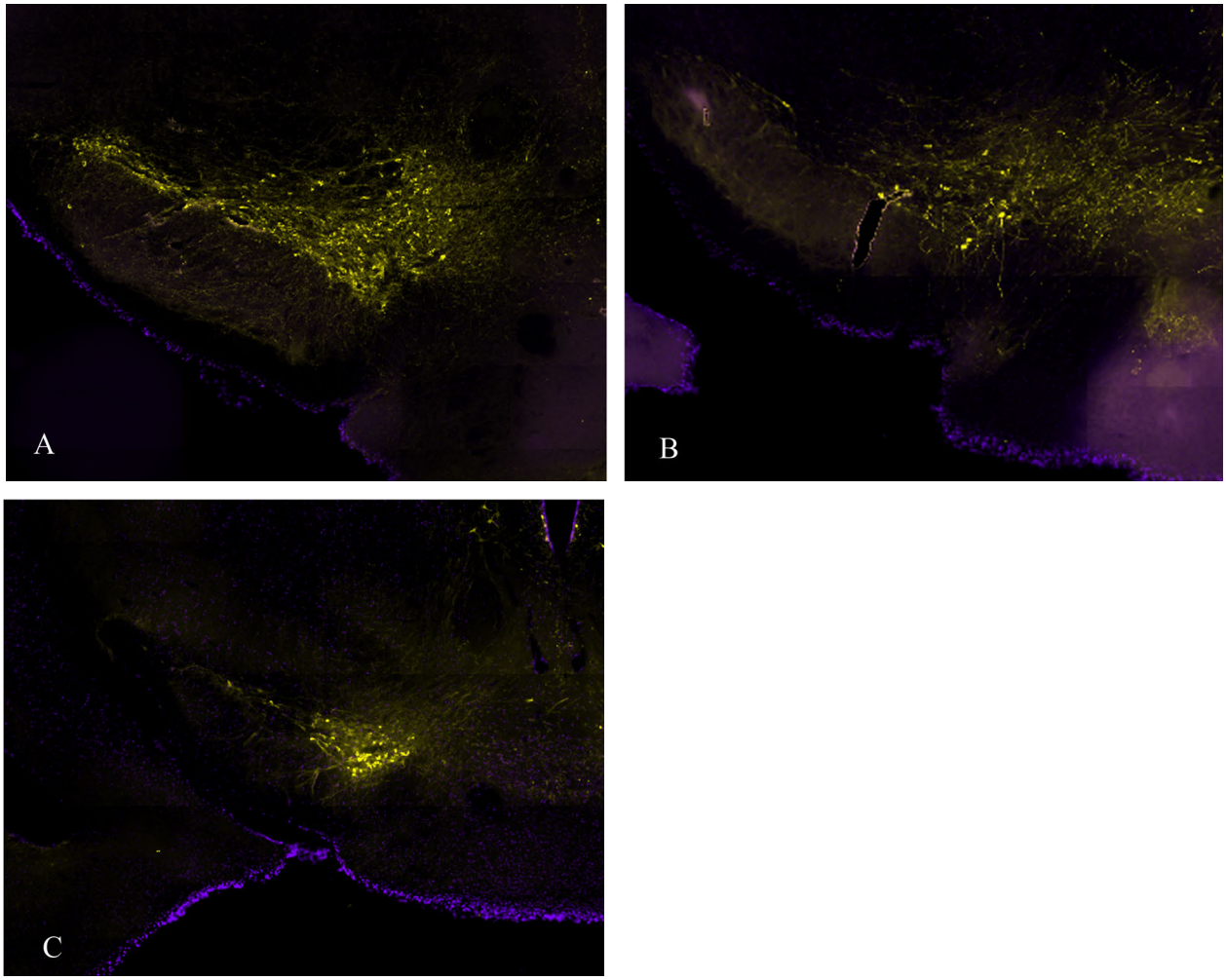

Figure 3.1. 10x image of immunofluorescent staining of tyrosine hydroxylase-positive neurons in 40 µm tissue sections containing the SNpc following treatment with a) saline b) 7 day MPTP-injection c) 14 day MPTP-injection. The time-dependent decrease in TH+ neurons indicates dopaminergic neurodegeneration following treatment with MPTP.

the problem of dual staining with two antibodies produced in the same host species. Since the secondary antibody containing the fluorescent marker is targeted against the host species of the primary cell, the secondary antibody hosts must be different in order to visually distinguish the antibody-antigen complexes. Therefore, we tried a variety of anti-TH antibodies to see if an antibody produced in a different species would effectively stain for TH and co-stain with MAP2. The first antibody we tested was a sheep anti-tyrosine hydroxylase (Millipore) at varying

concentrations. All concentrations resulted in a diffuse, unspecific binding. We then tested a chicken anti-tyrosine hydroxylase (Millipore) and found that it successfully co-localized with the rabbit anti-MAP2 at a concentration of 1:250, with an extra-heated sodium citrate antigen retrieval step. However, even with the higher concentration of the primary antibody and an vigorous antigen retrieval step, the results were variable. The protocol used to determine co-localization of these antigens using IHC has yet to be optimized.

Glial Activation in the SNpc

Neuroinflammation is now a well-established contributing factor in PD pathology (Tansey and Goldberg, 2010). Sub-acute intoxication with MPTP elicits a clear, dose-dependent increase in glial activation. In order to identify the presence of glial activation in MPTP toxicity, we used antibodies targeted toward two different proteins. Microglial activation results in the upregulation of a number of proteins and cell-signaling molecules, but many of them are also secreted by reactive astrocytes. Therefore, we used an antibody against ionized calcium-binding adapter molecule 1 (IBA-1, 1:500, Cell-Signaling), a calcium-binding protein that is specific to macrophages and is upregulated upon microglial activation, to identify the presence of microglial activation upon treatment with MPTP. In the assessment of gliosis, we also used an antibody targeted toward glial fibrillary acidic protein (GFAP, 1:500, Cell Signaling), an astrocyte-specific intermediate filament protein (Fig. 3.2). Double IHC staining protocol for these proteins has been previously optimized in our lab and therefore did not require any trial-and-error efforts.

Despite the use of multiple antibodies, the staining protocol for gliosis is less tedious and less time-consuming than that of tyrosine hydroxylase. We stained for IBA1 and GFAP in both

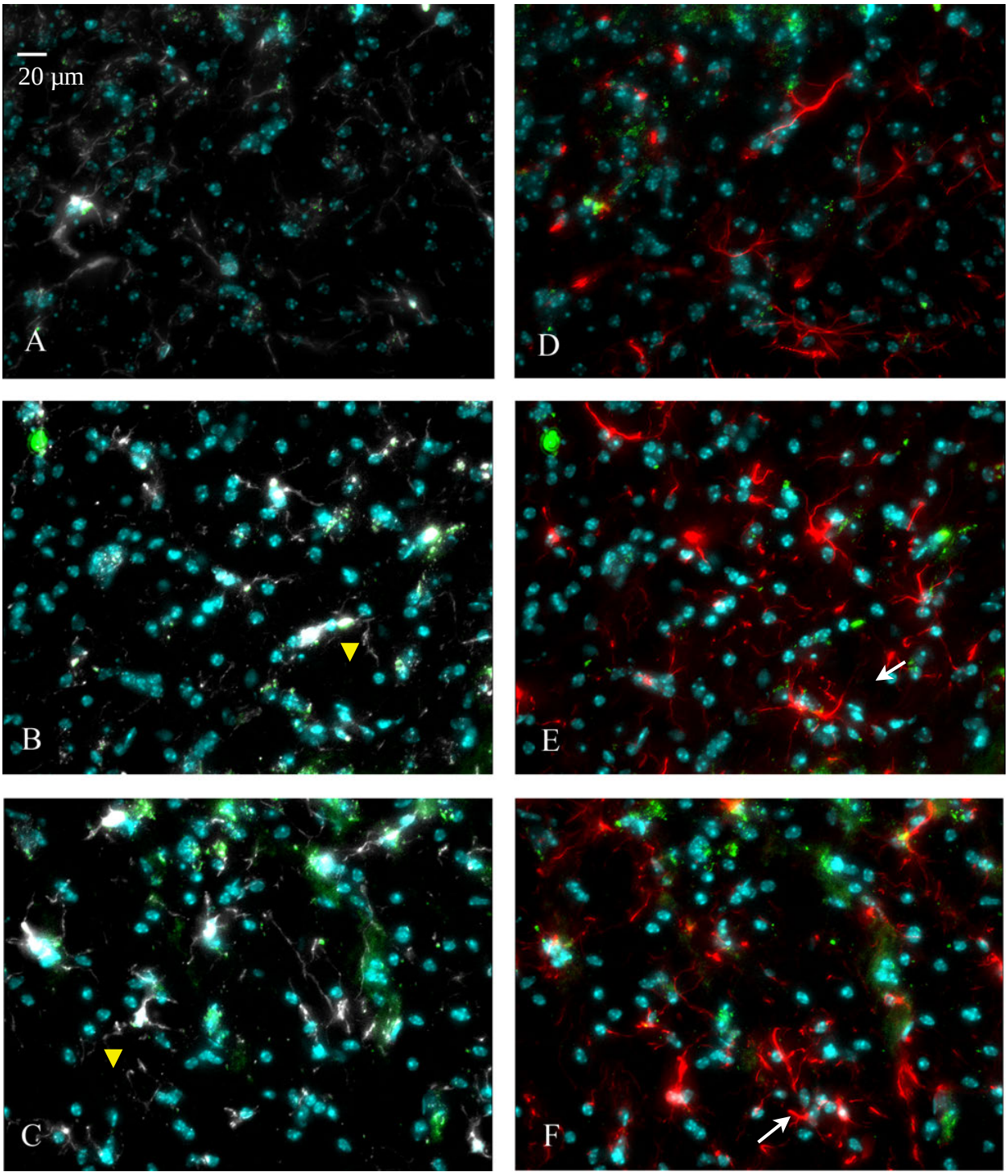

Figure 3.2. Fluorescent IHC stains of 40 µm tissue slices showing glial activation at 40x magnification. A-C: Time dependent microglial activation (yellow arrowheads) visualized by IBA-1 (white) following A) saline B) 7 days post-MPTP and C) 14 days post-MPTP treatment. D-F: Corresponding increase in astrogliosis (white arrows) as shown by enhanced GFAP expression (red) in D) saline E) 7 day MPTP and F) 14 day MPTP treatment.

striatal and nigral tissue, but we used only two samples per animal (from three animals per treatment group). The samples were selected based on morphology, tissue around the middle of the region was usually chosen, as it contains the most of that region.

Use of IHC in PD drug-development

IHC staining for gliosis in the striatum and the SNpc is a valuable tool for assessing the efficacy of therapeutics targeting neuroinflammation in Parkinson's Disease. A study published by our lab previously this year (for more information, refer to De Miranda, 2013) investigated the pharmacokinetics of a group of novel neuroprotective compounds, para-phenyl-substituted diindolmethanes (C-DIMs). These compounds were synthesized based on the structure of 3-3'-diindolylmethane, a metabolic product of phytochemical indole-3-carbinol digestion(De Miranda et al., 2013). The potential therapeutic properties of 3-3'-diindolylmethane have drawn a lot of attention to this class of compounds in the past decade as an anti-carcinogenic agent (De Miranda et al., 2013; Safe et al., 2008). As determined by previous *in vitro* studies from our lab, these compounds elicit their neuroprotective characteristics by suppressing NF-κB activity (Carbone et al., 2009; Tjalkens et al., 2008).

Other studies have shown that C-DIM compounds activate members of the NR4A receptor family, which recruit repressor complexes and decrease NF-κB-induced inflammation (De Miranda et al., 2013; Inamoto et al., 2008; Saijo et al., 2009). In this study, we used fluorescent IHC to stain for gliosis and assess the ability of various C-DIM compounds to alleviate MPTP-induced glial activation (Fig. 3.3).

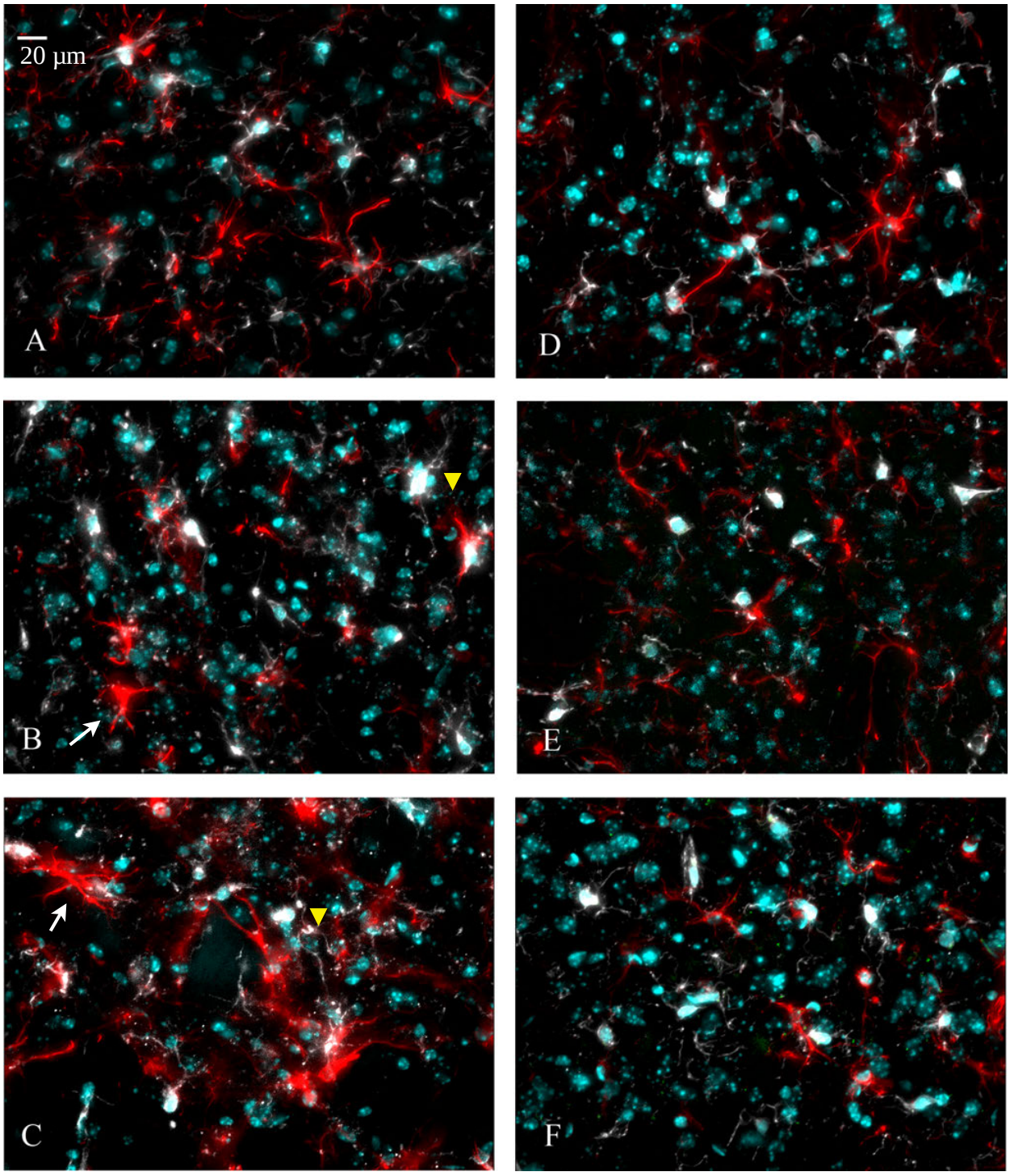

Figure 3.3. Fluorescent IHC stains showing the effects of C-DIM compounds on glial activation at 40x magnification. A-C: Progressive increase in microglial activation (yellow arrowheads) shown by IBA-1 expression (white) and astrocytosis (white arrows) shown by GFAP expression (red) in A) saline B) 7 days post-MPTP and C) 14 days post- MPTP treatment. D-F: Attenuated glial activation in MPTP-14 treated animals given D) C-DIM5 E) C-DIM8 and F) C-DIM12.

Following sub-acute treatment with MPTP for 7 days, mice were either terminated (MPTP-7) or kept until day 14 (MPTP-14). The mice in the MPTP-14+C-DIM treatment groups were gavaged once daily for another 7 days with 50 mg/kg of either C-DIM5, C-DIM8, C-DIM12, or corn oil for control (De Miranda et al., 2013). These mice were then terminated at day 14 and the neuroprotective efficacy of these compounds was evaluated. Although these IHC images alone do not reveal mechanistic data, they provide valuable qualitative insight into the physiological efficacy of these anti-inflammatory compounds *in vivo*.

TH and Intrinsic GFP Expression

Increased NF-κB expression has been seen in the brains and CSF of Parkinson's Disease patients and there is mounting evidence that activation of the pathway has deleterious effects on nigral neurons (McGeer et al., 1988; Tansey and Goldberg, 2010). We utilized the method of IHC staining with the NF-κB reporter mouse to determine whether the loss of dopaminergic neurons in the SNpc corresponds to NF-kB activation (Fig. 3.4). Following sub-acute treatment with MPTP, there was a progressive loss of TH+ neurons. While there was a definite increase in NF-κB expression from saline to MPTP-7 day mice, 14 day treated mice seem to have lower NF-kB and TH expression. This can most likely be explained by the loss of neuronal bodies and altered morphology due to the treatment.

Fluorescent IHC staining of tissue from *in vivo* studies with the reporter mouse also allows for visualization of NF-κB activation as it relates to other proteins and transcriptional products in neuroinflammation. However, there are a few limitations that must be addressed in order to optimize this model. The current process often fails to detect co-localization of GFP

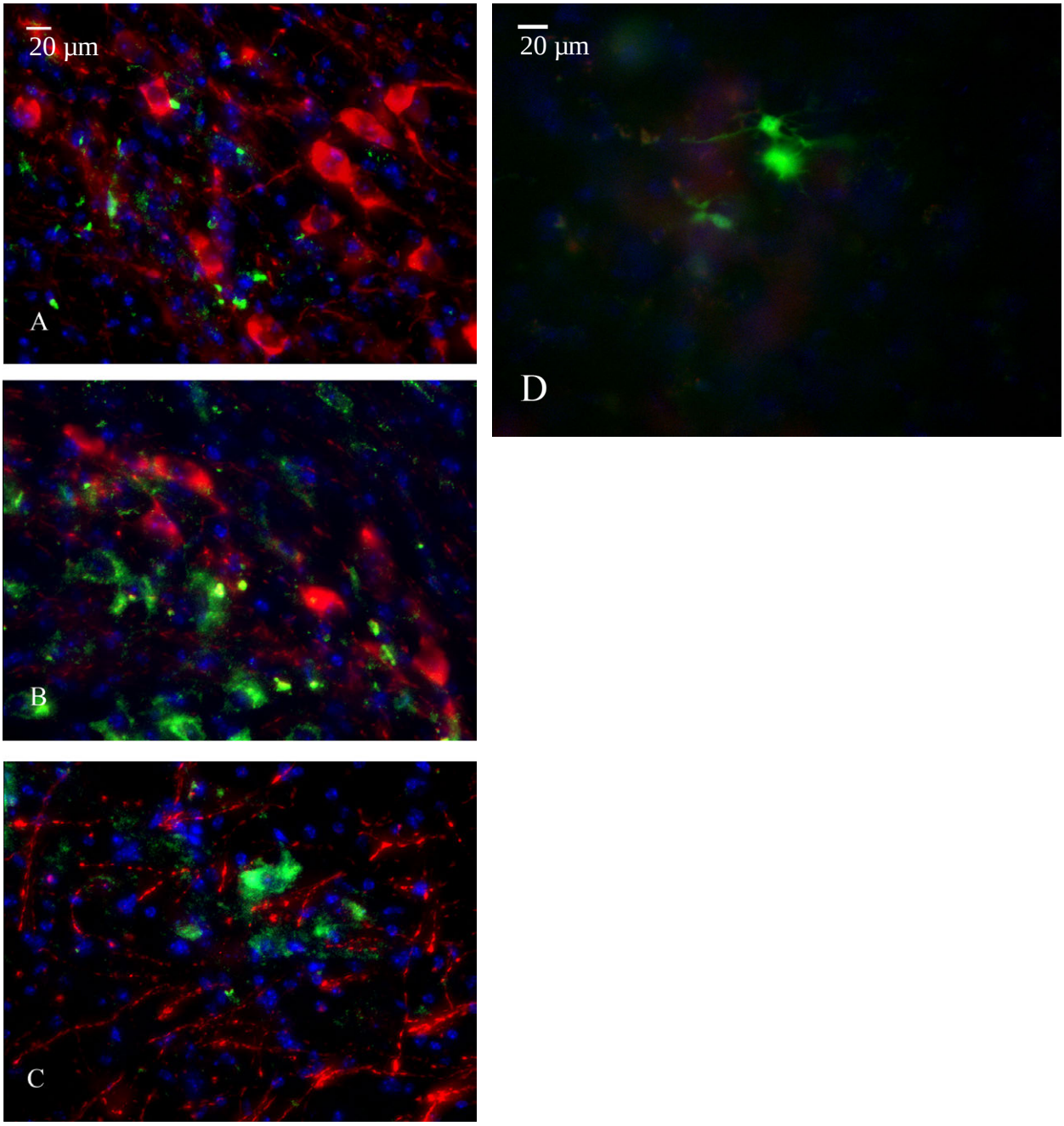

Figure 3.4. Fluorescent IHC stain for TH expression and intrinsic GFP expression. A-C: Loss of TH+ neurons (red) and NF-κB activation in A) saline B) MPTP-7 and C) MPTP-14 treated mice. D) NF-κB expression in a glial cell.

and the antibody-antigen complex formed in IHC. The most frequent problem that we encounter is that cells expressing both GFP and the protein of interest will appear to only express one or the other. Although there are alternative techniques that we can use in addition to IHC to identify and quantify protein expression, this limitation makes it difficult to obtain representative images of antigen co-localization with NF-κB.

The reason behind this conundrum is complex, however, we believe that there may be a few explanations. First, the transgenic mouse that we use was originally developed to study NF-κB expression in the liver, and therefore genetic penetrance of the reporter construct may vary in the brain (Magness et al., 2004). Thus, low GFP signals may be covered up by the signal of the fluorescent IHC stain. This would explain the difficulty in obtaining images in which GFP is co-localized with glial markers. Secondly, our method of fixing the tissue may play a role. It is well-established that the process of paraffin fixation destroys GFP expression in transgenic GFP-expressing mice and is therefore not an option for our studies (Jiang et al., 2005). GFP-targeting antibodies can be used to address this problem and have been used previously in our lab. The use of GFP antibodies are particularly advantageous if the GFP fluorescent signal is weak, as indirect IHC allows for amplification of this signal. However, there are limitations to that solution as well. Co-staining in fluorescent IHC becomes increasingly complicated as additional antibodies are used. Including another antibody against GFP adds another layer of complexity to the IHC protocol and this method will need to be further optimized.

The technique that we use to fix our tissue is cryosectioning, which is the preferred method of fixation for IHC as it is the best at preserving antigenity (Xiao Chen, 2010). However, it is possible that an unknown mechanism interferes with GFP and fluorescent IHC co-

localization in cryosectioned tissues. TH co-localization with GFP is particularly difficult in this model because the heat from antigen retrieval denatures intrinsic GFP and therefore it must be omitted when staining for co-localization of the two proteins. Since antigen retrieval drastically improves TH antigen availability, the number of antibody-antigen complexes are greatly reduced without this process, resulting in a lower fluorescent signal.

Discussion

This study explored the uses of fluorescent IHC staining of tissue from a transgenic *cis*-NF-κB^{eGFP}-containing reporter mouse to investigate the molecular mechanisms of NF-κB-mediated neuroinflammation in Parkinson's Disease. When used in conjunction with other analytical techniques, fluorescent IHC can provide both qualitative and quantitative information on the effects of NF-κB activity in PD pathology. In this study, we showed that IHC can be used to visualize neuronal loss and glial activation associated with sub-acute MPTP intoxication. Further optimization of this protocol is needed, particularly regarding co-localization of intrinsic GFP expression with other proteins. Our lab is experimenting with the use of alternative reagents and antigen retrieval methods to address the current limitations of this model. However, the protocol has the potential to provide valuable insight into the mechanisms of NF-κB in PD neurodegeneration, which may result in the development of therapeutics aimed to alleviate progression of the disease.

Chapter 4. Neuroinflammation in Drug-Induced Seizures

Introduction to Drug-induced Seizures

Drug-induced seizures are serious adverse drug reactions (ADRs) that can result from a number of pharmacological agents. Although the majority of reported drug-induced seizures result from compounds targeting the CNS, seizures are listed as an ADR in a wide range of drugs targeting many different systems (Easter et al., 2009). The current method used to assess the seizure-liability of a drug is purely observational and takes place in the late stages of pre-clinical trials. This method of assessment of seizure-liability categorizes compounds as either "convulsant," meaning that it elicits an observable change in phenotype, or as "proconvulsant," which cause an increase in the changes or intensity of a seizure (Easter et al., 2009). Many convulsant compounds are proconvulsant at lower doses. It is difficult to detect proconvulsant activity by behavioral phenotype, as it requires particular precursor events that may not be present in all test subjects and may not manifest as an observational change in behavior. Potentially proconvulsant compounds may create cellular changes that predispose the brain to excitotoxicity in latter events and are undetected during pre-clinical trials. The current method has low-sensitivity, is highly labor-intensive, and cost-ineffective. However, the molecular mechanisms involved in drug-induced seizures remain largely unknown, which currently makes it difficult to assess seizure-liability prior to the onset of convulsions. A deeper understanding of the cellular and molecular signaling involved in drug-induced seizures could not only provide new therapeutic targets, but could also transform the safety assessment protocol for new pharmaceutical agents.

NF-κB in Drug-Induced Seizures

Transcription factor NF-κB regulates many physiological processes in the CNS and there is substantial evidence supporting NF-κB activation in seizurogenesis. *In vitro* stimulation of hippocampal slices with kainic acid causes nuclear translocation of NF-κB and several studies have shown increased NF-κB following seizure-induction *in vivo* (Lubin et al., 2005; Prasad et al., 1994). Additionally, inhibition of NF-κB has been shown to increase susceptibility to seizure-induction, suggesting that NF-κB activation may be neuroprotective (Lubin et al., 2007). The neuroprotective properties of NF-κB activation suggest that it may be a useful indicator of stress response early on in seizurogenesis. Further understanding of the mechanisms involved in this response could make NF-κB activation a useful tool in detecting seizure-liable drugs early on.

Sub-Seizurogenic Dosing Regimen

In order to investigate the mechanisms of NF-κB expression with sub-seizurogenic exposure to kainic acid, *cis*-NF-κB^{eGFP}-containing transgenic mice were randomly separated into dosing groups of saline, 2.5 mg/kg kainic acid (2.5 KA), and 5 mg/kg kainic acid (5 KA). The sub-seizurogenic doses were tested prior to dosing and no behavioral signs of convulsions were observed. The mice were then further separated into subgroups of 1 dose (1x), 2 doses (2x), and 3 doses (3x), with 3 animals in each dose group. On the first day, the respective doses were administered intraperitoneally and again, no signs of convulsions were observed. Mice in the single dose group were terminated after the first dose, while mice receiving 2 and 3 doses were administered the second dose the following day. Similarly, mice in the 2 dose group were

terminated after the second dose and mice receiving 3 doses were given the last dose on the 3rd consecutive day and terminated afterwards.The mice were given multiple doses in this study to investigate changes in NF-κB activation in repeated, low-dose regimens. Number of doses did have behavioral effects on the mice while dosing, nor did it affect NF-κB expression within dose groups. Fluorescent microscopy of the hippocampus showed a clear, dose-dependent increase in NF-κB expression (Fig. 4.1), particularly in the CA1 and CA3 regions (Fig. 4.3 - Fig. 4.5). Although these images were taken on unstained tissue and therefore did not require any IHC staining, images of the reporter from unstained tissue allowed us to examine NF-κB activation following exposure to these low doses of kainic acid. Alternatively, IHC staining using a GFP antibody could be used to similarly show NF-κB activation in the hippocampus. However, the

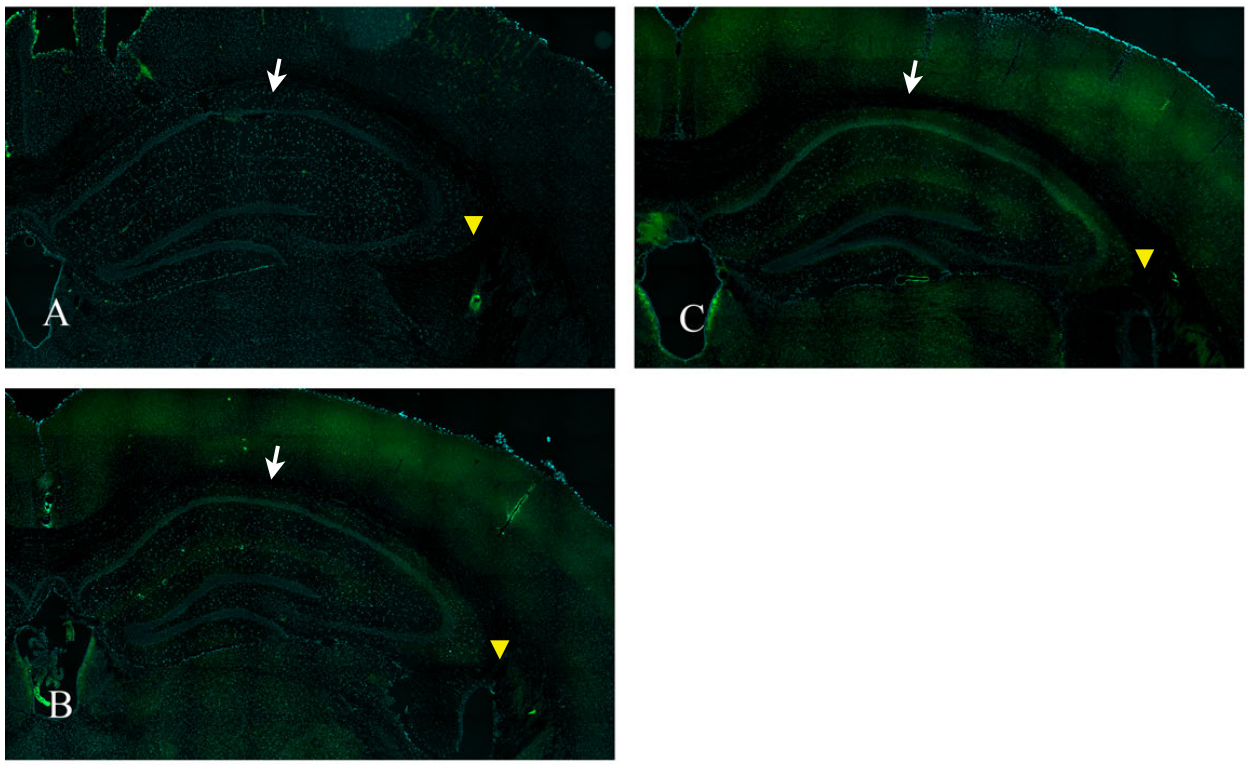

Figure 4.1. Dose-dependent increase in NF-κB activation in the hippocampus, particularly the CA1 (white arrows) and CA3 (yellow arrowheads) regions, after sub-seizurogenic exposure to kainic acid. Using the *cis*-NF-κB^{eGFP}- containing reporter mouse, NF-κB activation was assessed by examining GFP fluorescence after 3 doses of A) saline B) 2.5 mg/kg KA and C) 5 mg/kg KA.

intrinsic GFP expression, without the use of any external antibodies, was sufficient to show the dose-dependent increase in NF-κB activity. The tissue was prepared for microscopic examination by repeated washes with TBS before being mounted directly to the slides using DAPI-containing mounting medium (VectaShield).

NF-κB Activation and Neuronal Loss in Kainic Acid Toxicity

To assess NF-κB activation as it relates to kainic acid-induced neuronal loss, we used fluorescent immunohistochemical staining to determine morphologic integrity of the neurons. We utilized a rabbit polyclonal antibody against MAP2 (AbCam, 1:500) to visualize the hippocampal neurons in tissues following the sub-seizurogenic dosing regimen. MAP2 staining is more effective in the hippocampus than in the nigra, therefore requiring a lower concentration of anti-MAP2 antibody than that of the SNpc. In an attempt to preserve GFP fluorescence, we did not perform antigen retrieval during this process.

Consistent with the images of intrinsic GFP expression (Fig. 4.1), kainic acid activated NF-κB in a dose-dependent manner in both the CA1 and CA3, the areas most affects in kainic acid toxicity (Figure 4.3-4.5) (Wang et al., 2005). Although quantification of this data must be completed, the IHC images indicate that NF-kB expression increased substantially following three 2.5 mg/kg doses of kainic acid without affecting neuronal integrity. However, neuronal loss seemed to occur following three 5 mg/kg doses of kainic acid. This provides supporting evidence that NF-κB activation in sub-seizurogenic doses precedes neuronal death and may therefore be an early indicator of stress in the brain.

Despite our efforts, GFP/MAP2 co-localization was still absent in most of the images, although there is noticeable intrinsic GFP expression, as well as neuronal MAP2 staining present. In order to overcome this limitation and determine whether the NF-kB activation was neuronal, we used an antibody targeting GFP to co-stain for MAP2 and GFP (Fig. 4.2). This protocol yielded similar results to that of the MAP2/intrinsic GFP IHC stains, but showed co-localization of GFP and MAP2. Following 3 doses of 5 mg/kg kainic acid, GFP expression significantly increased in the CA3 region, while there was a slight disturbance in neuronal integrity. However, both the morphology of the staining and co-localization of the proteins indicate that the GFP expression is neuronal.

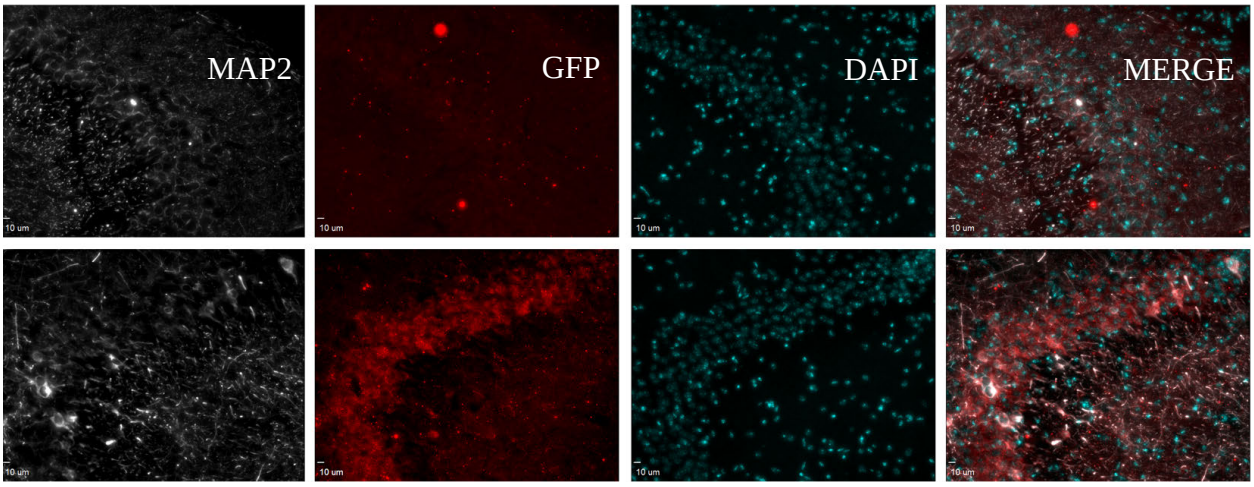

Figure 4.2. Co-localization of GFP and MAP2 using an antibody targeted to GFP. 40x images of immunohistochemical stains depicting MAP2 and GFP expression in the CA3 region of the hippocampus. MAP2 (white) and GFP (red) expression in mice treated with 3 doses of Saline (TOP ROW) and 5 mg/kg kainic acid (BOTTOM ROW).

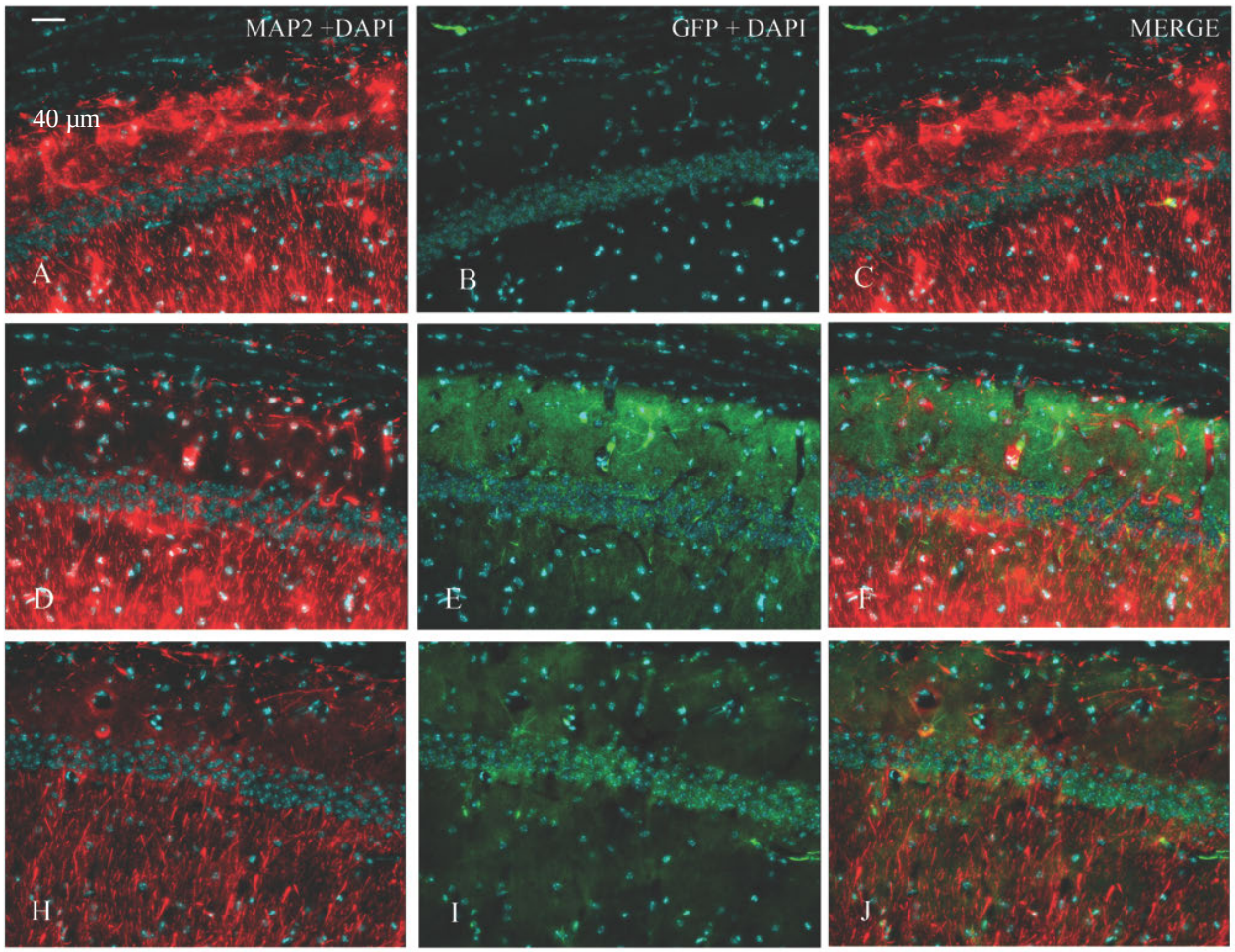

Figure 4.3. NF-κB activation in neurons following sub-seizurogenic doses of kainic acid. 20x images of immunohistochemical stains depicting MAP2 and intrinsic GFP expression in the CA1 region of the hippocampus. MAP2 (red) and GFP (green) expression in mice treated with 3 doses of A-C) Saline. D-F) 2.5 mg/kg KA H-J) 5 mg/kg KA.

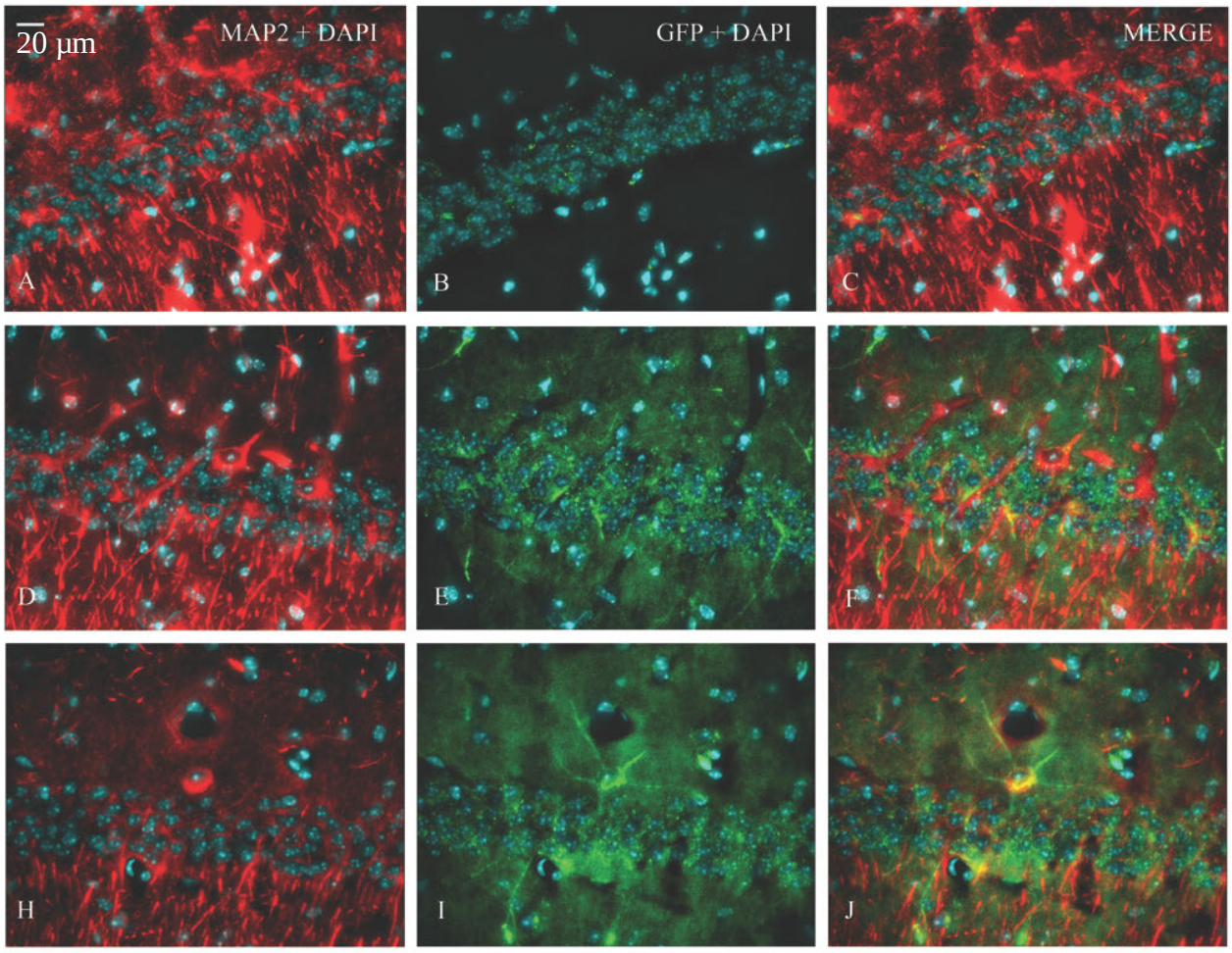

Figure 4.4. 40x images of fluorescent immunohistochemical stains showing MAP2 and intrinsic GFP expression in the CA1 region of the hippocampus. MAP2 (red) and GFP (green) expression in mice treated with 3 doses of A-C) Saline. D-F) 2.5 mg/kg KA H-J) 5 mg/kg KA.

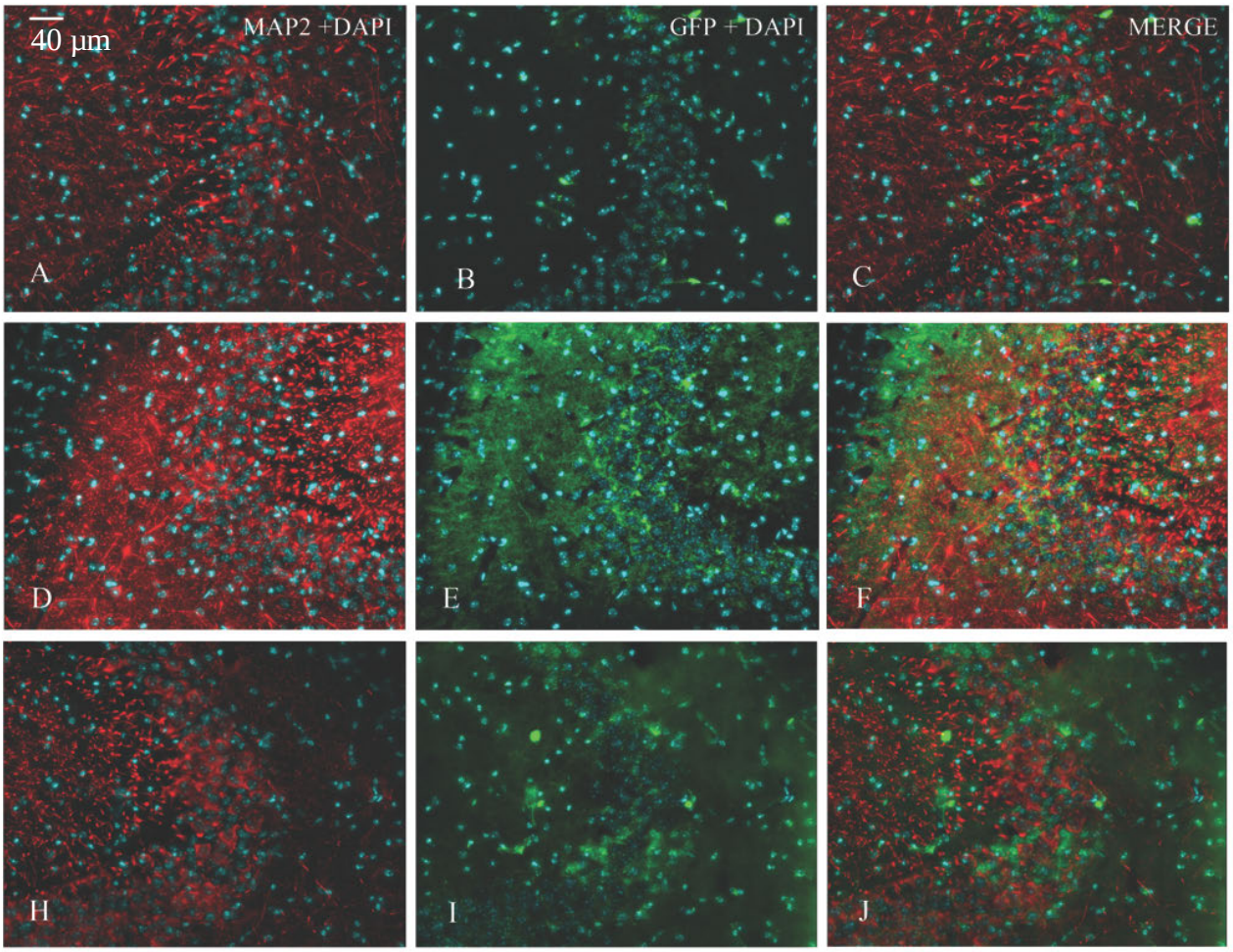

Figure 4.5. 20x images of immunofluorescent stains showing MAP2 and intrinsic GFP expression in the CA3 region of the hippocampus. MAP2 (red) and GFP (green) expression in mice treated with 3 doses of A-C) Saline. D-F) 2.5 mg/kg KA H-J) 5 mg/kg KA.

Fluorescent IHC was also used to assess NF-κB activation as an early stress response by examining hippocampal glial activity following treatment with proconvulsant doses of KA. In the event of neuronal injury, glial cells are recruited to the site of injury and release molecular signals which propagate the neuroinflammatory cascade. Research has shown that kainic acid-induced neuronal death is accompanied by a profound glial response (Wang et al., 2004).

In this study, we again used IHC to detect the presence of activated glia in the hippocampus (Figure 4.6 and Figure 4.7). We co-stained for the presence of reactive microglia and astrocytes by using a rabbit anti-IBA-1 antibody (Cell Signaling, 1:500) and a mouse anti-GFAP (Cell Signaling, 1:500) antibody, both proteins that are upregulated upon activation.

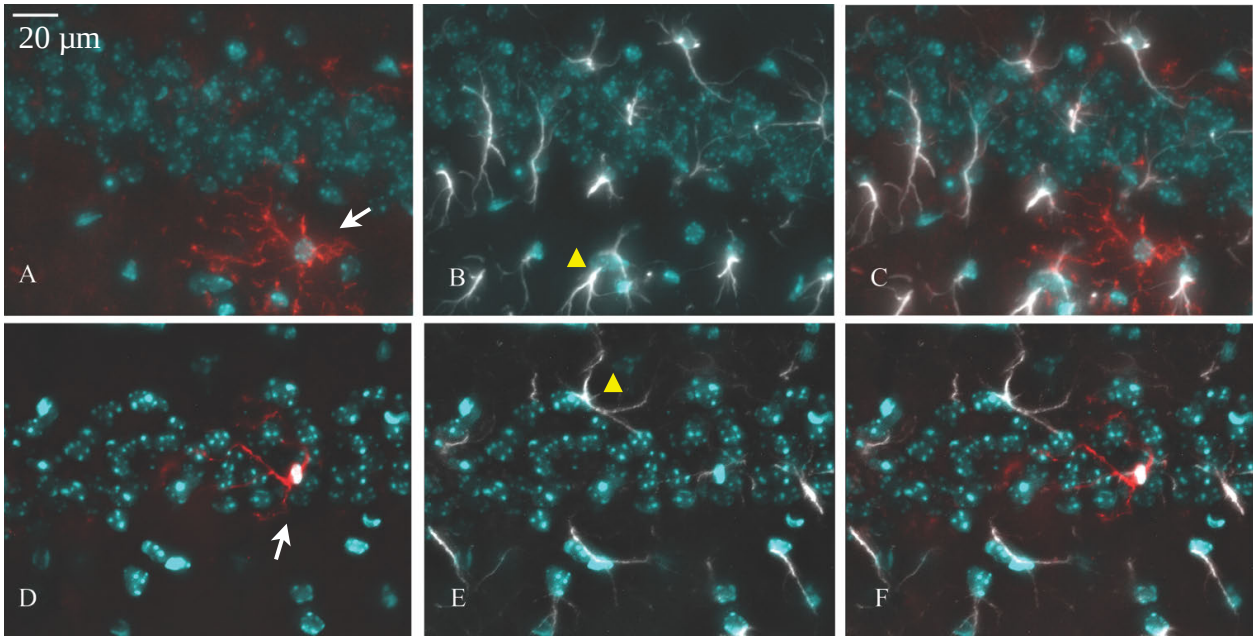

Figure 4.6. 40x images of immunofluorescent stains showing gliosis in the CA1 region of the hippocampus. Microglia (white arrows) visualized by use of IBA-1 (red) and astrocytes (yellow arrowheads) by used of GFAP (white) expression in mice treated with 3 doses of A-C: Saline A) IBA-1 B) GFAP C) IBA-1 + GFAP and D-E: 5 mg/kg KA D) IBA-1 E) GFAP F) IBA-1 + GFAP.

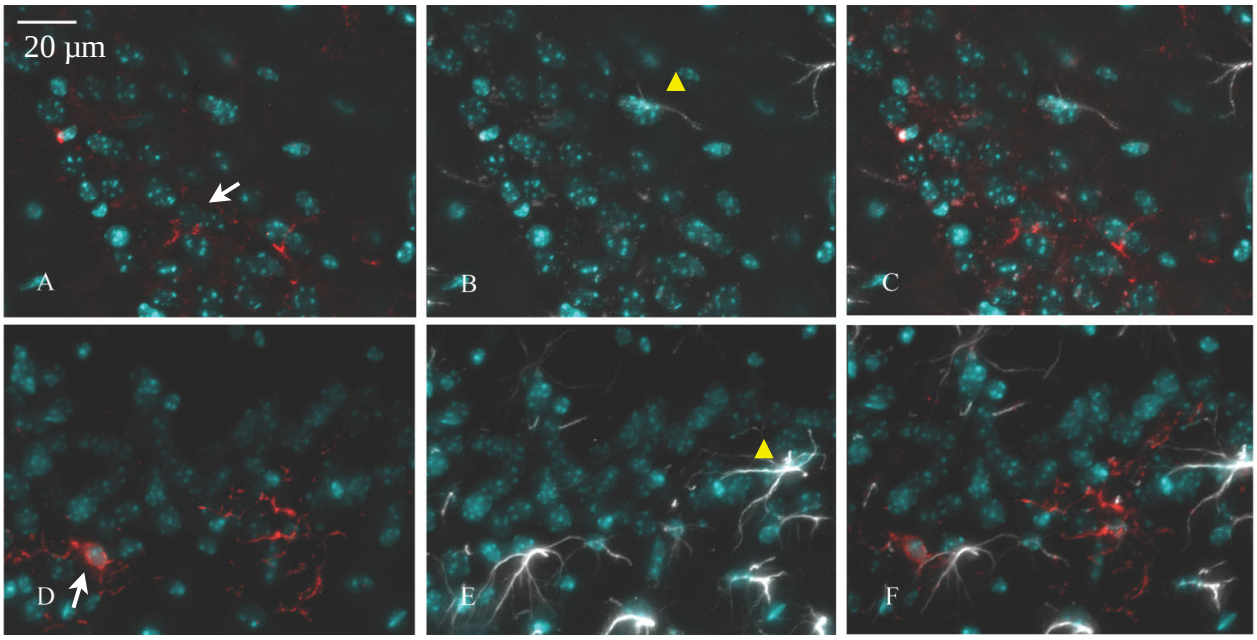

Figure 4.7. 40x images of immunofluorescent stains showing gliosis in the CA3 region of the hippocampus. Microglia (white arrows) visualized by use of IBA-1 (red) and astrocytes (yellow arrowheads) by used of GFAP (white) expression in mice treated with 3 doses of A-C: Saline A) IBA-1 B) GFAP C) IBA-1 + GFAP and D-E: 5 mg/kg KA D) IBA-1 E) GFAP F) IBA-1 + GFAP.

Staining for gliosis in the hippocampus follows an identical protocol to that of the SNpc, and there does not seem to be much of a difference in the efficacy of the stains between brain regions. Co-staining for gliosis in the hippocampus yielded no observable difference in glial activity between dose groups, indicating a lack of inflammatory signaling. The lack of gliosis is another indicator that the activation of NF-κB was not caused neuronal death from treatment with either doses of kainic acid. Since glial cells are the first-responders in the event of CNS-trauma, the lack of activation suggests that early NF-κB activation is not proinflammatory (Aloisi, 2001).

Stress Response Signaling in Early NF-κB Activation

To further assess the characteristics of early neuronal activation of inducible-NF-κB, we performed IHC staining with an antibody targeted against Nurr1. Nurr1 is an orphan receptor expressed in adult neurons which acts as a transcription factor by forming complexes with the retinoid x receptor (RXR) and binding to target genes (Saijo et al., 2009). Nurr1 seems to function in a variety of physiological responses, but emerging evidence suggests that the transcription factor may be important in neuronal survival and resistance to oxidative stress. Microarray analysis confirmed that Nurr1 over-expression resulted in the upregulation of several genes involved in cell survival and stress response (Sousa et al., 2007). Another study showed that Nurr1 regulates NF-κB-induced glial inflammatory responses by recruiting repressor complexes upon signaling (Saijo et al., 2009). In order to further evaluate the role of NF-κB activation in early stress response, we examined Nurr1 expression in the CA3 region of the hippocampus. Nurr1 is involved in a variety of physiological processes and is constitutively expressed in neurons (Sousa et al., 2007). We observed a dose-dependent increased in Nurr1 fluorescence following treatment with sub-seizurogenic doses of kainic acid (Figure 4.8). Studies have shown that Nurr1 activation may be under NF-κB regulation, and we observed an increase in Nurr1 that parallels that of NF-κB expression seen in the CA3 (Fig. 4.5) (McEvoy et al., 2002).

While fluorescent IHC is extremely useful in generating images and confirming the presence of proteins, additional methods of quantification are typically needed to determine statistical significance. Figure 4.8 B depicts a graphical representation of the increase in Nurr1 fluorescence in terms of MFI (Mean Fluorescent Intensity).

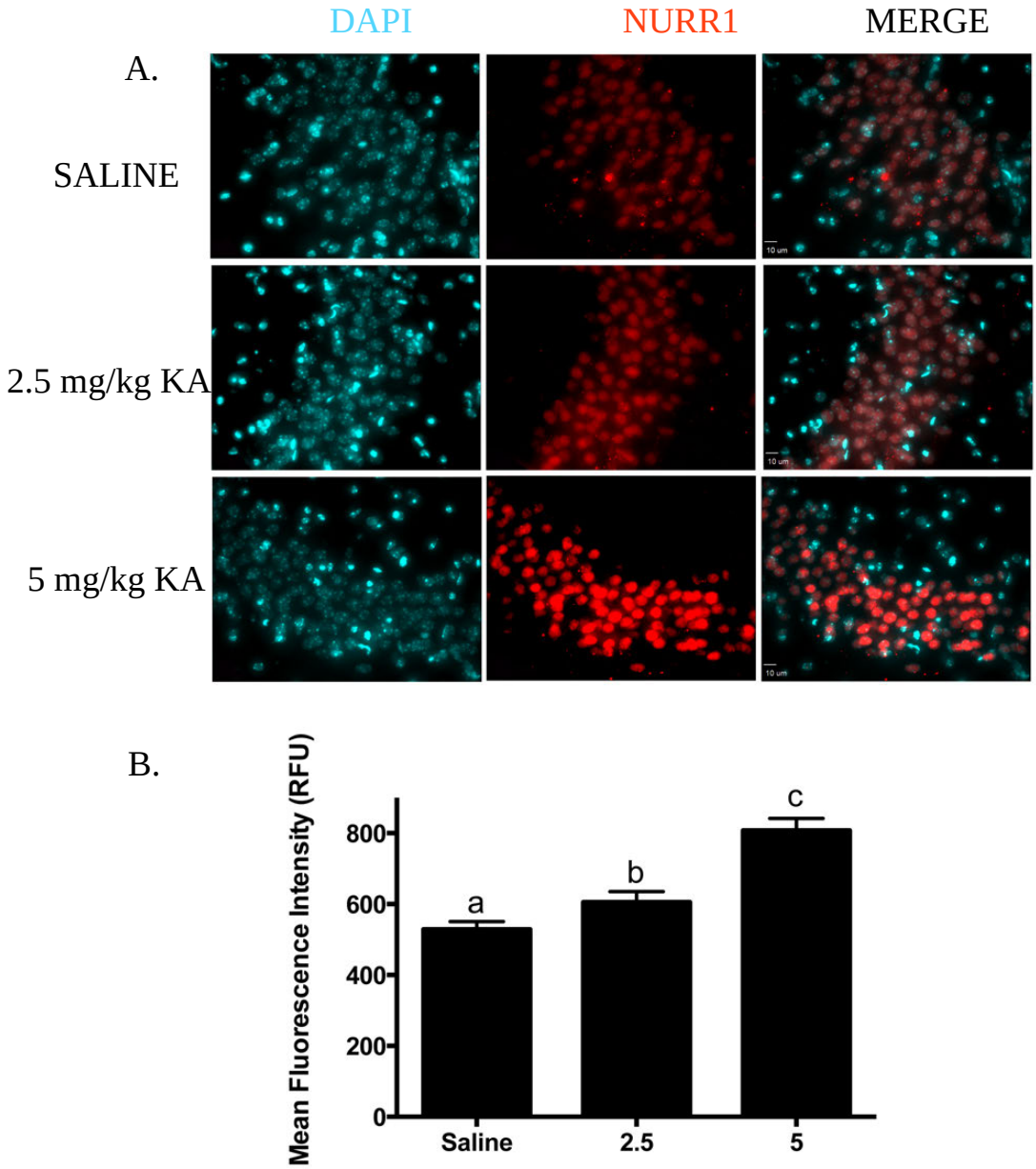

Figure 4.8. Sub-seizurogenic exposure to Kainic Acid results in a dose-dependent upregulation of Nurr1. A. Nurr1 expression was assessed by examining immunofluorescence in the CA3 with treatment with 3 doses of saline (top), 2.5 mg/kg KA (middle), and 5 mg/kg KA (bottom). B. Quantification of Nurr1 expression in terms of Mean Fluorescent Intensity (MFI) of saline (left), 2.5 mg/kg KA (middle), and 5 mg/kg (right) treated animals.

Quantification and statistical analysis of fluorescent intensity was completed by stereological analysis of 40x images of randomly placed counting frames (100 x 100 μm)(Miller et al., 2011). Consonant with the results obtained by IHC, there was a statistically-significant, dose-dependent increase in Nurr1 expression in terms of Mean Fluorescent Intensity (MFI) upon treatment with low-levels of kainic acid. These results act in agreement with the lack of glial activation from exposure at these doses and support a regulatory role of NF-κB in early stress response, prior to excitotoxic damage.

Discussion

The current method of safety assessment for drug-induced seizure liability is strictly behavioral and often fails to identify proconvulsant phenotypes which may later result in convulsions under certain physiological conditions (Easter et al., 2009). Understanding the cellular signaling that is involved in drug-induced epilepsy and the role of NF-κB is important in developing new safety assessment methods. In this study, we used fluorescent IHC to assess the role of NF-κB activation following sub-seizurogenic doses with kainic acid, a prototypic neurotoxin-induced model of status epilepticus. Our model utilized a transgenic *cis*-NF-κB^{eGFP}-containing reporter mouse and fluorescent immunohistochemical staining to visualize NF-κB activation and related proteins *in vivo.* Using this model, we showed that sub-seizurogenic exposure to kainic acid activates inducible NF-κB in hippocampal neurons even before neuronal death occurs.

This model is effective because it uses the transgenic NF-κB^{eGFP}-containing reporter mouse, which allowed us to visualize NF-κB activation as it occurred *in vivo.* Fluorescent IHC

staining of tissue from this model unveiled important mechanisms behind kainic acid-induced activation of the NF-κB pathway. Use of cell-specific antibodies in fluorescent IHC enabled us to identify the cellular features of NF-κB activation following administration of sub-seizurogenic doses of kainic acid. A MAP2 antibody was used to assess changes in cellular morphology, while antibodies targeting GFAP and IBA-1 were used to determine glial responses at such exposure levels. Co-staining with anti-MAP2 and anti-GFP antibodies showed co-localization of the two proteins and identified the origin of NF-κB activation to be neuronal. Use of an antibody targeting the prototypic stress response factor, Nurr1, showed that NF-κB activation corresponded with an increase in Nurr1 expression, suggesting that NF-κB activation may serve a neuroprotective role. Furthermore, Nurr1 induction is known to occur early on in cellular stress response (Saijo et al., 2009). Activation of this pathway following intoxication with sub-seizurogenic doses of kainic acid and corresponding to increased NF-κB expression further supports the role of NF-κB in early excitotoxic phenotypes. Therefore, use of NF-κB activation as a marker of early stress response could help to identify convulsant and proconvulsant compounds in the rudimentary stages of drug-development.

Concluding Statement

The method of fluorescent immunohistochemical staining of tissue from the transgenic *cis*-NF-κB^{eGFP}-containing reporter mouse has been extraordinarily helpful in unveiling the molecular mechanisms of NF-κB in Parkinson's Disease and drug-induced seizures. Although IHC is a widely used laboratory technique, the protocol has not yet been fully developed for studying NF-κB activity in the brain. The success of IHC staining is multifactorial and therefore a number of factors must be addressed in order to optimize the protocol. Furthermore, much remains unknown about NF-κB involvement in brain pathologies, and this model has many advantages as a research technique in this particular field. The transgenic *cis*-NF-κB^{eGFP}-containing reporter mouse enables investigation of NF-κB activation *in vivo*. The methods used to process, fix, and store the tissue effectively preserves antigenicity for use in IHC. Thus, fluorescent IHC staining of this tissue allows for real-time assessment of cellular and molecular changes. Indirect fluorescent IHC staining utilizes an amplified, fluorescent signal to identify a protein which does not require the presence of exogenous substrate. The signal is visualized by exciting the fluorophore conjugated to the secondary antibody with the corresponding wavelength of light and therefore the specificity of the signal is highly controlled. Furthermore, the fluorescent intensity of the signal can be easily analyzed. When used along with the NF-κB reporter mouse, this type of staining also reduces complexity of the method by removing the need for anti-GFP antibodies in many stains. Another important advantage of this technique as a whole is that it can be used to qualitatively identify cell-specific NF-κB activation in experimental models of brain pathologies. Identification of this activation may be a useful tool in drug development.

The limitations of this model are also important to address. Generally, when used alone, fluorescent IHC staining cannot be used to quantitatively analyze expression of a protein. Unless additional quantitative methods are used, e.g. fluorescent intensity, stereological counts, the results are purely qualitative. In this particular model, the GFP protein is denatured by antigen retrieval, so co-localization of intrinsic GFP and antigens which require antigen retrieval can be difficult to show via IHC. It is important to utilize this model in conjunction with other molecular biology techniques for laboratory research.

However, as a qualitative measure of NF-B activation and glial activation in brain pathologies, IHC can be a useful tool. Understanding NF-κB activation as both a neuroinflammatory and neuroprotective pathway is important in neuroscience research, as well as drug-development. Further characterization of this method has the potential to generate valuable insight into the mechanisms behind NF-κB activation and their implications in neuropathologies.

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