Roles of Cyclooxygenase-2 in Microglial Activation and Dopaminergic Cell Death

Rattanavijit Vijitruth

University of Kentucky, peacophil@hotmail.com

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ABSTRACT OF DISSERTATION

Rattanavijit Vijitruth

The Graduate School
University of Kentucky
2006
A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy in the College of Medicine at the University of Kentucky

By

Rattanavijit Vijitruth

Lexington, Kentucky

Director: Dr. Guoying Bing, Associate Professor of Anatomy and Neurobiology

Lexington, Kentucky

2006

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Accumulating evidence suggests that inflammation plays an important role in the progression of Parkinson’s disease (PD). Among many inflammatory factors found in the PD brain, cyclooxygenase (COX), especially the inducible isoform, COX-2, is believed to be the critical enzyme in the inflammatory response. Induction of COX-2 is also found in an experimental model of PD produced by administration of 1-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). To investigate whether inhibition of COX-2 by valdecoxib or deficiency in COX-2 could prevent dopaminergic neuronal toxicity and locomotor activity impairment, we injected MPTP into valdecoxib-treated C57BL/6N mice and COX-2 deficient mice, respectively. Both automated total distance and vertical activity measurements of the open-field test were significantly reduced in the vehicle-treated mice at two weeks post-MPTP injection. In contrast, valdecoxib treatment significantly attenuated these deficits. Similarly, COX-2 deficiency attenuated MPTP-induced loss of coordination on a rotarod assay. Valdecoxib or deficiency of COX-2 reduced microglial activation while preventing loss of tyrosine hydroxylase (TH)-positive neurons in the substantia nigra pars compacta (SNpc). The total number of activated microglia in the SNpc had a strong positive correlation with the level of COX-2 and dopaminergic neurodegeneration. The results of this study indicate that reducing the activity of COX-2
can mitigate the progressive loss of dopaminergic neurons as well as the motor deficits caused by MPTP neurotoxicity, possibly by suppressing the activation of microglia in the SNpc.

KEYWORDS: COX-2, microglia, neuroinflammation, Parkinson’s disease, MPTP

Rattanavijit Vijitruth

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By

Rattanavijit Vijitruth

Guoying Bing
Director of Dissertation

Dr. Gouglas Gould
Director of Graduate Studies

August 11, 2006
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DISSERTATION

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Dedicated to my parents

Mr. Wira Vijitruth
Mrs. Yosaluck Vijitruth
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Chapter One

Introduction

Roles of cyclooxygenase-2 in microglial activation and dopaminergic cell death

Parkinson’s disease

A major hallmark of Parkinson’s disease (PD) is the loss of dopaminergic neurons in the substantia nigra par compacta (SNpc) of the midbrain (Jellinger, 2001; Siderowf and Stern, 2003). It was first described by Dr. James Parkinson in 1812 (Parkinson, 2002). The cardinal symptoms of the disease are resting tremor, slowness in movement (hypokinesia/akinesia), rigidity, and postural instability (Collier and Sortwell, 1999; DeLong, 2000; Blum et al., 2001). The diagnosis of PD is still generally based on clinical symptoms, and the diagnosis is confirmed with neuropathological examination post-mortem. One important pathological feature of PD patients is the presence of neurofilament inclusions (Lewy bodies) in all affected brain stem regions (Rascol et al., 2003). There are over one and a half million North Americans diagnosed with Parkinson’s disease. About two to three percent of the population older than sixty-five years old has PD (Lang and Lozano, 1998; Mayeux, 2003; Siderowf and Stern, 2003). The most prevalent treatment is replacing the dopamine production loss of the SNpc due to dopaminergic neuronal cell death, by supplying the precursor of the dopamine, L-dopa. Unfortunately, the treatment is only effective for a certain amount of time. Eventually, the patients develop unusual movement (dyskinesia) as one of the major side effects of using L-dopa. Psychotic episodes also occur in patients taking L-dopa. In addition, L-dopa treatment does not stop or slow the progression of the
As a result, there is a need to find a new or an additional treatment for Parkinson’s disease. Nevertheless, finding the cure remains a difficult task because the etiology of PD is still unknown. There have been advances in research in this field and several factors, both genetics and environment, have been shown to induce PD or increase the risk of developing PD (Lindsay et al., 1993; Blum et al., 2001; Steece-Collier et al., 2002; Siderowf and Stern, 2003). However, the genetics factors are only 20% of the cause of PD. As opposed to a small amount of patients with familial PD, most of the patients have idiopathic PD.

**Inflammation in Parkinson’s disease**

Inflammation is thought to play an important role in etiology of several neurodegenerative diseases including PD, Alzheimer’s disease (AD), and amyotrophic lateral sclerosis (ALS) (Bojinov, 1971; McGeer et al., 1988b; Furukawa et al., 1992). First of all there are increased levels of inflammatory cytokines, including TNF-α, interleukin-6 (IL-6), and IL-1β in the brains of PD patients (Boka et al., 1994; Mogi et al., 1994b; Mogi et al., 1994a). In addition, microglia have been found to be highly concentrated in the SNpc (Lawson et al., 1990; Kim et al., 2000). Microglia are resident macrophages of the brain sharing similar properties (Sedgwick et al., 1991; Perry et al., 1995) and constituting 10% of brain cells (Lawson et al., 1990). Once activated, these microglia transform from striated bodies into big round, amoeboid, bodies with short thick processes. Activated microglia share many surface molecules with macrophages (Flaris et al., 1993). In PD, activated microglia in the SNpc have been found expressing the major histocompatibility (MHC) II complex HLA-DR (McGeer et al., 1988b), the macrophage marker EMB11 (Banati et al., 1998), tumor necrosis factor-α (TNF-α) (Boka et al., 1994; Hunot et al., 1999), interferon-γ (IFN-γ), the low affinity immunoglobin (Ig) E receptor CD23 (Hunot et al., 1999), inducible nitric oxide synthase (iNOS) (Hunot et al., 1996; Knott et al., 2000), cyclooxygenase-2 (Knott et al., 2000), complement 3 receptor (Banati et al., 1998; Mirza et al., 2000),...
and increased ferritin (Mirza et al., 2000). The widely used indicators for microglial activation in research encompass the multifunctional nature of activated microglia, which include the up-regulation of cell surface markers such as the macrophage antigen complex-1 (Mac-1), phagocytosis, and the production of cytotoxic molecules, including reactive oxygen species (ROS), nitric oxide (NO), and a variety of proinflammatory cytokines such as interleukin-1β (IL-1β) (Banati et al., 1993; Gehrmann et al., 1995; Hopkins and Rothwell, 1995). These activated microglia are able to phagocytose other cells, physically help in immunological process, or release substances that can directly or indirectly promote survival or death of the adjacent, surrounding, nearby, or distant cells. They constitutively express complement receptors and transform to activated immunocompetent antigen-presenting cells in response to various stimuli (Cross and Woodroofe, 2001). These activated microglia cluster around dopaminergic neurons in phagocytosis (McGeer et al., 1988a; McGeer et al., 1988b; Banati et al., 1998; Langston et al., 1999; Knott et al., 2000; Vila et al., 2001). Vulnerable dopaminergic neurons display nitrosylation of proteins (Giasson et al., 2002), receptors for TNF-α (Boka et al., 1994), and downstream intracellular effector molecules like the nuclear transcription factor NFκB (Hunot et al., 1997). These data indicate an aggressive, selective and active inflammatory process of cell death in PD.

**MPTP model of Parkinson’s disease**

Neurotoxin 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) selectively injures the SNpc (Langston et al., 1999). Its was first clinically discovered in drug addicts who unknowingly injected it over a week as a contaminant of meperidine (pethidine) and developed PD-like symptoms (Langston et al., 1983). Postmortem examination of MPTP-intoxicated humans years after exposure showed dopaminergic cell loss, without Lewy body formation, and marked microglial reaction in the SNpc (Langston et al., 1999). The presence of this chronic inflammatory reaction years after the original
toxic exposure supports the idea that environmental insult to the brain can promote a self-perpetuating process of neurodegeneration mediated by localized inflammatory processes.

MPTP readily enters the brain and is metabolized by astrocytes to the active metabolite 1-methyl-4-phenylpyridinium (MPP+). MPP+ enters cells via the dopamine transporter and inhibits complex I of the mitochondrial electron transport chain (Du et al., 2001), producing SNpc cell death, depletion of striatal dopamine and activation of microglia in both regions (Liberatore et al., 1999). Microglia not only activate, as evidenced by altered morphology from ramified to macrophage-like, but also increase their number, suggesting recruitment and/or proliferation. Expression of cell surface markers and production of toxic cytokines, NO, and ROS seem to occur prior to maximal neuronal damage with the microglia contributing to the demise of SNpc neurons (Liberatore et al., 1999).

The MPTP model of PD is recognized as the best experimental of sporadic PD and replicates most of the biochemical and pathological features seen in the clinical conditions (Jackson-Lewis et al., 1995). MPTP is an effective dopaminergic neurotoxin in animals and humans. It significantly decreases the levels of dopamine and its metabolites, the number of dopaminergic terminals in the striatum (Burns et al., 1983; Zuddas et al., 1992), and the amount of tyrosine hydroxylase (TH) in the SNpc (Ricaurte et al., 1987). MPTP-treated C57BL/6 mice are prevalently used as a model for elucidating the neurotoxicity of MPTP (Ricaurte et al., 1987)

**Cyclooxygenase-mediated Inflammation in Parkinson’s disease**

Both isoforms of cyclooxygenase, namely COX-1 and COX-2, are the rate limiting enzymes in arachidonic acid (AA)-derived prostaglandin production (Samuelsson, 1991; Wenzel, 1997) due to their cyclooxygenase reaction in producing prostaglandin H2 (PGH₂), which is the first step of the AA
pathway (Langenbach et al., 1995; Morham et al., 1995). While COX-1 is constitutively expressed in most tissues, COX-2 is induced during pathophysiological responses by inflammatory stimuli such as bacterial endotoxin, IL-1, and various growth factors (Kujubu et al., 1991; Needleman and Isakson, 1997). During the process of prostaglandin production, however, reactive oxygen species are generated as by-products (Nikolic and van Breemen, 2001), which in addition to endotoxin, mitogens, cytokines and certain inflammatory agents, can activate microglia (Nakamura, 2002). Microglia can also be activated by oxidative stress (Czlonkowska et al., 2002). Microglia activation, in turn, cause release of free radicals and inflammatory cytokines, including IL-1β, IL-6 and TNF-α (Liu and Hong, 2003). In normal circumstances, such response by microglia is protective in fighting off pathogens like bacteria, for example. In contrast, under pathological conditions induced by certain insults, including oxidative stress, excitotoxicity from ion imbalance or trauma, microglia can be over-stimulated and produce excess cytotoxic agents that damage neurons, which stimulate overexpression of neuronal and microglial COX-2 (Liu and Hong, 2003), which has been implicated as a major responsible COX during inflammation (Vane and Botting, 1995, 1998a, b). Thus, a cycle is created, and such co-propagation of COX-2 and microglia may continue to damage neurons and the surrounding cellular environment until, in most cases, a symptom of a neurodegenerative disease is unveiled, such as dementia in Alzheimer’s disease (AD) and bradykinesia in PD. Whatever the etiology of PD or other neurodegenerative diseases, an interruption to the positive feed-back loop between COX-2 and microglia may prevent the secondary injury due to excess inflammation and oxidative reaction.

Regulation of cyclooxygenase-2 gene expression

Potential COX-2 transcription regulatory sequences are a TATA box, a C/EBP motif, two AP-2 sites, three SP1 sites, two NFκB sites, a CRE motif, and an Ets-1 site (Ward et al., 1996; Lukiw et al., 1998; Tang et al., 2001). Inducible transcription factor NFκB can be activated by many stimuli including
ROS, cytokines, JNK, and p38 (Schulze-Osthoff et al., 1997) and regulates expression of genes implicated in the cellular response to pathogen stimulation or injury (Ward et al., 1996), including COX-2 (Bauer et al., 1997; Newton et al., 1997).

JNK and p38 MAPK influence activation and transactivation of NFκB in the cytosol and in the nucleus, respectively (Schulze-Osthoff et al., 1997). JNK has been reported to be involved in the apoptotic death of dopaminergic neurons (Luo et al., 1998), and its inhibition attenuates MPTP-induced death of SNpc dopaminergic neurons (Xia et al., 2001). Since 1996, p38 MAPK has been recognized and suggested to play a role in pro-inflammatory signaling system in mammals and in inflammation-mediated neurotoxicity, respectively (Kyriakis and Avruch, 1996; Han et al., 1997). It is activated in the brains of Alzheimer’s disease patients (Hensley et al., 1999) and following ischemia (Walton et al., 1998), and it has been implicated in LPS-induced death of mesencephalic dopaminergic neurons (Jeohn et al., 2002). In addition, p38MAPK has been linked to the expression of COX-2 (Guan et al., 1998; Newton et al., 2000; Surh et al., 2001) as well as iNOS (Chen et al., 1999), both of which have been implicated in promoting tissue damage in progressive and chronic inflammation.

A major product of COX metabolism, prostaglandin D2, is converted into prostaglandins of the J series, including 15-deoxy-Δ12,14-prostaglandin GJ2 (PGJ2). PGJ2 is a natural ligand of peroxisome proliferator-activated receptor-γ (PPARγ) and thus participates in the feedback mechanism (Shibata et al., 2002) since PPARγ can inhibit NFκB (Delerive et al., 2000; Delerive et al., 2001).

**Cyclooxygenase-2-deficient mice**

COX-1 and COX-2 mice were established by Dr. Langenbach at the National Institute of Environmental Health Science, Research Triangle Park, NC (Langenbach et al., 1995; Morham et al.,
COX-2-deficient mice have been used in only a number of investigations involving dopaminergic neuroprotection (Feng et al., 2002; Feng et al., 2003; Teismann et al., 2003b). Besides these studies, there have been reports on a significant reduction in the brain injury produced by middle cerebral artery occlusion (Iadecola et al., 2001) and transient forebrain ischemia in COX-2-deficient mice (Sasaki et al., 2004). This protection may be due to attenuation of excitatory glutamate neurotoxicity, a critical factor in the initiation of ischemic brain injury and to reduction of the deleterious effects of post-ischemic inflammation, a process contributing to the secondary progression of the damage (Nagayama et al., 1999; Iadecola et al., 2001; Sasaki et al., 2004). Since C57BL/6 mice are sensitive to MPTP (Zuddas et al., 1992), COX-2 deficient mice from C57BL/6 genetic background will be a useful model in which to study the role of inflammation in the pathogenesis of PD.

**Cyclooxygenase-2 inhibitor**

Conventional nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit both isoforms of COX (Laneuville et al., 1994; O'Neill et al., 1994; Gierse et al., 1995; Smith and Dewitt, 1996; Berg et al., 1997; Miralpeix et al., 1997; Wong et al., 1997). It was hypothesized that selective COX-2 inhibitors would have the advantages of conventional NSAIDs but would not interfere with GI protection or homeostasis (DeWitt, 1999; Prasit et al., 1999). Celecoxib (Celebrex®; Pharmacia, Peapack, NJ; Pfizer, New York, NY) was approved by the Food and Drug Administration in December 1998 (Chandrasekharan et al., 2002), rofecoxib (Vioxx®; Merck, Whitehouse Station, NJ) in May 1999, and valdecoxib (Bextra®; Pharmacia) in November 2001 (Ormrod et al., 2002). Selectivity of COX-2 inhibitors may be analyzed in several different *in vitro* assays, but the most relevant is by the drug-enzyme interaction in whole-blood assays (Patrignani et al., 1994; Warner et al., 1999). Measuring thromboxane B2 generated in clotting whole blood and prostaglandin E2 (PGE2) production after incubation of whole blood with lipopolysaccharide are validated measurements of COX-1 and COX-2
activity, respectively (Brideau et al., 1996; Young et al., 1996). Whole-blood assays have shown the following COX-2/COX-1 selectivity ratios of celecoxib, valdecoxib, and rofecoxib to be 7.6, 30, and 35, respectively (Chen et al., 1999; Riendeau et al., 2001). However, the clinical relevance of whole-blood assays and the relationship of COX-2 selectivity to patient outcome remains to be established (Patti et al., 2002).

**Cyclooxygenase-2-deficiency vs. cyclooxygenase-2 inhibitors**

We have postulated our hypotheses of the progression of PD and a way to stop it, and there are many tools available to test them. Genetically, COX KO mice can be used. MPTP treated COX-2 deficient mice show less tyrosine hydroxylase positive (TH+) cell loss (Feng et al., 2002; Teismann et al., 2003b), reduced oxidative damage (Teismann et al., 2003b) and decreased activation of microglia (see preliminary studies) than the wild-type in an established MPTP mouse model of PD.

Pharmacologically, nonsteroidal anti-inflammatory drugs (NSAIDs) can be used due to protective effects from several epidemiological studies that NSAIDs reduce risk of having neurodegenerative disease AD (Nakamura, 2002; Etminan et al., 2003). However, conventional NSAIDs are not selective to COX-2, and many side-effects including gastrointestinal and renal problems can occur. Recently approved selective COX-2 inhibitors, celecoxib (Celebrex), rofecoxib (Vioxx) and valdecoxib (Bextra), have shown improved anti-inflammatory performance and safety compared to NSAIDs (Furst, 1997; Tive, 2000; Kalgutkar and Zhao, 2001; Matheson and Figgitt, 2001).

Our recent studies with these new COXIBs in an MPTP model show behavioral improvement in COXIBs-treated mice compared to vehicle treated mice (Chapter Three and Four). The studies also provided a hint that COXIBs may interact with the dopamine (DA) system in addition to other functions reported with NSAIDs, such as inhibiting NFkB (Niederberger et al., 2003), suppressing
cPLA2 expression (Yuan et al., 2000) or activating peroxisome proliferator-activated receptor-γ (PPAR-γ) (Combs et al., 2000), all of which reduce COX-2 levels. PPAR-γ regulates carbohydrate and lipid metabolism, and it has been recently known to have anti-inflammatory function through inhibiting NFκB (Delerive et al., 2000; Delerive et al., 2001). PPAR-γ agonists, thus, are used to study protective effects in diabetes type II (Stumvoll, 2003) and anti-inflammation (Landreth and Heneka, 2001). Evidently, both deficiency in COX-2 (Feng et al., 2002; Teismann et al., 2003b) and inhibition of COX-2 by COXIBs (Teismann et al., 2003b) like valdecoxib or PPAR-γ agonists like pioglitazone (Breidert et al., 2002) are protective in MPTP model. The comparative efficacy of each and the respective underlying mechanisms for advantage seen in each have not been published. The overlapping function is the depletion of COX-2 activity, but COX-2 deficient mice may have developmental plasticity over time, while COX-2 inhibitors can interact with other non-COX-2 targets.

Compensatory and gene-dose effects of cyclooxygenases

Early in vivo studies of compensatory effects of COXs in each COX deficient naive mice reveal negative results (LANGENBACH et al., 1999); the authors, however, did not state which tissues were tested in what amount or how it was done. Under pathological and certain conditions, in contrast, the compensatory effects are uncovered by inflammatory stimuli (Kirtikara et al., 1998), pain stimulation (Ballou et al., 2000), and uterine preparation for implantation (Reese et al., 1999). The interesting aspect about the compensation is that it is tissue-specific (Zhang et al., 2002). While heart, lung, spleen, kidney, stomach, and liver samples show no compensation or only induction of COX-1 in COX-2 KO or vice versa, brain tissue and peritoneal macrophages reveal significant reciprocal compensation (Zhang et al., 2002).
The results above reflect the different roles of COXs and the need of function that either isoforms can perform when another is absent in various tissues. Thus, the induction of COX-1 to compensate for the loss of COX-2 and *vice versa* in the brain and macrophages suggest amount-requirement rather than need of specific COX-2 products. However, the study by Langenbach et al. (1999) didn’t specify which part of the brain was used. Unlike many other tissues, the brain is very heterogeneous; it has different populations of neurons and different concentrations of macroglia and microglia. In addition, localization and induction of COXs may not be identical throughout the brain. Previous studies also did not include heterozygous COX-1 or COX-2 genotypes. We have studied the complementary effect in striatum and substantia nigra *pars compacta* (SNpc) in all COX-1 or COX-2 genotypes in MPTP mouse model and pharmacologically in mice treated with valdecoxib (Bextra).

**Concluding remarks**

Parkinson’s disease (PD) is a chronic and progressive motor disorder marked by degeneration of dopaminergic neurons in the substantia nigra *pars compacta* (SNpc). Increased inflammation and oxidative stress have been implicated in this neuronal death, as elevated levels of cyclooxygenase-2 (Teismann et al., 2003b) and reactive microglia (McGeer et al., 1988b) have been found in PD brains. Cyclooxygenase, present as COX-1 and COX-2 isoforms, is the rate-limiting enzyme in arachidonic acid-derived prostaglandin production (Samuelsson, 1991; Wenzel, 1997). While COX-1 is constitutively expressed in most tissues, COX-2 is induced during pathophysiological responses to inflammatory stimuli such as bacterial endotoxin, interleukin-1 (IL-1), and various growth factors (Kujubu et al., 1991; Needleman and Isakson, 1997).

During the process of prostaglandin production, reactive oxygen species are generated as by-products (Nikolic and van Breemen, 2001) which, in addition to endotoxin, mitogens, cytokines, and certain
inflammatory mediators, can activate microglia (Nakamura, 2002). Microglia are also activated by oxidative stress (Czlonkowska et al., 2002). Microglial activation causes the release of free radicals (Arimoto and Bing, 2003) and of inflammatory cytokines, including IL-1β, IL-6, and tumor necrosis factor-α (Liu and Hong, 2003). Under normal circumstances, a response by microglia is protective in fighting off pathogens; however, under pathological conditions induced by certain insults, including oxidative stress, excitotoxicity from ion imbalance, and trauma, microglia can be over-stimulated and produce excessive cytotoxic agents that damage neurons, stimulating overexpression of neuronal and/or microglial COX-2 (Minghetti and Levi, 1998; Gebicke-Haerter, 2001; Arimoto and Bing, 2003; Liu and Hong, 2003; Teismann et al., 2003b; Teismann et al., 2003c; Teismann et al., 2003a; Minghetti, 2004; Wang et al., 2005). Co-propagation of COX-2 expression and microglial activation may cause secondary damage to neurons and the surrounding cellular environment; therefore, pharmacological intervention to stop the positive feed-back loop between COX-2 and microglial activation may prevent secondary injury induced by an excessive inflammatory response and oxidative stress.

In several epidemiological studies, nonsteroidal anti-inflammatory drugs have shown protective effects in reducing the risk of neurodegenerative disease such as Alzheimer’s disease (Nakamura, 2002; Etminan et al., 2003) and PD (Chen et al., 2003). In the present study, we tested the hypothesis that excessive COX-2 aggravates MPTP-induced toxicity by perpetuation of the inflammatory response, which leads to secondary neuronal cell death in the SNpc. This study was designed to explore the role of COX-2-related inflammation in the pathogenesis of PD and to test the possibility of COX-2 inhibitors as a potential therapeutic drug for PD. Using an MPTP mouse model, C57BL/6N mice treated with therapeutic doses of valdecoxib showed improved cellular survival and behavioral function compared to vehicle controls. Similar results were obtained using COX-2-deficient mice. Both inhibition of COX-2 and genetic deficiency of COX-2 reduced SNpc microglial activation and mitigated MPTP-induced neurotoxicity on dopaminergic neurons in the SNpc.
All studies presented are designed to supplement each other. Although each study can stand alone, analyzing all results as a whole will give a complete picture whether COX-2 inhibitors can protect, or at least delay, neurodegeneration in PD.

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Chapter Two

Materials and Methods

**Animals and treatments:**

The development of COX1 and COX-2 knockout mice has been previously described (Langenbach et al., 1995; Morham et al., 1995). COX-1 and COX-2-deficient C57BL/6 mice were established at the National Institute of Environmental Health Science, Research Triangle Park, NC, USA, from which breeders were obtained to produce new breeding colonies at the University of Kentucky. Mice were kept on a 12:12 hour light:dark cycle and fed *ad libitum*. All COX-1 and COX-2 knockout (KO) -/-, heterozygous (HT) +/-, and wild type (WT) +/+ controls used were male littermates from a number of simultaneous matings and were seven to nine months old, weighing 25-35 grams. The genotype was determined by PCR. In addition to these sets of mice, male retired C57BL/6N breeders (aged seven to nine months, weighing 25-35 grams, Charles River Breeding Laboratories) were also used.

For each study, 8-12 mice per group received MPTP·HCL (Sigma-Aldrich, St. Louis, MO) at a dosage of $4 \times 15$ mg/kg i.p. at 1.5 hr intervals and were killed one or two weeks after the last injection. The non-MPTP treated controls received a comparable volume of 0.9% saline. MPTP handling and safety measures were in accordance with our Division of Laboratory Animal Resources Standard Operating Procedure and the Institutes of Health procedure for working with MPTP or MPTP-treated animals. Administration of valdecoxib (Bextra; Pharmacia, Chicago, IL), celecoxib (Celebrex; Pfizer, NY) and rofecoxib (Vioxx; Merck, NJ) was modified from a published method (Jantzen et al., 2002): Daily amount of 10, 30 or 50 mg/kg of valdecoxib, 30 mg/kg of celecoxib, or 30 mg/kg rofecoxib was mixed
with and administered as a cheese pellet, at 24-hour intervals from two weeks before MPTP injection until the end of the experiment. All procedures involving animals are approved by the Institutional Animal Care and Use Committee at the University of Kentucky and are in strict accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

**Genotyping of COX-2-deficient mice:**

We performed genotyping with a standard protocol to identify wild-type, heterozygous, and homozygous COX1 and COX-2-deficient mice. Four weeks after birth, segments of about three to five millimeters of mouse tails were digested with lysis buffer and proteinase-K at 55°C overnight (Genomic DNA purification kit, Gentra systems, Minneapolis, MN). After RNase treatment, DNA was separated by phenol-chloroform extraction and ethanol precipitation. PCR was performed with the following COX1 and COX-2 specific primers (invitrogen, Carlsbad, CA):

- COX-1 WT Forward: 5’–AGG AGA TGG CTG CTG AGT TGG-3’
- COX-1 KO Forward: 5’–GCA GCC TCT GTT CCA CAT ACA C-3’
- COX-1 Reverse: 5’–AAT CTG ACT TTC TGA GTT GCC-3’
- COX-2 WT Forward: 5’–ACA CAC TCT ATC ACT GGC ACC-3’
- COX-2 KO Forward: 5’–ACG CGT CAC CTT AAT ATG CG-3’
- COX-2 Reverse: 5’–TCC CTT CAC TAA ATG GCC TC-3’

The thermal cycler (Eppendorf Mastercycler gradient, eppendorf, Hamburg, Germany) was programmed as follows: one cycle at 95°C for five minutes, and 30 cycles of 94°C for 30 seconds, 60°C for one minute, and 72°C for 90 seconds, followed by a final extension cycle of 72°C for seven
minutes. PCR is expected to yield fragments of 600 and 800 bp for COX-1 and 760 and 900 bp for the COX-2 wild-type and mutant alleles, respectively.

**Immunohistochemistry:**

Brains were sectioned at 30 µm thickness on a sliding microtome for free-floating tissue sections. Every sixth section from a given area was stained with polyclonal antibodies (Ab) against neuronal TH (1:2000 Pelfreez, Roger, AR) or Mac-1 (1:1000 Serotec, Oxford, UK). Sections were incubated in 4% normal serum in PBS for 30 minutes. After this blocking step, the sections were incubated overnight in PBS containing 0.025% Triton X-100, 1% normal serum, and the primary antibodies at 4 ºCelsius. The avidin-biotin immunoperoxidase method with 3,3’-diaminobenzidine tetrahydrochloride as the chromagen was used to visualize immunoreactive cells (ABC Kits, Vector Laboratory, Burlingame, CA). For Nissl-staining, SNpc sections were stained with cresyl violet. Sections were then mounted on gelatinized slides, left to dry overnight, dehydrated in ascending alcohol concentrations, and mounted on Permount (Fisher Scientific, Fair Lawn, NJ).

**Western blot analysis:**

Cellular proteins were extracted from the striatal samples with an extract buffer containing 0.5% Triton X-100 and protease-inhibitor cocktail (1:1000, Sigma-Alsrich, St. Louis, MO). The tissues were homogenized in this buffer with the Fisher model 100 sonic dismembranator and put on ice for one hour. The soluble extracts were separated by centrifugation at 11,500 rpm for five minutes at 4 ºCelsius. Equal amounts of protein samples (20 µg) were mixed with the loading buffer (60 mM Tris-HCl, 2% SDS, and 2% β-mercaptoethanol, pH 7.2), boiled for 4 minutes, resolved by SDS-
polyacrylamide gels, and transferred to a nitrocellulose filter (Millipore, Bedford, MA) using a semidry blotting apparatus (Bio-Rad Laboratories, Hercules, CA). After blocking with a solution containing 5% nonfat milk, the filters were incubated with TH (1:1000 Boehringer-Mannheim, Indianapolis, IN), COX-1 (1:250, Research Diagnostics Inc, NJ) and COX-2 (1:500, Research Diagnostics Inc, NJ) or β-actin (Sigma, St. Louis, MO) antibodies for detection of the level of dopaminergic neuronal terminals, and for normalization of the loading protein. The signal was visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Biosciences, Little Chalfont Buckinghamshire, England) by employing a goat anti-rabbit or goat anti-mouse secondary antibody conjugated with hydrogen peroxidase (Sigma-Aldrich, St. Louis, MO). Signal specificity was insured by omitting each primary antibody in a separate blot, and loading errors were corrected by measuring β-actin immunoreactive bands in the same membrane. The density measurement of each band was performed with Scion image software (Scion Corporation, Frederick, MD). Background samples from an equivalent area near each lane were subtracted from each band to calculate mean band density.

**Cell counting:**

The total number of TH- and Nissl-stained SNpc neurons and Mac-1-stained SNpc activated microglia were counted in sections from six to eight mice per group using the optical fractionator method for unbiased cell counting. The optical fractionator method of cell counting combines the optical dissector with fractional sampling, and is unaffected by the volume of reference (i.e., SNpc) or the size of the counted elements (i.e. neurons) (West, 1993). Cell counts were performed by using a computer-assisted image analysis system consisting of a Zeiss Axioskop2Plus photomicroscope equipped with a MS-2000 (Applied Scientific Instrumentation, Eugene, OR) computer-controlled motorized stage, a Sony DXC-390 (Japan) video camera, a DELL GX260 workstation, and the Optical Fractionator
Project module of the BIOQUANT Stereology Toolkit Plug-in for BIOQUANT Nova Prime software (BIOQUANT Image Analysis Corporation, Nashville, TN). Cell counting was done on both sides of SNpc of every sixth section throughout the entire extent of the SNpc. Each midbrain section was viewed at low power (×10 objective), and the SNpc was outlined by using a set of anatomical landmarks. The cell numbers were counted at high power (×40 objective). Adjacent sections immediately caudal and rostral to the sections used for TH staining were stained and counted for Nissl-stained neurons and Mac-1-stained activated microglia. TH- and Nissl-stained neurons were counted only when their nuclei were optimally visualized within one focal plane. Nissl-stained neurons were differentiated from non-neuronal cells by the clearly defined nucleus, cytoplasm, and a prominent nucleolus. After all of the cells were counted, the total numbers of neurons or activated microglia in the SNpc were automatically calculated by the module using the formula described by West et al. (West, 1993).

**Behavioral analysis and evaluation of locomotor activity**

**Apparatus**

During the light period, locomotor activity was assessed using four automated activity chambers (Model RXYZCM-8, Accuscan Instruments, Columbus, OH). Each chamber consisted of a 41×41×31-cm³ Plexiglas box with a grid of infrared beams mounted horizontally every 2.5 cm and vertically every 4.5 centimeters. The monitors were connected to a Digiscan Analyzer (Model DCM-8, Accuscan Instruments) that transmitted the number of beam breaks (activity data) to a computer. During operation, the pattern of beam interruptions was recorded for six consecutive 5-minute periods and analyzed by the computer.
Behavioral measures

Prior to valdecoxib administration, animals were allowed to habituate to the locomotor activity chambers during daily 30-min sessions over six consecutive days. Two measures of overall locomotor activity were obtained during the behavioral sessions: total distance traveled and vertical activity. Total distance traveled is quantified as the sum of the distance measured (in centimeters) across the 30-min recording period. Vertical activity is quantified as the sum of the number of vertical photobeam interruptions across the six 5-minute intervals.

Rotarod testing:

The Rotarod treadmill (MED Associates Inc, St. Albans, VT.), designed to measure motor performance and coordination, consists of a 3.6-cm diameter cylindrical treadmill connected to a computer-controlled stepper motor-driven drum that can be programmed to operate at a constant speed or in a defined acceleration mode. When the animal falls off the rotating drum, individual sensors at the bottom of each separate compartment automatically record the amount of time (in seconds) spent on the treadmill. Mice were trained two consecutive days before MPTP injections in acceleration mode (2-20 rpm) over five minutes. The training was repeated with a fixed speed (16 rpm) until the mice were able to stay on the rod for at least 150 seconds. On day 2, 4, and 6 after MPTP injections, mice were assessed for their coordination capability with a maximum recording time of 150 seconds. Rotational speeds of 16, 20, 24, 28, and 32 rpm were recorded in succession, and the overall rod performance (ORP) for each mouse was calculated by the trapezoidal method as the area under the curve in the plot of time on the rod versus rotation speed (Rozas et al., 1998).
**High-performance liquid chromatography (HPLC) assay**

Left striatum was dissected en masse from adult (7–9 months old, \( n = 8-12 \) mice/group) C57BL/6 or COX-2-deficient mice. All brain tissue samples were weighed and snap-frozen. For analysis, samples were processed in pH 4.1 buffer, and monoamines were separated by reverse-phase high pressure liquid chromatography with electrochemical detection (HPLC-EC) using dihydroxybenzylamine (DHBA) as an internal standard. DA, NE and DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were detected by a dual-coulometric electrochemical detector. Catecholamine levels were expressed in units of ng/g of the striatum per wet weight of tissue.

**Statistical analysis:**

All data were analyzed using an IBM-based personal computer statistical package (SYSTAT 10, SPSS Inc, Chicago, IL). Except for the correlation analyses, all values are expressed as the mean ± SEM, and differences among means were analyzed by using one- or two-way analysis of variance (ANOVA) with time, treatment, or genotype as the independent factor. When ANOVA showed significant differences, pairwise comparisons between means were tested by Bonferroni post hoc testing. Statistical significance was set at \( p < 0.05 \) for all analyses.
Chapter Three

Effects of different selective COX-2 inhibitors in reducing MPTP-induced behavioral impairments

Summary

The study in Chapter Three explores the effects of three different selective COX-2 inhibitors, valdecoxib, celecoxib, and rofecoxib, on the spontaneous horizontal total distance traveled and vertical activity of mice before and after MPTP treatment compared to the non-inhibitor-treated groups. This study is the basis for later studies in which a selected COX-2 inhibitor and appropriate behavioral setup and schedule will be use accordingly in Chapter Four. After several pilot studies, it became clear that regarding behavioral outcome, valdecoxib is the best drug, followed by celecoxib, and rofecoxib the least. The appropriate time points for behavioral testing are one day and then two weeks after MPTP injection provides. The behavioral benefits from valdecoxib can last even four weeks, suggesting a stable effect of the drug. Protection of motor performance and functional improvement or is the goal of the treatment for PD, so that patients can do regular daily activity with ease. Thus, the ability to improve or protect motor function is one of the first and foremost criteria that need to be tested before performing analysis at the cellular level.
Background

COX-2 inhibitors represent a significant therapeutic development because of their improved side effect profile compared with conventional NSAIDs (Peura, 2002). Several other COX-2 inhibitors are currently under development. A few early studies reported that the risk of developing Alzheimer’s disease (AD) was reduced among NSAID users, especially in those who had taken the medications for more than two years (McGeer et al., 1996; Stewart et al., 1997; in t’ Veld et al., 2001). In later epidemiological studies, NSAIDs demonstrated protective effects in reducing the risk of neurodegenerative disease such as AD (Nakamura, 2002; Etminan et al., 2003) and PD (Chen et al., 2003). A possible mechanism for this effect is a reduction in inflammation that may promote neuronal degeneration.

The open-field test is the most prevalently used behavioral method in assessing behavioral deficits after MPTP injection. The experimental set up and scheduling of the test, however, can be quite different from laboratory to laboratory. Some investigators start the test only an hour after MPTP injection while others start one day, one week, or even two months later. Some groups perform repeated measurements of the mouse locomotor activity while others do only one. The key to successful behavioral testing is to conduct the test with the set up and schedule that is appropriate for the respective experimental design. This may take a trial and error approach at the early stage since there are several factors that can affect the behavior of the mice, and thus affect the behavioral results of the study. The different factors not only affect the behavior, but also make it difficult to directly compare results between laboratories as their experimental paradigms differ in age, strain, MPTP administration schedule and regimen, gender, size and shape of open field, trial length, number of trials, habituation to open field prior to testing, or interval between MPTP injection and testing (Sedelis et al., 2001).
We have done several pilot studies and have found that the open-field test is appropriate for the MPTP regimen that we currently use (4×15 mg/kg, 1.5 hr interval, i.p.). This paradigm induces transient hyperactivity of mice at the early stage, and mice then develop slow movement and hypoactivity at a later stage. In past experiments, locomotion of mice became stable after a week. Thus, for studies in Chapter Three and Four in which C57BL/6 mice were treated with selective COX-2 inhibitors, the end point of behavioral measurement is chosen. As mentioned above, many factors determine the best behavioral test for a particular study. Genetic background of mice is also one of these factors, and for unclear reasons, COX-2-deficient mice and their littermates did not exhibit the same result as in the pure C57BL/6 mice from the vendor. The behavioral differences between experimental treatments were more difficult to reach or distinguish. The rotarod test, however, is a good test for our COX-2-deficient study (Chapter Five) while it is not as appropriate to use for the pure C57BL/6 mice. Part of the reason is that C57BL/6 mice tend to be able to stay on the rod better than other strains even after experimental manipulation such as receiving MPTP injection. This reinforces the fact that the appropriate test is needed for a particular experimental design to be able to appropriately assess behavioral impairments of mice.
Results

Selective COX-2 inhibitors alleviate MPTP-induced loss in spontaneous locomotor activity of C57BL/6 mice.

During pre-MPTP days, there were no statistically significant differences among the experimental groups in the total distance (cm) [shown as % control here] mice traveled during each 30 minute session (Figure 3.1). By the end of the pre-MPTP treatment period, mice in all groups traveled similar distances (7 and 3 days before MPTP injection). MPTP was administered at day zero. In contrast, after MPTP treatment (Figure 3.2), it became difficult for mice to initiate spontaneous locomotor activity and to travel the distances they were capable of performing prior to MPTP administration. The vehicle-treated mice lost over 80% distance compared to the control and their own base-line. The selective COX-2 inhibitor (COXIB)-treated mice lost only about 50 percents. On average, COXIB (valdecoxib, celecoxib or rofecoxib)-treated mice performed 50% better than the vehicle treated mice. For this particular experiment, distance traveled was measure twice a week post-MPTP injection (day 5, 7, 12, and 14). The total distances after MPTP injection presented here were based on the average of the minimum distance traveled post-MPTP injection by each behaviorally impaired mouse. Compared to the vehicle-treated mice, all selective COX-2 inhibitor-treated mice moved for statistically higher total distances after MPTP injection (One-way ANOVA, *p < 0.05).

MPTP-induced deficit in vertical activity is decreased by selective COX-2 inhibitors.

After MPTP injection, mice dramatically lost their vertical (rearing) ability. An objective measure of the vertical activity, recorded by an automated locomotor activity testing machine, revealed the ability
of valdecoxib and other COXIBs to maintain behavioral movement closer to that of control-saline treated mice (Figure 3.4) and of their baseline prior to MPTP injection (Figure 3.3). The vehicle-treated mice lost about 90% while the selective COX-2 inhibitor-treated mice lost about 75% of their vertical activity. The numbers used for the average calculation for each group were the minimum vertical activity measured on day 5, 7, 12, and 14 of each mouse. In concordance with the total distance result, vertical activity of COXIB-treated mice were statistically higher than the vehicle-treated after MPTP injection (One-way ANOVA, *p < 0.05).

**Selective COX-2 inhibitors decrease the incidence of MPTP-induced severe immobility in C57BL/6 mice.**

COXIBs help reduce immobility. After MPTP injection, there were many mice with behavioral deficits. However, there were a number of mice that have severe immobility (high immobility scores), whose total distance moved were less than two standard deviations from average total distance of the control. Almost 25% of the vehicle-treated mice had severe immobility (Figure 3.5). With all of the COXIB-treated mice combined, there were approximately 12% of mice with severe immobility. When analyzed separately, there were around 17% and 15% mice with severe immobility in celecoxib- and rofecoxib-treated mice, respectively. Valdecoxib-treated mice had the least numbers of mice with severe immobility, as only 5% of all MPTP+valdecoxib-treated mice exhibited the deficits (*p < 0.05).

Correlation analysis of the immobility scores, total distance, speed and vertical activity was tested. The immobility scores were based on the percent distance moved by each mouse. The total distance of each mouse was normalized to the average total distance of the control saline; then the minimum total distance from day 1-7, 12 and 14 was calculated. The immobility scores were calculated as follow: If the minimum total distance (% of control) was <5%, <10%, <20%, <30%, <40%, <50%, <60%, <70%, and ≥70%, then the immobility scores would be 24, 20, 16, 12, 8, 4, 2, 1, and 0, respectively. The
immobility scores have a strong negative correlation with the total distance and vertical activity. The Spearman correlation coefficient \((r_s)\) between the immobility scores and the total distance was about -0.89 while the difference between the immobility scores and the vertical activity was about -0.63 (Table 3.1). The \(r_s\) value between the total distance and the vertical activity was 0.67 while speed had no strong correlation with immobility scores, total distance or vertical activity. The speed is the total distance per total time the mouse moved; thus, a severe mouse that moved the least may still exhibit a normal speed because they also spent the least movement time. The significantly lower percentage of impaired mice in each COXIB or all COXIBs agrees with the results that COXIB-treated mice have better horizontal and vertical activity counts \((*p < 0.05)\).

**Selective COX-2 inhibitors reduce MPTP-induced hyperactivity**

As described above, mice were treated with a COX-2 inhibitor (Bextra, Celebrex, or Vioxx) (30 mg/kg daily in cheese pellets) for the duration of the study, starting 2 weeks before MPTP injection. Habituation was conducted in the testing cages on experimental days 1 through 6 (day -20 to day -15). Testing began on day -10 (experimental day 11) and continued through day 7 (experimental day 28). MPTP was administered on experimental day 21, 2 weeks following initiation of COX-2 inhibitor treatment; thus, tests were conducted before MPTP injection for baseline measures (day -10 to day -1) and after MPTP injection for measures of hyperactivity and recovery (day 5 -day 14). Following MPTP administration (15 mg/kg 4 times at 1.5 hr intervals, i.p.), we observed exaggerated, continuous, uncoordinated running and repetitive jumping in many MPTP-treated mice. These behaviors were less pronounced in the MPTP+COX-2 inhibitor groups, particularly in the valdecoxib- and celecoxib-treated groups, and absent in the control animals. For total distance moved during the testing period, mean values ± SEM are presented (Figure 3.6). On days -20 through day -7, \(n = 9-14\) mice; on days 12 and day 14, \(n = 8-12\) mice due to MPTP-induced mortality. Time course values were analyzed for
significant main effects using a repeated measures analysis of variance. Post hoc comparisons were made using Bonferroni test. On testing day 5, valdecoxib and celecoxib significantly reduced the total distance moved during testing as compared to mice receiving MPTP+vehicle (*p < 0.05). The COXIB-treated mice behaviors were not significantly deviated from the control while the vehicle-treated mice were significantly hyperactive than the control (**p < 0.01).

**Valdecoxib still protects mice from MPTP-induced behavioral impairments at four weeks.**

To study the long term effects of selective COX-2 inhibitors, we tested behavior of mice twice a week after seven days post-MPTP injection for a one-month end point. In addition, mice were tested daily during the first week post-MPTP administration starting a day after the injection. The habituation tests and the valdecoxib effect tests pre-MPTP were carefully included in the design of the experiment. As expected, all mice habituated and had similar horizontal and vertical activity pre-MPTP treatment (Figure 3.7 and 3.8). Selective COX-2 inhibitors valdecoxib significantly attenuated MPTP-induced hyperactivity in wild-type mice within the first few days after MPTP injection. MPTP-treated mice reduced their movement down to 11% of control at day one and then had increasing level of horizontal activity from about 154% of control up to about 800% of control in the vehicle-treated mice and from about 78% to 525% in the valdecoxib-treated mice (Figure 3.7). Valdecoxib significantly reduced the mice from behaving more hyperactive than normal on day 2 to day 5 after MPTP-injection. Later on, hyperactive mice became hypoactive or inactive starting around day 6 after MPTP injection. Valdecoxib appeared to slow down the progression of abnormal hyperactivity. The activity of vehicle-treated mice dropped significantly and remained significantly less than that of the control, and that of the valdecoxib-treated mice, from one week to four weeks after MPTP injection.
Around day 6 after MPTP treatment, hyperactive mice became hypoactive or inactive, and vertical activity changed quickly with the horizontal activity. Before day 7, the vertical activity was unstable. For example, vertical activity of the vehicle-treated mice from day 1 to day 3 may range from 3% to 51% and down to 25% of control in about 24 hours interval (Figure 3.8). From day 7 post-MPTP intoxication, the vertical activity became more stable, and valdecoxib-treated mice had stable vertical activity significantly higher than that of the vehicle-treated mice. The activity of vehicle-treated mice dropped significantly and remained significantly less than that of the control and that of the valdecoxib-treated mice even at the twenty–eighth day post-MPTP injection.
Discussion

In this study we demonstrated that selective COX-2 inhibitors, valdecoxib, celecoxib, and rofecoxib, reduce MPTP-induced loss in total distance traveled and vertical activity of C57BL/6 mice. Among the three COXIBs, valdecoxib exhibits the best behavioral protection, with celecoxib second, and rofecoxib third. Valdecoxib had the least number of behavioral impaired mice and attenuated hyperactivity at early post-MPTP injection and hypoactivity at late post-MPTP injection more effectively than the other COXIBs did. In addition, the beneficial effects of valdecoxib can still be seen one month post-MPTP injection, suggesting the reliability of the drug and the behavioral test.

The behavioral tester should consider the dynamic of the mice behavior after MPTP treatment when designing experiments or analyzing data. First of all, MPTP toxicity to the CNS takes time and MPTP can directly and indirectly cause death of cells that control movements. There is individual difference in MPTP-vulnerability, maybe due to the underlying developmental differences or technical errors. Even within an experimental group, mice develop hyperactivity and hypoactivity at different rates. Using minimum values or maximum values to assess the hypokinetics or hyperkinetics of mice treated with MPTP may be more appropriate than the traditional approach of testing one time point. However, if a complete assessment of the behavioral change of mice has been conducted, appropriate testing dates can be assigned prior to the beginning of the later experiments. MPTP-induced hyperactivity has been observed in other laboratories as well (Chia et al., 1996). Hyperactivity of MPTP-treated mice is usually ignored or even viewed as an incorrect result or an error. However, this hyperactivity can be reproduced reliably and repeatedly with our experimental setup. This hyperactivity is one of the most accurate behavioral markers in predicting which mice will develop hypoactivity or inactivity later. Vehicle-treated mice had high numbers of hyperactive mice whose behavioral performances were scored as immobile. Valdexocib-treated group had significantly less numbers of hyperactive mice, and
thus also have significantly less numbers of mice with low immobility scores. The immobility score was based on the minimum distance traveled from day 5 to 14 of mice compared to the control mice. As a result, the total distance was positively correlated with the immobility scores of each mouse. The vertical activity, in turn, is related but not strongly correlated to the distance traveled at least during the first few days post-MPTP injection when some mice are so hyperactive that they continuously, uncontrollably, and swiftly move horizontally so that they had very few moments to stop and rear. Evidently, when the vehicle-treated mice were hyperactive on day 5, their vertical activity was extremely low. However, these mice could jump very high, many of the times hitting the top of the Plexiglas testing box. Instead of rearing, when they have a moment to stop moving, they often jump repeatedly and quickly. This jumping behavior is almost never observed in the control mice.

The transient hyperactivity is very interesting to pursue in future experiments; an ability to measure change in cellular or molecular levels while testing behavior of mice is needed. Nonetheless, we suspect that the hyperactivity may be related to the glutamate excitatory or dopaminergic neurotransmission, as these two systems has been manipulated by several drugs and toxins to induce hyperactivity in mice. Many PD patients had active life styles when at the earlier stages of their lives, such as the movie star Michael J. Fox, boxer Muhammad Ali, and Pope John Paul the third. Due to the nature of our experimental set up, processes that may take years to occur or to be noticed in humans may be quickly seen in the animal model, especially when the cause of these processes is modified, in this case by MPTP-induced neurotoxicity. Because the current trend is to find a treatment for hypokinesia and bradykinesia of PD patients, we will focus on the hypoactive phases of MPTP-treated mice. Accordingly, behavioral tests will be conducted on day 1 and 14 post-MPTP injection. This hyperactivity was not noticed in COX-2-deficient mice. So they did not show noticeable differences among genotypes on open-field test. As mentioned earlier in the background of this chapter, different
paradigms need different behavioral tests. As a result, COX-2-deficient mice were tested on their rotarod performance (Chapter Five).

An objective measure of spontaneous total horizontal distant movement can be feasibly recorded by an automated locomotor activity testing machine, and we have observed the ability of valdecoxib and other COXIBs to maintain locomotor behavior close to that of normal control-saline treated mice and their baseline before MPTP injection. The ability of COXIBs to exert such effects leads to the exciting possibility that COX-2 inhibitors may have a function closely related to the glutamate or dopamine system regulation that other drugs or COX-2 deficiency does not possess.
Figure 3.1. Total distance in percent of the control saline pre-MPTP administration of various COXIB-treated C57BL/6 mice. MPTP was administered two weeks after the start of selective COX-2 inhibitors (COXIBs) treatment (day zero). COXIBs treatment started at day -14 and continued until the end of the experiment. Before MPTP treatment, there were no statistically significant differences among the experimental groups in the total distance (cm) [shown here as % of the control] mice traveled during each 30 min session. By the end of the pre-MPTP treatment period, all mice traveled very similar in distance in centimeters (day -7 and day -3). Data are means ± SEM for eight to twelve mice per group, One-way ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 3.2. Total distance in percent of the control saline of various selective COX-2 inhibitor-treated C57BL/6 mice post-MPTP injection. All MPTP-treated mice lost the ability to initiate spontaneous locomotor activity although the effect was less severe in mice receiving selective COX-2 inhibitors (valdecoxib, celecoxib or rofecoxib). On average, selective COX-2 inhibitor-treated mice performed 50% better than the vehicle-treated mice. The total distances after MPTP injection presented here were based on the average minimum distance traveled by behaviorally impaired mice. Compared to the vehicle treated mice, all selective COX-2 inhibitor treated mice demonstrated a statistically significant enhanced ability to execute spontaneous movements (*p < 0.05 compared to the MPTP+vehicle mice, One-way ANOVA with subsequent Bonferroni for multiple comparisons). Data are means ± SEM for eight to twelve mice per group.
Figure 3.3. Vertical activity of various selective COX-2 inhibitor-treated C57BL/6 mice pre-MPTP treatment. Vertical activity of mice was recorded simultaneously with the total distance (cm) traveled by each mouse [shown here as % of the control saline]. As in the total distance measurement, mice were habituated for six consecutive days (day -20 to day -15) before the treatment of the selective COX-2 inhibitors was initiated (day -14). MPTP administration began on day zero. In agreement with the total distance result, vertical activity of COXIBs-treated mice was not statistically different from the other groups of mice (Data are means ± SEM for eight to twelve mice per group, One-way ANOVA with subsequent Bonferroni for multiple comparisons).
Figure 3.4. Vertical activity of various selective COX-2 inhibitor-treated C57BL/6 mice post-MPTP treatment. MPTP induced loss of vertical activity. After the MPTP injection, the selective COX-2 inhibitor-treated mice had 10-20% higher vertical activity than the vehicle-treated group [shown here as % of the control saline]. As in the total distance post-MPTP treatment, the numbers used for the average calculation for each group were the minimum vertical activity measure on day 1-7, 12, and 14 of each mouse. In agreement with the total distance result, vertical activity of COXIB-treated mice was statistically less impaired. Data are means ± SEM for eight to twelve mice per group (One-way ANOVA with subsequent Bonferroni for multiple comparisons, *p < 0.05).
Figure 3.5. The incidence of MPTP-induced severe immobility in C57BL/6 mice. COXIBs help reduce immobility. After MPTP injection, there were many mice with behavioral deficits. However, there are a number of mice that have severe immobility (high immobility scores), whose total distance moved were less than two standard deviations from average total distance of the control. Almost 25% of the vehicle-treated mice have severe immobility. Overall, there were about 12% of mice with severe immobility for all mice treated with COXIBs. Specifically, there were about 17% and 15% mice with severe immobility in celecoxib- and rofecoxib-treated mice, respectively. Only about 5% of valdecoxib treated mice had severe immobility. Data calculated from eight to twelve mice per group (Pearson chi-square statistic, *p < 0.05).
Table 3.1. The correlation of MPTP-induced severe immobility, total distance, speed and vertical activity of C57BL/6 mice. The immobility scores have a strong negative correlation with the total distance ($r_s = -0.886$) and vertical activity ($r_s = -0.627$). Between the total distance and vertical activity the $r_s$ was $0.667$, and speed had no strong correlation with other parameters ($r_s = -0.02$, 0.178 or 0.102, Spearman correlation statistic, $p < 0.05$). Data calculated from eight to twelve mice per group.
Figure 3.6. Early hyperactivity and later hypoactivity of C57BL/6 mice treated with MPTP.

Selective COX-2 inhibitors valdecoxib and celecoxib significantly attenuate MPTP-induced hyperactivity in wild-type mice. Five days following MPTP administration, valdecoxib and celecoxib significantly reduced the total distance moved during testing as compared to mice treated with MPTP only (*p < 0.05, One-way ANOVA with subsequent Bonferroni for multiple comparisons). Data are means ± SEM for eight to twelve mice per group.
Figure 3.7. **Long term effect of valdecoxib on total distance.** Selective COX-2 inhibitors valdecoxib significantly attenuate MPTP-induced hyperactivity in wild-type mice in the first few days after MPTP injection. On day 5, when hyperactivity of the MPTP+vehicle mice peaked, valdecoxib-treated mice activity was not as high on average. Later on, hyperactive mice became hypoactive or inactive around day 6 after MPTP injection. The activity of vehicle-treated mice dropped significantly and remained significantly less than that of the control and that of the valdecoxib-treated mice. Data are means ± SEM for eight to twelve mice per group (*p < 0.05 and **p < 0.01, Two-way repeated measures ANOVA with subsequent Bonferroni for multiple comparisons).
Figure 3.8. Long term effect of valdecoxib on vertical activity. Selective COX-2 inhibitor valdecoxib significantly attenuated MPTP-induced deficit in rearing in wild-type mice in the first few days after MPTP injection. The rearing activity depended on the horizontal activity of mice, which was unstable and changed drastically in the early days post-MPTP injection. Around day 6 after MPTP injection, hyperactive mice became hypoactive or inactive. From day 7 post-MPTP intoxication, the vertical activity became more stable and only valdecoxib-treated mice had stable vertical activity close to that of the control, which was significantly higher than that of the vehicle-treated mice. The activity of the vehicle-treated mice dropped significantly and remained significantly less than that of the control and that of the valdecoxib-treated mice. Data are means ± SEM for eight to twelve mice per group (*p < 0.05 and **p < 0.01, Two-way repeated measures ANOVA with subsequent Bonferroni for multiple comparisons).
Chapter Four

The Selective COX-2 inhibitor valdecoxib reduces MPTP-induced neurotoxicity and motor deficits

Summary

In the previous chapter, we demonstrated that COXIBs reduced MPTP-induced behavioral deficits in C57BL/6 mice. In addition, valdecoxib was determined to be the best drug regarding functional protection of MPTP-treated mice, which can still be observed at one month. This chapter, we continue the study using valdecoxib to pharmacologically test the hypothesis that reducing COX-2 level is beneficial in inhibiting microglial activation and decreasing dopaminergic neuronal death and behavioral anomaly. Immunohistochemical and stereological data indicate that inhibiting COX-2 by valdecoxib reduces dopaminergic neurodegeneration and microglial activation with strong positive correlation among them. In addition, behavioral studies reveal that MPTP-treated mice are protected from developing loss of horizontal or vertical activity if they have been given valdecoxib 30 mg/kg daily starting two weeks prior to MPTP-intoxication. These results support the use of selective COX-2 inhibitors as a therapeutic intervention of the progression of the PD.
Background

Although loss of substantia nigra pars compacta (SNpc) dopaminergic neurons is the pathological characteristic of Parkinson’s disease (PD), the cause of PD is still unknown. However, in agreement with other laboratories, we have found that wild-type mice treated with COXIBs have a higher tolerance to MPTP-induced dopaminergic neurodegeneration than their control counterparts. COX-2 is thought to play a protective role in normal inflammatory response. We have found, however, that excessive COX-2 expression is detrimental to cells due to the ability of COX-2 to induce oxidative stress and over activate microglia, which in turn, causes a microglia-mediated release of neurotoxic agents, such as reactive oxygens, inflammatory agents and toxic cytokines. These agents can induce apoptotic genes and, eventually, cell death. Thus, inhibition of COX-2 should give protective results in reducing neuronal death. We therefore hypothesize that selective COX-2 inhibition can be used as a therapeutic intervention in neurodegenerative progression of Parkinson’s disease. This study evaluated the ability of a recently FDA approved selective COX-2 inhibitor, valdecoxib (Bextra), to reduce cellular injury and behavioral abnormality in an MPTP mouse model of PD.

The purpose of this study is to test the efficacy of COXIBs in a PD model of MPTP-treated mice. MPTP treated mice can show pathology and behavioral impairment similar to that observed in humans (Jackson-Lewis et al., 1995). SNpc dopaminergic neurodegeneration and behavioral impairment is well mimicked in the MPTP mouse model (Arai et al., 1990; Jackson-Lewis et al., 1995; Schmidt and Ferger, 2001). To be justified as an effective drug, COX-2 inhibitors should reduce SNpc dopaminergic neuronal damage/loss and improve locomotor impairment following MPTP intoxication. Valdecoxib (Bextra) is chosen for this specific study not only because it produces less side effects (Ormrod et al., 2002; Alsalameh et al., 2003), but also because our previous studies have shown repeatedly that valdecoxib can reduce locomotor deficit and increase observable recovery rate (Chapter Three). The
need to conduct this experiment originates from the gap in the literature regarding the additional benefit
gained from COX-2 deficiency (Chapter Five) vs. selective COX-2 inhibitors-mediated extra benefit in
MPTP tolerance. We lack knowledge of developmental benefits of deficiency in COX-2 (from
plasticity and compensatory effects by other genes including COX-1) and also the ability of exogenous
COXIBs to perform other functions besides inhibiting its intended target, COX-2. Such fundamental
information can provide a substantial linking of COX-2 inhibitor as a treatment for PD if it can also
benefit PD patients in a way that COX-2 deficiency or other anti-inflammatory drugs cannot
accomplish.

If reducing COX-2 mediated inflammation is protective to dopaminergic neurons, and valdecoxib
treatment inhibit COX-2, then valdecoxib treated C57BL/6 mice should have lower microglial
activation, higher dopaminergic neuronal survival and better behavioral performance after MPTP
treatment.
Results

Valdecoxib treatment attenuates MPTP-induced dopaminergic neurodegeneration.

To determine the neuroprotective effect of COX-2 inhibitors against MPTP-induced neurotoxicity, TH-positive and Nissl-stained neurons in the SNpc were stereologically quantified. Treatment with valdecoxib did not affect the number of TH-positive and Nissl-stained neurons in the SNpc (Figure 4.1 and 4.3), and a stereological analysis showed no significant difference among the saline-injected groups (Figure 4.2 and 4.4). Fourteen days after the MPTP injections, there was a clear MPTP-associated toxic effect on the SNpc as revealed by diminished TH- and Nissl-stained neurons in sections from MPTP-treated mice, and the loss was significantly reduced by treatment with valdecoxib (Figure 4.1). Treatment with MPTP induced about 78% TH-positive cell loss while the various valdecoxib pretreatment groups showed only about 42-68% loss of TH-positive neurons. Compared to the saline+vehicle control, there was significant loss of TH-positive neurons in the MPTP-treated groups (**p < 0.001) (Figure 4.2). The numbers of TH-positive neurons remaining in the SNpc after MPTP injection in the higher doses of valdecoxib-treated mice (30 and 50 mg/kg) are statistically higher than in the MPTP+vehicle group (#p < 0.05 and ###p < 0.001, Figure 4.2). Nissl stain showed similar trends and statistical results (Figure 4.3 and 4.4), suggesting an actual TH-positive neuronal loss instead of a loss of TH expression. To determine whether valdecoxib could prevent not only MPTP-induced loss of SNpc dopaminergic neurons but also the loss of striatal dopaminergic fibers, we assessed the density of TH immunoreactivity in the striata of the different mice (Figure 4.5). MPTP significantly reduced striatal TH immunoreactivity compared to the saline control by 70% in the MPTP+vehicle (**p < 0.01) and only about 30% in the MPTP+valdecoxib mice (Figure 4.6). These findings
demonstrate that valdecoxib protects the nigrostriatal pathway against the MPTP-induced degeneration of dopaminergic neurons.

**The selective COX-2 inhibitor valdecoxib or COX-2 deficiency abates microglial activation.**

To investigate potential mechanism of secondary dopaminergic neuronal death, we performed immunohistochemistry using a microglia-specific antibody (anti-Mac-1 antibody). A large number of activated microglia, which had expanded cell bodies and poorly ramified short and thick processes, were seen in the MPTP+vehicle group but were not observed in the MPTP+valdecoxib group or saline-treated groups (Figure 4.7). This supports our hypothesis that inhibition of COX-2 expression during injury stimuli (MPTP) can reduce microglial activation, which may lead to secondary degeneration and progressive cell loss. In sections with numerous activated microglia, the SNpc can be distinguished from the surrounding areas as the activated microglia tend to stay within the SNpc or along the border of the SNpc. MPTP-induced microglial activation was clearly observed in the vehicle-treated mice, but to a lesser extent in the 10, 30 or 50 mg/kg valdecoxib-treated mice (Figure 4.7). Some activated microglia could be seen in the saline-treated animals, but the number of activated microglia was very small compared to the MPTP-treated WT mice (***p < 0.001, Figure 4.8). These findings suggest that COX-2 may play a role in modulating microglial activation.

**Dopaminergic neuronal survival is inversely correlated to COX-2 and microglial activation.**

To determine the relationship between the TH-positive neurons and microglia activation, we performed immunohistochemistry of adjacent SNpc sections of each brain, counted the cell numbers and studied statistical correlations among them. The sections counted for dopaminergic neurons (Figure 4.1 and
4.2) were 30 µm caudal relative to the sections evaluated for Mac-1 immunoreactivity (Figure 4.7 and 4.8) and 30 µm rostral relative to the Nissl-stained sections (Figure 4.3 and 4.4) of the same mouse brain. The Pearson correlation matrix shows the graphic representation of pooled data values for the number of TH- and Nissl-stained neurons and Mac-1-stained activated microglia of each mouse from the valdecoxib study (Figure 4.9). TH-positive neuron counts were highly correlated with Nissl-stained neuron counts (r > 0.94), while microglial activation had strong negative correlations with both TH- and Nissl-cell counts (both with Pearson correlation statistic r ≈ -0.80; p < 0.05; Table 4.1). These results suggest a strong co-existence of progressive dopaminergic neuronal degeneration with activation of microglia.

The relationship of COX-2 inhibition or expression to the TH-positive neuronal survival and microglia activation can be inferred from Figures 4.1 and 4.7. Statistically, the correlation of COX-2 to the number of TH- and Nissl-stained neurons and Mac-1-stained activated microglia can be determined by the correlation analysis. We ranked the data as 0, 10, 30 or 50 in accordance with the mg/kg amount of valdecoxib each mouse received daily. In Figure 4.10 and Table 4.2, the correlation was analyzed as in Figure 4.9 and Table 4.1 only using the MPTP-treated samples where COX-2 and microglial activation is induced by MPTP (Teismann et al., 2003b). From the COX-2 inhibitor study (Figure 4.10 and Table 4.2), the number of TH-positive neurons was strongly positively correlated with the number of Nissl-stained neurons and the amount of the COX-2 inhibitor administered per day (r_s ≈ 1.0 and r_s = 8.0, respectively). There was also a strong negative correlation with the Mac-1-stained reactive microglia (r_s ≈ -0.90, p < 0.05, Spearman correlation statistic, Table 4.2). It is important to note that the Mac-1-stained, reactive microglia counts, showed a strong negative correlation with the level of the COX-2 inhibitor (r_s ≈ -0.80, p < 0.05, Spearman correlation statistic, Figure 5D). These results imply that decreased activity of COX-2, due to inhibition by valdecoxib, strongly correlates with higher survival of dopaminergic neurons and decreased microglial activation. These results indicate a strong
correlation of progressive dopaminergic neurodegeneration with increased COX-2 and increased microglial activation.

**Valdecoxib reduces MPTP-induced locomotor activity deficits**

To determine the behavioral manifestations of the observed cellular changes, we assessed spontaneous locomotor activity by measuring the total distance traveled and vertical activity for 30 minutes using an automated open-field activity apparatus. There were no differences among the mice before MPTP injection (before day 0, Figure 4.11 and 4.13), and horizontal distance and rearing frequency were similar among all groups. A sharp reduction in distance from the first day of the behavioral testing (day -20) to a lesser but consistent baseline level at later days (from day -19), indicated a habituation effect (Figure 4.11 and 4.13). Two weeks (day -14) before MPTP injection, Valdecoxib treatment was started. To determine the effect of valdecoxib on pre-MPTP mouse behavior, mice were tested three times during valdecoxib treatment alone (day -10, -7 and -3) and showed no differences from non-valdecoxib-treated groups or their own baselines (the distance traveled during day -17 to day -15). On day zero, mice were injected with MPTP or saline. At day 1 post-MPTP injection (Figure 4.12 and 4.14), a marked reduction in spontaneous activity was observed (**p < 0.001). However, the reduction of spontaneous activity was likely due to systemic MPTP side-effects. Nevertheless, on day 1, the valdecoxib-treated mice, especially the ones treated with higher dosages of 30 or 50 mg/kg per day, were able to initiate spontaneous locomotor activity more than the vehicle-treated mice (##p < 0.01, Figure 4.12). Behavioral performance was tested on multiple days to ensure the validity of the results. Our previous studies revealed that MPTP-treated mice recovered from MPTP-induced hypoactivity after a few days, and their locomotor activity fluctuated and then stabilized around two weeks (unpublished observation). At two weeks post-MPTP injection, 30 or 50 mg/kg valdecoxib-treated mice recovered to the level of saline controls while the MPTP-treated vehicle group remained hypoactive (**p < 0.001) and had a statistically lower total ambulating distance and decreased rearing
frequency compared to the valdecoxib-treated group (##p < 0.01, Figure 4.12 and #p < 0.05 and ##p < 0.001, Figure 4.14, respectively). These behavioral results reflect the cellular protective effects of the COX-2 inhibitor when provided in sufficient amounts.
Discussion

Our hypothesis predicted that selective COX-2 inhibitors are a potential therapeutic treatment for PD due to their ability to prevent secondary damage to dopaminergic neurons caused by over activation of microglia and release of cytotoxic agents, including free radicals and inflammatory/apoptotic cytokines mediated by COX-2. As a result, we expect to see better survival of dopaminergic neurons, less activation of microglia, and improved locomotor impairment in valdecoxib-treated mice. Indeed, we observed this phenomenon, which suggests that our hypothesis is supported by our results.

Administering the drug through a cheese pellet is our original idea for a novel free-will drug-intake method. If the mice did not or could not eat cheese, an orally gavaging method would be used to administer valdecoxib dissolved in olive oil solution (vehicle).

In addition to the fact that they are readily available and economically validated, the use of old C57BL/6 mice is justified because of their most susceptible to MPTP-induced PD symptoms. Moreover, MPTP-related works have been conducted mostly with C57BL/6 mice, and the goal is to focus on the effect of drugs not the genotypic differences as in study in Chapter Five.

Considering the efficacy of a COXIB like valdecoxib (Bextra) on in an MPTP model, the next logical step is to compare its efficacy to other anti-inflammatory drugs. Many anti-inflammatory drugs could be used, but pioglitazone is justified for the following reasons. First of all, like Bextra, pioglitazone is already in use clinically (Lawrence and Reckless, 2000; Willson et al., 2000). Both drugs can be orally administered and the same dose can be used. Using drugs that are already approved by the FDA will facilitate the approval for using in Parkinson’s disease treatment. Moreover, pioglitazone has been shown protective for SN dopaminergic neurons in the MPTP mouse model (Breidert et al., 2002).
Secondly, PPAR-γ agonists like pioglitazone can indirectly block COX-2 (Bauer et al., 1997), iNOS (O'Neil and Kaltschmidt, 1997; Bernardo et al., 2000) and IL-1β (O'Neil and Kaltschmidt, 1997; Fahmi et al., 2001) through the NFκB pathway. Therefore, it will be valuable to compare the effect of its broad anti-inflammatory inhibition with that of a narrower effect of Bextra. Interestingly, NSAIDs can act as an agonist (Lehmann et al., 1997) or antagonist (Bishop-Bailey and Warner, 2003) of PPAR-γ. Bextra may also show either effect, and thus it will be interesting to compare these two drugs since they can cross-talk through NFκB and either one may inhibit one another. Finally, it will be interesting to compare these two drugs from the “endogenous vs. exogenous COX-2 inhibitors” aspect. PPAR-γ is, more or less, an endogenous COX-2 inhibitor, and its activation can be induced by its ligand pioglitazone.

Future research will also include the novel approach of testing effects of the proposed drugs on cytokines, apoptotic genes, and signaling pathways in the MPTP model. Measurement of cytokines, apoptotic genes and signaling molecules will give insights to roles of inflammation, activated microglia and PPAR-γ activation, death-effectors and anti-apoptotic genes, and inflammation and oxidative stress response, respectively. All of which, in turn, will help to determine the etiology and unveil the molecular mechanism underlying the potential treatment of PD.

Because PPAR-γ regulates metabolism of carbohydrate and lipids in addition to inflammation, the effects we will observe due to pioglitazone may be due to alteration in metabolism instead of, or in addition to, induced anti-inflammation response. The extra activities of pioglitazone will not be a focus of the study. If it is proven that pioglitazone is significantly better than COX-2 inhibitor in all aspects of their various actions, we will further study the mechanism of the differences, starting from metabolic regulation. Pioglitazone may improve MPTP-treated mice cellularly (Breidert et al., 2002), but behaviorally it may not. Other thiazolidinediones, such as rosiglitazone or troglitazone, can be used.
Although the former is already FDA approved like pioglitazone and Bextra (Sood et al., 2000), the latter is preferred because of its superior specificity (Oates et al., 2002). In addition, innate PPAR-\(\gamma\) agonist PGJ2 or, interestingly, conventional NSAIDs (preferred indomethacin, due to its ability to increase almost 6.5 fold activation) can be used to confirm that the result is not specific to pioglitazone, but to general PPAR-\(\gamma\) agonists.

Future studies with another anti-inflammatory drug appear interesting. However, it may provide little new information and major points that have already been demonstrated in this study. First of all, TH-positive neurons are more resistant to MPTP in mice treated with a selective COX-2 inhibitor (valdecoxib). Secondly, MPTP induced microglial activation is inhibited by the selective COX-2 inhibitor valdecoxib. Thirdly, TH-positive neuronal counts have strong negative correlation with the number of Mac-1-stained activated microglia and COX-2. Last but not least, selective COX-2 inhibitors alleviate MPTP-induced loss of spontaneous horizontal locomotion and vertical activity of C57BL/6 mice.

Chapter Three combined with this chapter outlines a series of experiments we have conducted to test the efficacy of the recently FDA-approved COXIBs in preventing PD-like symptoms. The advantage of using newly developed selective COX-2 inhibitors, as opposed to aspirin or other previous COX-2 inhibitors is that they can selectively inhibit COX-2 and leave COX-1 functional. This can reduce the unwanted side-effects like bleeding of the digestive tract or renal problems, which sometimes occur in patients taking drugs that inhibit both forms of COX. Our preliminary results guide us to use valdecoxib (Bextra) for further experiments due to its ability of alleviating behavioral deficits better than the other drugs at the same dosage. We mimic the normal chronic use of COX-2 inhibitors by daily administering valdecoxib to mice in food supplements, starting two weeks before MPTP injection.
and continuing until the end of experiment. We injected MPTP into the abdominal space of retired male mouse breeders.

The main findings of this study concluded that both automated total distance- and vertical activity measured were significantly reduced in the non-valdecoxib-treated mice at two weeks post-MPTP injection. In contrast, valdecoxib-treatment significantly alleviated these deficits. In addition, valdecoxib reduced the elevation of the immunoreactivity of the SNpc COX-2 and the marker of microglial activation Mac-1, while preventing loss of immunoreactivity of tyrosine hydroxylase (TH), an enzyme essential in production of dopamine.

Our finding is novel because we are the first to show the inverse relationship of reducing microglia activation through inhibiting COX-2 and increasing the working TH and dopaminergic neurons. It is interesting because our finding suggests that activation of COX-2 occurred prior to activation of microglia. Clinically, our findings may add PD to the list of many diseases COX-2 inhibitors can cure or prevent. The most famous of COX-2 inhibitor, aspirin, is already proven to be effective in reducing heart disease, even stopping the onset of the heart attack, and in lowering various forms of cancer in different tissues (most notably in esophagus, stomach and colon). This supports the use of COX-2 inhibitors in PD clinical trials. Regarding the basic sciences, these findings introduce a new way of reducing microglial activation in research. The fact that we have used retired breeder mice is both novel and appealing in PD research because periodic PD usually occurs after the time when men have had children and many sexual incidents. Many PD patients are not just aged, but they are most likely “retired” from sexual activities.

The results of this study indicate that retired breeders with reduced COX-2 activity have a reduced progressive loss of motor control caused by MPTP, possibly because of suppression of damage caused
by activation of microglia. These mice have lower microglial activation and higher neuronal survival after MPTP injection. Future research is needed to determine whether inhibiting COX-2 after MPTP injection can also stop the death of neurons. COX-2 inhibitors may not be as effective in curing PD once excessive oxidative stress and inflammation have already damaged dopaminergic neurons. Nevertheless, these drugs may still save other neurons from dying, which may reduce the severity of PD. The prevention route is more promising, and perhaps COX-2 inhibitors will one day be recognized as a daily supplement for patients seeking to alleviate neurological symptoms of PD and frequent visits to the neurologist.

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Figure 4.1. Photomicrographs of representative SNpc sections stained with an antibody against TH of C57BL/6 mice. The SNpc tissues were collected 14 days post-MPTP injection. The MPTP (4×15 mg/kg, 1.5 hr interval, i.p.)-treated mice have fewer TH-positive neurons compared to the saline groups. However, valdecoxib treatment reduced the neuronal loss, especially at a higher dose (30 or 50 mg/kg daily). Scale bar, 100 µm.
Figure 4.2. Number of TH-positive neurons of various dosages of valdecoxib after MPTP injection. MPTP administration led to significant loss of TH-positive neurons numbers by 78 percent for vehicle and by only about 68, 56, and 42 percent for 10, 30 and 50 mg/kg valdecoxib-treated mice, respectively. Among the MPTP-injected mice, the valdecoxib-treated mice had 10 to 32 percent more TH-positive neurons than the vehicle-treated mice. Data are means ± SEM for six to eight mice per group, ***p < 0.001 compared to saline+vehicle group; #p < 0.05 and ###p < 0.001 compared to MPTP+vehicle group, by ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 4.3. Photomicrographs of representative Nissl-stained SNpc sections of valdecoxib-treated C57BL/6 mice. Nissl stain shows similar trends as TH stain. The SNpc tissues were collected 14 days post-MPTP injection. The MPTP (4×15 mg/kg, 1.5 hr interval, i.p.)-treated mice have fewer Nissl-positive neurons compared to the saline groups. However, valdecoxib treatment reduced the neuronal loss, especially at a higher dose (30 or 50 mg/kg daily). Scale bar, 100 µm.
Figure 4.4. Number of Nissl-positive neurons of mice treated with various dosages of valdecoxib after MPTP injection. Nissl stain shows similar trends as TH stain. MPTP administration led to significant loss of Nissl-positive neurons numbers. Among the MPTP-injected mice, the valdecoxib-treated mice had more Nissl-positive neurons than the vehicle-treated mice. Data are means ± SEM for six to eight mice per group, ***p < 0.001 compared to saline+vehicle group; #p < 0.05 and ###p < 0.001 compared to MPTP+vehicle group, by ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 4.5. TH-stained Western blot of representative striatal tissues from valdecoxib-treated mice after MPTP injection. The striatal tissues were collected 14 days post-MPTP injection.

Inhibition of COX-2 reduced the MPTP-induced loss of the striatal TH immunoreactivity. The β-actin was used to normalize the TH immunoreactivity.
After MPTP treatment, the vehicle-treated mice had significantly reduced TH immunoreactivity compared to the saline-treated mice (**p < 0.001). Among the MPTP-injected mice, the valdecoxib (30 mg/kg daily)-treated mice had at least 30% higher TH immunoreactivity than the vehicle-treated mice. Data are means ± SEM for six to eight mice per group, **p < 0.01 and ***p < 0.001 compared to saline+vehicle group; ##p < 0.01 compared to MPTP+vehicle group, by ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 4.7. Inhibition of MPTP-induced microglial activation by valdecoxib. At 14 days post-MPTP injection, there was a high level of microglial activation in the SNpc. Unlike the vehicle group, mice treated with valdecoxib have diminished microglial activation. Pictures on the right are magnified photographs of the pictures on the left side. In contrast to inactivated striped microglia in MPTP+valdecoxib and control saline sections, activated microglia in MPTP+vehicle sections have a rounder body, fatter processes and denser stain. Scale bar, 100 µm.
Figure 4.8. Number of Mac-1-positive microglia of mice treated with various dosages of valdecoxib after MPTP injection. MPTP treatment leads to a significant increase in the number of activated microglia in mice receiving vehicle or the lowest dose of valdecoxib (10 mg/kg daily) but not the higher dose of valdecoxib (30 or 50 mg/kg daily). Activated microglia in the SNpc were bilaterally counted under a 40X objective. Data are means ± SEM for six to eight mice per group, ***p < 0.001 compared to saline+vehicle group; ###p < 0.001 compared to MPTP+vehicle group, by ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 4.9. Correlation matrix of TH-, Nissl- and Mac-1-stained cells of C57BL/6 mice. The results from the correlation matrix shown are tabulated in Table 4.1. Pearson correlation statistic, n = 6-8 per group.
Table 4.1. Correlation analysis of TH-, Nissl- and Mac-1-stained of C57BL/6 mice. As expected, the number of viable TH-positive neurons was strongly correlated with the number of neurons counted with the Nissl stain ($r > 0.90$). Importantly, the number of activated microglia was strongly negatively correlated with TH- and Nissl-stained neurons, both $r \approx -0.80$ ($p< 0.05$, Pearson correlation test, $n = 6-8$ per group).

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Figure 4.10. Correlation matrix of TH-, Nissl-, Mac-1-stained cells and valdecoxib dosages after MPTP injection. The results from the correlation matrix shown are tabulated in Table 4.2. Spearman correlation statistic, n = 6-8 per group.
Table 4.2. Correlation analysis of TH-, Nissl-, Mac-1-stained cells and valdecoxib dosages after MPTP injection. As expected, the number of viable TH-positive neurons was strongly correlated with the number of neurons counted with the Nissl stain (r_s > 0.90). Importantly, the number of activated microglia was strongly negatively correlated with TH- and Nissl-stained neurons, both r_s ≈ -0.90 (p < 0.05, Spearman correlation test, n = 6-8 per group). Similar analysis as in Figure 4.9 and Table 4.1 was used, but included only MPTP-treated values and assigned value of 0 for no treatment and 10, 30 and 50 for increasing dosage of valdecoxib. The results were similar to those of Figure 4.9 and Table 4.1 with a positive correlation of the amount of daily valdecoxib to TH- and Nissl-stained neuronal numbers (both r_s ≥ 0.80) and a strong negative correlation of the dosage of valdecoxib to microglial activation (r_s = -0.841, p < 0.05 Spearman correlation statistic, n = 6-8 per group).
Figure 4.11. Total distance (cm) pre-MPTP administration of valdecoxib-treated C57BL/6 mice.

On pre-MPTP days, there were no statistically significant differences among the experimental groups in
the total distance (cm) mice traveled during each 30-min session. By the end of the pre-MPTP
treatment period (3 days before MPTP injection), mice in all groups traveled similar distances. MPTP
was administered at day zero. Data are means ± SEM for eight to twelve mice per group pre-MPTP
treatment. Statistical significance was assessed by two-way repeated measures ANOVA with
Bonferroni post hoc test.
After MPTP treatment, mice initiated less spontaneous locomotor activity than they had prior to MPTP administration. On average, valdecoxib-treated mice performed up to 25% better than the vehicle-treated mice. Data are means ± SEM for six to eight mice per group post-MPTP treatment. Statistical significance was assessed by two-way repeated measures ANOVA with Bonferroni post hoc test, **p < 0.01 and ***p < 0.001 compared to the saline+vehicle group; ##p < 0.01 compared to MPTP+vehicle group.
Figure 4.13. Vertical activity pre-MPTP administration of valdecoxib-treated C57BL/6 mice. On pre-MPTP days, there were no statistically significant differences among the experimental groups in the vertical activity during each 30-min session. By the end of the pre-MPTP treatment period (3 days before MPTP injection), mice in all groups traveled similar vertical activity. MPTP was administered at day zero. Data are means ± SEM for eight to twelve mice per group pre-MPTP treatment. Statistical significance was assessed by two-way repeated measures ANOVA with Bonferroni post hoc test.
Figure 4.14. MPTP-induced deficit in vertical activity is decreased by selective COX-2 inhibitors.

An objective measure of the vertical activity, recorded by an automated locomotor activity testing machine, revealed the ability of valdecoxib to maintain rearing activity closer to that of control-saline-treated mice and to their baseline prior to MPTP injection (Figure 4.13). In agreement with the total spontaneous horizontal distance results, vertical activity of valdecoxib-treated mice was statistically less impaired. Data are means ± SEM for eight to twelve mice per group pre-MPTP treatment and six to eight mice per group post-MPTP treatment, and were analyzed by two-way repeated measures ANOVA with Bonferroni post hoc test, **p < 0.01 and ***p < 0.001 compared to the saline+vehicle group; #p < 0.05 and ##p < 0.01 compared to MPTP+vehicle group.

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Chapter Five

COX-2-deficient mice are resistant to MPTP-induced neurotoxicity and behavioral impairments

Summary

In previous chapters, we demonstrated that COXIBs reduced MPTP-induced behavioral deficits in C57BL/6 mice. In addition, valdecoxib was determined to be the best drug regarding functional protection of MPTP-treated mice, which can still be observed at one month. This chapter, we continue the study using COX-2-deficient mice to genetically test the hypothesis that reducing COX-2 level is beneficial in inhibiting microglial activation and decreasing dopaminergic neuronal death and behavioral anomalies. Immunohistochemical and stereological data indicate that COX-2 deficiency reduces dopaminergic neurodegeneration and microglial activation with strong positive correlations among them. In addition, behavioral studies reveal that MPTP-treated mice are protected from developing loss of horizontal or vertical activity if one or both alleles of cox-2 gene have been deleted prior to MPTP-intoxication. These results support the use of selective COX-2 inhibitors as a protection or risk reduction of developing PD.
**Background**

Our hypothesis predicted that selective COX-2 inhibitors are a potential therapeutic treatment for PD due to their ability to prevent secondary damage to dopaminergic neurons caused by over-activation of microglia and release of cytotoxic agents including free radicals and inflammatory/apoptotic cytokines mediated by COX-2. As a result, we expect to see better survival of dopaminergic neurons, less activation of COX-2 and microglia, and a reduction in locomotor impairment in COX-2-deficient mice, as seen in valdecoxib-treated mice (Chapter Four). This study, however, can also assist in testing the hypothesis that the effects seen with COX-2 inhibitor are different from COX-2 KO mice. We have to actually test two major hypotheses before concluding that valdecoxib gives more benefits than COX-2 deficiency does. This is because we need to verify that either one is beneficial first: specifically, COX-2 KO mice, as well as valdecoxib treated mice, should show protection. Comparison between valdecoxib and vehicle treated COX-2 KO mice is the key to for interpreting whether valdecoxib can give extra benefit and perform other functions besides binding to COX-2. It is difficult to predict whether deficiency in COX-2 or valdecoxib treatment will cause more or less expression of individual cytokines, apoptotic genes or signaling molecules. However, in general, valdecoxib is expected to bestow extra benefits due to its potential to interact with NFκB. In addition, valdecoxib is expected to have added effects on DA system, especially dopamine transportor (DAT), due to its ability to reduce hyperactivity as shown in our previous data.

Induction of apoptotic genes or toxic cytokines is not necessary for cells to die. Nonetheless, it is important to derive the underlying mechanism of the COX-2 inhibitor in neuroprotection so that comparisons can be made with that of the COX-2-deficient mice. The distinction between necrosis and apoptosis as a pathway for neuronal death is not the focus of this study. Rather, because harmful effects from the induction of apoptotic genes and toxic agents released by microglia may vary in
different cell types, specific cell markers may be used if localization of certain proteins of interests are not easily identified.

One time point may not give a true indication of what may happen at other time points, i.e. 1-6 days earlier or 2 and 4 weeks later, but the 1 week time point is a good start for studies with COX-2-deficient mice. First of all, several other studies have done MPTP related works with 7 days post-MPTP injection as the end of the experiments, including studies most related to us (Teismann and Ferger, 2001; Feng et al., 2002; Teismann et al., 2003b). Thus, it will be easier to compare our results with their data. Future studies will include different time points and a comparative look at the results. End points measurement of a dynamic system as DA system with HPLC may not be able to reflect what really happens when mice are hyperactive and when mice are unable to move. In vivo microdialysis may be a more effective way to verify what happens and can repeatedly measure live mice at different time points during behavioral activity measures.
Results

**COX-2 deficiency attenuates MPTP-induced loss of TH-positive neurons and neuronal terminals in the nigrostriatal system**

Because of possible unintended confounding effects associated with oral administration of COX-2 inhibitors (Tegeder et al., 2001), we validated the pharmacological approaches using a genetic approach with COX-2-deficient mice. Immunohistochemical studies revealed no differences between saline-treated mice of different COX-2 genotypes (Figure 5.1). However, among the MPTP-treated mice, the COX-2 knockout (KO) mice exhibited the least TH-positive cell loss, while the wild-type (WT) mice had the most loss (Figure 5.2). The heterozygous (HT) mice showed TH-positive neuronal survival comparable to the KO mice. MPTP significantly reduced the number of the TH-positive neurons in the SNpc, and both the HT and KO mice had significantly more (20-30%) TH-positive neurons than the WT mice (#p < 0.05 and ##p < 0.01, respectively). Nissl staining showed similar trends and statistical results (Figure 5.3 and 5.4), which indicates a true loss of the TH-positive neurons rather than a decrease in TH expression. To determine whether deleting the *cox-2* genes can prevent MPTP-induced SNpc dopaminergic neuron loss as well as the loss of striatal dopaminergic fibers, we assessed the TH immunoreactivity in striata from the different groups of mice by Western blot analysis (Figure 5.5). MPTP significantly reduced striatal TH immunoreactivity compared with the control by 80% in the WT (*p < 0.05) but by less than 50% in the HT and KO mice. Compared to the MPTP-treated WT mice, both the MPTP-teated HT and KO mice had statistically higher striatal TH immunoreactivity (#p < 0.05, Figure 5.6). These results support the effects of dopaminergic neuronal protection observed with the selective COX-2 inhibitor valdecoxib and demonstrate consistency between the pharmacological and genetic approaches.
The selective COX-2 inhibitor valdecoxib or COX-2 deficiency abates microglial activation.

To further evaluate the role of COX-2 in modulating microglial activation in a COX-2 dose-responsive manner, we performed Mac-1 staining and counted the number of activated microglia in COX-2-deficient mice receiving only saline. Saline-treated mice showed no differences among the COX-2 WT, HT and KO genotypes. MPTP-induced microglia activation was again observed in the WT mice but was comparatively less in the HT or KO mice (Figure 5.7). A large number of activated microglia, which had expanded cell bodies and poorly ramified short and thick processes, were seen in the MPTP-treated WT group but were not observed in the MPTP-treated COX-2-deficient group or saline-treated groups (Figure 5.7). This supports our hypothesis that inhibition of COX-2 expression during injury stimuli (MPTP) can reduce microglial activation, which may lead to secondary degeneration and progressive cell loss. In sections with numerous activated microglia, the SNpc can be distinguished from the surrounding areas as the activated microglia tend to stay within the SNpc or along the border of the SNpc.

The numbers of activated microglia in the MPTP-injected HT and KO mice were significantly (four and five times) lower than the MPTP-injected WT mice (###p < 0.001, Figure 5.8). These findings suggest that COX-2 may play a role in modulating microglial activation.

Dopaminergic neuronal survival is inversely correlated to COX-2 and microglial activation.

To determine the relationship between the TH-positive neurons and microglia activation, we performed immunohistochemistry of adjacent SNpc sections of each brain, counted the cell numbers and studied statistical correlations among them. The sections counted for dopaminergic neurons (Figure 5.1 and 5.2) were 30 µm caudal relative to the sections evaluated for Mac-1 immunoreactivity (Figure 5.7 and
5.8) and 30 µm rostral relative to the Nissl-stained sections (Figure 5.3 and 5.4) of the same mouse brain. The Pearson correlation matrix shows the graphic representation of pooled data values for the number of TH- and Nissl-stained neurons and Mac-1-stained activated microglia of each mouse from the COX-2 deficiency study (Figure 5.9). TH-positive neuron counts were highly correlated with Nissl-stained neuron counts ($r > 0.94$), while microglial activation had strong negative correlations with both TH- and Nissl-cell counts (both with Pearson correlation statistic $r \approx -0.80$; $p < 0.05$; Table 5.1). These results suggest a strong co-existence of progressive dopaminergic neuronal degeneration with activation of microglia.

The relationship of COX-2 inhibition or expression to the TH-positive neuronal survival and microglia activation can be inferred from Figures 5.1 and 5.7. Statistically, the correlation of COX-2 to the number of TH- and Nissl-stained neurons and Mac-1-stained activated microglia can be determined by the correlation analysis. It has been suggested that the level of COX-2 in the HT mouse is half of the WT (LANGENBACH et al., 1999); therefore, we assigned the WT as having a full expression capability of COX-2 and designated the amount of COX-2 in the WT, HT and KO mice as 1.0, 0.5 and 0.0, respectively. In Figure 5.10 and Table 5.2, the correlation was analyzed as in Figure 5.9 and Table 5.1 only using the MPTP-treated samples where COX-2 and microglial activation is induced by MPTP (Teismann et al., 2003b). Similar results to the COX-2 inhibitor study were obtained in the COX-2 deficiency study (Figure 5.10 and Table 5.2), which showed that the number of TH-positive neurons strongly positively correlated with the number of Nissl-stained neurons ($r_s \approx 0.9$) and strongly negatively correlated with the number of Mac-1-stained reactive microglia and with the level of COX-2 ($r_s \approx -0.80$ and $r_s \approx -0.90$, respectively, $p < 0.05$, Spearman correlation statistic, Table 5.2). Mac-1-stained reactive microglia counts strongly positively correlate with the level of COX-2 ($r_s \approx 0.90$, $p < 0.05$, Spearman correlation statistic, Table 5.2). These results indicate a strong correlation of progressive dopaminergic neurodegeneration with increased COX-2 and increased microglial activation.
**MPTP-induced loss of locomotor coordination is attenuated in COX-2-deficient mice.**

To characterize behavior resulting from underlying biological changes in the COX-2-deficient mice, we assessed locomotor coordination and balance in 150-second sessions using an automated Rotarod testing apparatus. All mice were trained and were able to stay on the rotating rod at a fixed speed of 16 rpm for 150 seconds. Two days after MPTP injection (Figure 5.11A), the WT mice spent significantly less time overall on the rotating rod at various speeds. This is reflected in a low overall rod performance (ORP) score (*p < 0.05), which implies a loss of motor coordination. The COX-2-deficient mice significantly retain postural balance at several speeds for longer periods of time than the WT mice (#p < 0.05). For both the MPTP-treated HT and KO mice, the ORPs appeared to be similar to the saline controls. After day 4, statistical significance for the difference in ORP between HT and WT mice was lost. On day 4 (Figure 5.11B) and day 6 (Figure 5.11C), the HT mouse performance was between that of the WT and the KO mice. The sensitivity of this assay may be suboptimal due to a ceiling effect from the maximum trial length of 150 seconds, the ability of some coordination-impaired mice to cling onto the rotating treadmill without falling, and nonspecific effects of high rotation speeds on both impaired and control mice. The saline WT mice and the MPTP KO mice remain relatively stable with respect to time spent on the rod at the different speeds throughout the experiment. These behavioral data implicate a beneficial effect of reduced COX-2 levels in COX-2-deficient mice.
Discussion

COX-1 and COX-2 are closely related isoenzymes. In some studies, they show compensatory effects when one of them is deficient (Kirtikara et al., 1998; Reese et al., 1999; Ballou et al., 2000; Zhang et al., 2002). Our laboratories (data not shown) and others have found that there is no COX-1 genotypic difference for MPTP tolerance (Feng et al., 2002; Teismann et al., 2003b). Nevertheless it is still possible that the beneficial effect in COX-2 deficient mice or COX-2 inhibition is due to overexpression of COX-1. Thus, in future studies, we will investigate the underlying mechanism behind the protective effect by inhibiting COX-2 against MPTP toxicity in mice with different COX-1 genotypes. The heterozygous mice are included to study the gene dose response of the complementary effects. COX-1 KO mice with valdecoxib are needed to study the results of depleting both COXs since double COX-1/COX-2 null mice are 100% not viable (Langenbach et al., 1999), which seems to be due to failure of closure and remodeling of arterial connection that directs blood flow away from the pulmonary circulation (Loftin et al., 2001).

There are questions that need experimental settings to answer: What are the effects of inhibiting both COXs with and without MPTP? Does a compensatory phenomenon occur in the striatum and/or SNpc under normal situations and pathophysiological conditions induced by MPTP? Also, is there a dose response due to deficiency in COX-1 as COX-2 is inhibited? The new approach of comparing MPTP treated male mice of different COX-1 genotypes treated with selective COX-2 inhibitors will give insights for these questions.

The comparison between COX-1 KO mice and COX-1 KO mice with valdecoxib with and without MPTP is the key for interpretation whether non-selective COX-2 inhibitors are harmful and whether non-COX mediated events occur following MPTP injection. On the other hand, comparing COX-1 WT
mice and COX-1 KO with and without valdecoxib will give a better clue on complementary effects of COX-1. From our previous Western blot studies, both inhibition of COX-2 (Chapter Four) and COX-2 deficiency (Chapter Five) did not appear to induce change in COX-1 level in our experimental set up (data not shown).

If compensatory effects of COX-2 occur in COX-1 deficient mice, we should see genotypic differences in MPTP treated COX-1 WT, HT and KO mice: if COX-1 HT or KO has higher COX-2 levels to compensate for the loss of COX-1, and lower level of COX-2 protects mice from MPTP toxicity, then COX-1 WT mice, which have less COX-2, would have shown less susceptibility for MPTP toxicity. We have not observed this regarding locomotor activity and HPLC measurement of DA and DA metabolites levels (data not shown). On the other hand, if the protective effects seen in COXIBs treated mice are due to higher elevated level of COX-1 to compensate the loss of COX-2, and different genotypes of COX-1 mice are treated with MPTP, then we should observe more protection in COX-1 WT than HT than KO. Again, we have not seen this. So, it seems like there is no compensatory effect of COX-2 in COX-1 mice in MPTP toxicity, unless of course, the compensatory level is not observable in MPTP-induced PD paradigm or, at least, in behavioral tests and HPLC assay. Nonetheless, the induction of COX-2 due to absence of COX-1 is much less than the other way around (Reese et al., 1999), which may explain the results observed above, and this is why we are focusing on giving COX-2 inhibitor to COX-1 deficient mice, not vice versa, and not giving selective COX-1 inhibitor to COX-1 mice. The goal is not to test every combination possible. The induction may be developmentally dependent (not drug-induced) or take a long time for a COXIB to show effects. Testing the hypothesis using mice with different COX-2 genotypes will help in answering this speculation.

It is difficult to predict whether COX-1 deficient or valdecoxib-treated COX-1 WT mice will have more or less expression of certain cytokines, apoptotic genes or signaling molecules. However, in
general, COX-1 KO with valdecoxib is expected to have negative outcomes due to its total loss of COXs, and our pilot study has shown that COX-1 KO mice treated with valdecoxib have lower DA level (data not shown).

Comparing studies of different null mice may be difficult due to flanking gene problems, especially if they are on different chromosomes as in the case for cox genes (Loftin et al., 2001), but our COX mice have been back crossed for more than 10 generations, and published solution for genetic background problems has been used (Wolfer et al., 2002).

Part of the reason that effects of COX-2 compensation in COX-1 mice are difficult to see is that COX-2 is the inducible form of the COXs. Thus, even with compensation, under pathological conditions, all COX-1 mice (with COX-2 alleles intact) will over-induce COX-2 and harm all genotypes of COX-1 mice similarly. Constitutively overexpressed COX-1 mice crossing with COX-2 deficient mice or vice versa will help clarify this problem.

Both COX-1 and COX-2 activities can be assessed with enzyme immunoassay (EIA) kits to further verify the difference in their activities. These are commercially available (Cayman Chemical Company) and have previously been used by other laboratories (Teismann et al., 2003b), The activity of COXs, however, is expected to be directly proportional to the level of COX-2 protein, as demonstrated in another laboratory (Kirtikara et al., 1998).

Aside from related future studies, this study has confirmed the previous pharmacological data that reduction of COX-2 is protective to dopaminergic neurons from activated microglia. This study, combined with studies in previous chapters, also hints that selective COX-2 may have extra benefit that COX-2 deficiency may not include. First of all, valdecoxib protects higher percentages of
dopaminergic neuronal soma and terminals. Secondly, the reduction of microglial activation is less in the valdecoxib-treated mice but still gives more dopaminergic neurons, suggesting non-microglial inactivation activity of valdecoxib that adds additional survival percentage of the TH-positive neurons. Thirdly, the correlation analysis of the valdecoxib study has stronger values in all correlations except between the inhibitor with TH-, Nissl- and Mac-1 stained cells, implying the presence of non-COX-2 inhibition extra-activity of valdecoxib. When such activity occurred, less amount of valdecoxib was needed for dopaminergic neuronal protection. The results support the hypothesis that COX-2 inhibitors can give extra benefit due to the nature of their chemical structures, compared to COX-2 deficiency, and that extra benefit may exceed the developmental changes of COX-2-deficient mice. Nonetheless, the results can only suggest the relative benefit between the two studies but not the absolute or general truth since the experimental studies used different sets of mice and were conducted at different times, although remaining variables were held constant. Adminstration of valdecoxib to COX-2 KO mice would make the interpretation of these studies stronger.

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Figure 5.1. Photomicrographs of representative SNpc sections stained with an antibody against TH of COX-2-deficient mice. The SNpc tissues were collected 7 days post-MPTP injection. The MPTP (4×15 mg/kg, 1.5 hr interval, i.p.)-treated mice have fewer TH-positive neurons compared to the saline groups. However, COX-2 deficiency reduced the neuronal loss, especially in ablation of COX-2. In COX-2-/- (KO) mice, dopaminergic neurons are protected from MPTP neurotoxicity. Representative TH immunocytochemistry shows a marked loss of TH-positive neurons in SNpc of COX-2 +/+ (WT) mice compared to both COX-2 -/- (KO) and COX-2 +/- (HT) mice after MPTP treatment. Scale bar, 100 µm.
Figure 5.2. Number of TH-positive neurons of COX-2-deficient mice after MPTP injection.

MPTP treatment leads to a significant loss in number of TH-stained neurons. TH-positive cells in the SNpc were bilaterally counted under 40X objective. Data are means ± SEM for six to eight mice per group, **p < 0.01 and ***p < 0.001 compared to saline+vehicle group; #p < 0.05 and ##p < 0.01 compared to MPTP+vehicle group, by ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 5.3. Photomicrographs of representative Nissl-stained SNpc sections of COX-2-deficient mice. The SNpc tissues were collected 7 days post-MPTP injection. The MPTP (4×15 mg/kg, 1.5 hr interval, i.p.)-treated mice have fewer Nissl-positive neurons compared to the saline groups. However, COX-2 deficiency reduced the neuronal loss, especially in ablation of COX-2. In COX-2-/- (KO) mice, dopaminergic neurons are protected from MPTP neurotoxicity. Representative Nissl staining shows a marked loss of TH-positive neurons in SNpc of COX-2 +/- (WT) mice compared to both COX-2 -/- (KO) and COX-2 +/- (HT) mice after MPTP treatment. Scale bar, 100 µm.
Figure 5.4. Number of Nissl-positive neurons of COX-2-deficient mice after MPTP injection.

MPTP treatment leads to a significant loss in number of Nissl-stained neurons. Nissl-positive cells in the SNpc were bilaterally counted under 40X objective. Data are means ± SEM for six to eight mice per group, **p < 0.01 and ***p < 0.001 compared to saline+vehicle group; #p < 0.05 and ##p < 0.01 compared to MPTP+vehicle group, by ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 5.5. TH-stained Western blot of representative striatal tissues from COX-2-deficient mice after MPTP injection. The striatal tissues were collected 14 days post-MPTP injection. Inhibition of COX-2 reduced the MPTP-induced loss of the striatal TH immunoreactivity. The β-actin was used to normalize the TH immunoreactivity. COX-2 deficiency reduced the MPTP-induced loss of striatal TH immunoreactivity.
Figure 5.6. Optical density measurement of the striatal TH immunoreactivity of COX-2-deficient mice after MPTP injection. MPTP-treated WT mice had significantly reduced TH immunoreactivity compared to the saline WT (*p < 0.05). Among the MPTP-treated mice, the COX-2-deficient mice had at least 30% higher TH immunoreactivity than the WT mice. Data are means ± SEM for six to eight mice per group, *p < 0.05 compared to saline+vehicle group; #p < 0.05 compared to MPTP+vehicle group, by ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 5.7. Inhibition of MPTP-induced microglial activation by COX-2 deficiency. Seven days after MPTP treatment, COX-2 +/+ mice had a local increase of microglial activation in the SNpc, which is shown with immunohistochemical stains for Mac-1, compared to saline-treated or MPTP-treated COX-2 +/- and -/- mice. The magnified right panels show activated microglia. In contrast to inactivated striped microglia in MPTP-treated COX-2-deficient and control saline sections, activated microglia in MPTP-treated COX-2 wild-type have a rounder body, fatter processes and denser stain. Scale bar, 100 µm.
Figure 5.8. Number of Mac-1-positive microglia of COX-2-deficient mice after MPTP injection.

MPTP treatment leads to a significant increase in the number of activated microglia in the WT relative to the HT and KO mice. Activated microglia in the SNpc were bilaterally counted under a 40X objective. Data are means ± SEM for six to eight mice per group, ***p < 0.001 compared to saline+vehicle group; ###p < 0.001 compared to MPTP+vehicle group, by ANOVA with subsequent Bonferroni for multiple comparisons.
Figure 5.9. Correlation matrix of TH-, Nissl- and Mac-1-stained cells of C57BL/6 mice. The results from the correlation matrix shown are tabulated in Table 5.1. Spearman correlation statistic, \( n = 6-8 \) per group.
Table 5.1. Correlation analysis of TH-, Nissl- and Mac-1-stained of C57BL/6 mice. As expected, the number of viable TH-positive neurons was strongly correlated with the number of neurons counted with the Nissl stain ($r > 0.90$). Importantly, the number of activated microglia was strongly negatively correlated with TH- and Nissl-stained neurons, both $r \approx -0.80$ ($p< 0.05$, Pearson correlation test, n = 6-8 per group).
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Figure 5.11. Overall rod performance (ORP) of COX-2-deficient mice after MPTP-injected mice.

Animals were trained on the rod for two consecutive days before intraperitoneal MPTP (4×15 mg/kg, 1.5 hr interval) or saline injection. Mice were assessed for their Rotarod performance on day 2 (A), 4 (B) and 6 (C) after MPTP injection. Motor deficit is observed in the MPTP-treated animals, but deficiency in COX-2 significantly prevents this impairment. Deficiency in COX-2 does not affect baseline motor function in the saline-injected COX-2 HT and KO mice. Data are means ± SEM for six to eight mice per group post-MPTP treatment and analyzed by two-way repeated measures ANOVA.
with Bonferroni post hoc test, *p < 0.05 compared to the saline+vehicle group and #p < 0.05 compared to MPTP+vehicle group.
Chapter Six

General Discussion and Conclusions

The major finding of all of our previous studies is that the selective COX-2 inhibitor valdecoxib or deficiency of COX-2 inhibits microglial activation and protects the nigrostriatal dopaminergic system against MPTP-induced neurotoxicity and behavioral deficits. These studies were conducted in attempt to fill in the gap within the literature on the effects of COX-2 inhibition in protecting SNpc dopaminergic neurons from MPTP-induced neurotoxicity. We inhibited COX-2 using both pharmacological and genetic approaches in aged mice (7-9 months old). The HT mice were included to determine the effects of heterozygosity for COX-2 on MPTP-induced cellular toxicity and behavioral impairments. This is because COX-2 is an inducible enzyme, and MPTP may or may not be able to induce the same magnitude of COX-2 in WT mice as in heterozygous mice. We are the first to show a potential link between COX-2 and microglial activation in COX-2 heterozygous mice in the demonstration of correlations between the numbers of TH- and Nissl-stained neurons, Mac-1-stained microglia, and of all genotypes or the three inhibitor dosages of COX-2. We also show that the behavioral benefits of COX-2 inhibition and deficiency correlate well with the observed cellular protection.

Non-biased stereological cell counting indicated that MPTP-treated mice have severe loss of TH-positive neurons in the SNpc while valdecoxib-treated mice, in a dose-responsive manner, have reduced MPTP-associated degeneration of dopaminergic neurons. The concordance between the reduction of TH expression and neurodegeneration was made distinctive by the neuronal loss revealed by quantification of Nissl-stained neurons from adjacent sections caudal to the TH-stained sections. Thus, it appears that selective COX-2 inhibition can attenuate MPTP-induced dopaminergic
neurodegeneration. This result is supported by other investigators that used slightly different experimental setups (Teismann and Ferger, 2001; Sugama et al., 2003a; Teismann et al., 2003b). To ensure that the results obtained with the COX-2 inhibitor are specifically related to COX-2, we conducted an analogous experiment with COX-2-deficient mice. In concurrence with other laboratories (Feng et al., 2002; Teismann et al., 2003b), COX-2 deficiency protects TH-positive neurons in the SNpc from MPTP-induced neurotoxicity. The efficacy of an inhibitor to inhibit COX-2 is likely to give a result intermediate to that of the COX-2 heterozygous (HT) or knock-out (KO) mice. The TH-stained neuronal bodies in the SNpc were protected as well as the striatal TH-stained fibers/terminal, which suggests protection of the nigrostriatal pathway.

Mounting evidence has demonstrated induction of microglial activation in neurodegenerative diseases (Gebicke-Haerter, 2001; Koutsilieri et al., 2002; Liu and Hong, 2003), including PD (Czlonkowska et al., 2002; Hartmann et al., 2003) and PD animal models (Sugama et al., 2003a; Sanchez-Pernaute et al., 2004). It is controversial whether such activation of microglia is beneficial or detrimental to neurons. Our research sheds light on the role of activated microglia in neurodegeneration, as we observed the neuroprotection afforded by selective COX-2 inhibition or deficiency of COX-2, which correlated with attenuation of microglial activation. Using selective COX-2 inhibitors, earlier investigators reported either no inhibition of activated microglia in the mouse MPTP-induced PD model (Teismann et al., 2003b) or decreased activation of microglia in the rat 6-hydroxy dopamine-induced PD model (Isacson et al., 1985). We show for the first time a direct correlation between COX-2 and microglia activation in the mouse MPTP model. Using the optical fractionator method to estimate total cell number, we found a substantial decrease in the activation of microglia within the SNpc in MPTP-treated COX-2 HT mice and an even further reduction in KO mice relative to the MPTP-treated WT mice. From these results we speculate that COX-2 may play some role in suppressing the chronic inflammation and microglial activation that is observed years after MPTP exposure (Langston et al., 1999; McGeer et al., 2003;
Barcia et al., 2004). This secondary inhibition of microglia activation will be expected to attenuate the progressive cell loss induced by the inflammatory response.

Using adjacent SNpc sections to stain and count the number of TH- and Nissl-stained neurons and Mac-1-stained activated microglia, we performed a correlation analysis of TH- and Nissl-stained neurons, COX-2 and Mac-1-stained activated microglia. With all the data analyzed or with only the data from the MPTP-treated animals, we can see that the number of TH-stained neurons has a high positive correlation with the number of Nissl-stained neurons. This implies that the lower TH count is due to degeneration of neurons rather than to reduction of TH expression. The strong negative correlation of Mac-1 to TH- or Nissl-stained neurons, with or without data from the non-MPTP-treated animals, implicates a high number of activated microglia from an MPTP insult co-exists with dopaminergic neuronal cell death. From the valdecoxib study, the number of TH-positive neurons was strongly correlated with the number of Nissl-stained neurons and the magnitude of COX-2 inhibition, but had a strong negative correlation with the Mac-1-stained reactive microglia. Thus, inhibition of COX-2 correlates with reduced degeneration of the dopaminergic neurons. We demonstrate a clear correlation of COX-2 gene expression to the number of Mac-1-stained, activated microglia or to the degeneration of dopaminergic neurons (inferred from TH and Nissl). The WT, HT and KO animals with full (1.0), half (0.5) and zero (0.0) availability of COX-2 had a linear positive relationship with the number of activated microglia (Mac-1) but had an inverse relationship with the numbers of TH- and Nissl-stained neurons. In addition, Mac-1-stained reactive microglia counts strongly positively correlate with COX-2 levels. These results suggest that inhibition or deficiency of COX-2 correlates well with the amount of microglial activation and with the degeneration of the SNpc dopaminergic neurons that result from MPTP-induced neurotoxicity.
Animal behavior is a result of underlying cellular physiology, and the results observed at the cellular level are augmented by the behavioral analysis. We thoroughly assessed motor movement using an automated locomotor activity test and Rotarod apparatus. For the total distance and vertical activity assessment, mice were able to habituate to the testing protocol, and the results from the habituation period suggest that mice adjust to the setup very quickly and that the habituated level of activity is much lower than the first-time exposure. Once adjusted, the level of spontaneous horizontal travel and vertical activity were constant for non-MPTP treated animals throughout the experiment and for all animals prior to MPTP injection. Moreover, valdecoxib does not affect the movement of mice pre-MPTP treatment. Our results differ from those of other investigators who have only reported behavioral testing one day post-MPTP injection or earlier (Ferger et al., 1998b; Ferger et al., 1998a; Shiozaki et al., 1999; Ferger et al., 2000; Teismann and Ferger, 2001). This is likely because we included behavioral measurements at two weeks when the locomotor activity of each mouse became stable and was more consistent within each experimental group. Our present study demonstrates that MPTP significantly reduces total distance traveled and vertical activity of the mice, and that valdecoxib decreases MPTP-induced behavioral impairments.

MPTP-induced loss of locomotor balance and coordination was measured using the Rotarod test after the mice were able to habituate to the machine and the procedure. Pre-training ensured that all the mice could walk on the rod for at least 150 seconds at 16 rpm. As a result, the performance differences we observed post-MPTP injections were unlikely due to individual differences in adapting to a new environment or in strength, but were rather due to the MPTP-induced loss of locomotor coordination or balance. Consistent with the protection seen at the cellular level, the COX-2 HT mouse performance was intermediate to the WT and KO, although the HT mice behaved generally closer to the KO than the WT mice. The heterozygous mice have half the WT level of COX-2 (LANGENBACH et al., 1999), and this may explain the pharmacological benefits of reducing COX-2 activity because they exhibit less
MPTP-induced Parkinson’s disease-like pathology and symptoms as shown in this study. Therefore, it is reasonable to infer that 50% inhibition of COX-2 activity may be sufficient for a protective benefit in this model.

We hypothesized that COX-2 inhibition or deficiency mediates effects involved in the neuroprotection of the SNpc dopaminergic neurons in MPTP-induced mouse parkinsonism. This means that the differences in neuronal degeneration or microglial activation are not due to any effects of COX-2 inhibition on MPTP metabolism or MPP⁺ accumulation but are due to inhibition of COX-2 mediated neurotoxicity. This is because it has been shown that the ratio between the amount of MPTP given and the amount of MPTP reaching the brain is constant and that concentrations of 1-methyl-4-phenylpyridinium (MPP⁺) in the striatum are similar regardless of age (Ricaurte et al., 1987), with various kinds of COX-2 inhibitors used at different doses (Teismann and Ferger, 2001; Teismann et al., 2003b), or in COX-2 KO mice (Teismann et al., 2003b).

Based on earlier studies, we assume that microglial activation occurs before massive death of the dopaminergic neurons (Sugama et al., 2003b). From our study, activated microglia appear in COX-2 KO and WT mice injected with saline, which demonstrates that COX-2 is not required for all microglial activation. We defined this as basal microglial activation; however, we did observe a significant reduction in MPTP-induced microglial activation in the COX-2 KO and inhibitor-treated mice relative to the MPTP-treated WT mice. COX-2 has been proposed to mediate microglial activation through the generation of reactive oxygen species (Czlonkowska et al., 2002). Therefore, we speculate that COX-2 inhibition may mediate secondary microglia activation, which perpetuates the chronic inflammatory response seen in MPTP-induced PD. Studies from our group and from others have shown that activated microglia can cause dopaminergic neuronal death by releasing nitric oxide (Minghetti and
Levi, 1998; Hunot et al., 1999; Wang et al., 2002; Arimoto and Bing, 2003), superoxide free radicals (Gao et al., 2002), or proinflammatory cytokines like tumor necrosis factor-α (Boka et al., 1994).

Microglia can also induce neuritic beading (Takeuchi et al., 2005) or synaptic stripping along dendrites (Schiefer et al., 1999) leading to synaptic disconnection and loss of trophic support and cell death (Isacson et al., 1985; Volpe et al., 1998). Thus, activation of microglia may play an important role in secondary injury by releasing cytokines, reactive oxygen species, and nitric oxide which is important in the progressive loss of neurons and the perpetuation of the inflammatory response observed in PD. In microglial culture, COX-2 inhibitors reduce inducible nitric oxide synthase expression in lipopolysaccharide-activated microglia (Minghetti et al., 1997); therefore reducing nitric oxide production, which suggests a positive modulatory effect of exogenous COX-2 inhibitor on activated microglial toxic substances release. The dopaminergic neurons of the SNpc are vulnerable to inflammation-induced oxidative stress because dopamine metabolism and autoxidation generate reactive oxygen species (Stokes et al., 1999). Consequently, the COX-2-mediated enzymatic reaction contributes to dopaminergic neuronal death by oxidizing dopamine to a reactive dopamine quinone (Hastings, 1995), by increasing DNA oxidation (Nikolic and van Breemen, 2001), or through increased microglial activation leading to chronic inflammation.

To what extent is dopaminergic neuronal death attributable to microglial activation as opposed to a direct effect of cyclooxygenase-mediated reactions? Using the activated microglial inhibitor minocycline, Przedborski’s group showed that activated microglia contribute to about 20% of the MPTP-induced TH-positive cell death (Wu et al., 2002). The same group also showed 30-40% neuroprotection by the COX-2 inhibitor rofecoxib leaving 74-88% neuronal survival after MPTP injection but failed to show inactivation of microglia by COX-2 inhibition or deficiency (Teismann et al., 2003b). The differences between their findings and ours may be due to different experimental settings, procedures, or technical variables, such as using different COX-2 inhibitors, pre-treatment
time with the inhibitors, drug/toxin dosages, or unequal ages of mice. In addition, the previous work examined microglial activation at early stages following MPTP administration; thus, it is possible that examination of pathology at later stages is a better indicator of microglial activation. Moreover, a direct neurotoxic role of COX-2 activation cannot explain why COX-2 inhibitors may be protective or toxic in different PD models or systems (Sairam et al., 2003; Teismann et al., 2003b). As Wang et al. suggested, the final effect of inflammation may vary depending on the balance between neurotrophic and neurotoxic factors released by activated microglia in different systems or approaches, and the discrepancy thus may be due rather to an indirect role of COX-2 in neurotoxicity through regulation of inflammation (Wang et al., 2005). The current result could be because of differences in the persistence of neuronal abnormalities or microglial activation.

Our studies suggest a temporal and spatial relationship between microglial activation and neurodegeneration. Our data is supported by others who also reported this relationship which is accompanied by COX-2 induction (Wu et al., 2002; Sugama et al., 2003a; Teismann et al., 2003b). This suggests that COX-2 may mediate microglial activation and play a key role in amplifying the inflammatory response and other toxic effects, which ultimately exacerbates dopaminergic neuronal loss. From Teismann et al. and Sugama et al. (Sugama et al., 2003a; Teismann et al., 2003b), we can infer that COX-2 and activated microglia play an important role in secondary injury of dopaminergic neurons and in the perpetuation of inflammatory responses since their levels became noticeably high a few days after the MPP+ exposure had already induced the primary loss of the dopaminergic neurons. Damaged neurons can activate microglia, and as Wu et al. showed, the blockade of microglial activation by minocycline prevents MPTP-induced neurotoxicity with evidence of reduced microglial-derived cytotoxic mediators, such as the formation of mature interleukin-1β, the activation of NADPH-oxidase and inducible nitric oxide synthase (Wu et al., 2002). This suggests that microglial activation and release of toxic substances occurs before the secondary or progressive death of the dopaminergic
neurons. Our data as well as those of Sugama et al. suggest that a prolonged oxidative and inflammatory environment, which follows the initial toxic insult, leads to the subsequent loss of neurons that have been compromised but may have potentially reversible damage (Sugama et al., 2003a). In our MPTP paradigm, about 50% of the compromised dopaminergic neurons with reversible damage will die within one to two weeks of the initial injury. In this study, we believe that valdecoxib treatment rescues this population of compromised neurons, which is why we observed the neuroprotective properties afforded by valdecoxib (Figure 9). Therefore, inhibition of COX-2 by valdecoxib or deficiency of COX-2 appears to be able both directly and indirectly to reduce dopaminergic neurodegeneration and progression to behavioral deficits induced by MPTP, possibly through the attenuation of microglia activation.

We have not repeated those experiments done by previous groups of investigators, which support our studies. From the previous works by Teismann et al. and others (Sugama et al., 2003a; Teismann et al., 2003b), we have not overlooked another possible interpretation of our results: that microglia may become inactive faster without COX-2. In general, activated immune cells including microglia become inactive over time through normal regulatory processes. Indeed, it has been shown that peak activation of microglia occurs around day 2-3 after MPTP injection, after which microglial activation dissipates (Sugama et al., 2003a; Teismann et al., 2003b). Our results show extended activation of microglia two weeks after MPTP injection although this degree of activation is less than that seen during the first few days by other investigators. We also show much lesser amounts of activated microglia in COX-2-deficient or COX-2-inhibited mice. We conclude that COX-2 plays a role in sustaining microglial activation, or that activated microglia may be excluded persistent activation if COX-2 is lacking. In other words, our results showed that COX-2 inhibition or deficiency may be related to decreased microglia activation. With time, activation of microglia declines as COX-2 inhibition helps to reduce the perpetuation of a vicious circle that leads to chronic inflammation and secondary neurodegeneration.
Conclusions

Our results provide strong support for the hypothesis that an exacerbated inflammatory process, potentially as a result of COX-2 mediated microglial activation, is detrimental to dopaminergic neurons; and that inhibition of COX-2 prevents progression of PD-like pathology and symptoms by breaking a vicious circle of perpetual microglial activation, thus producing the neuroprotective properties we observe. This is based on the strong correlations we find between COX-2 levels and microglial activation or dopaminergic neurodegeneration. We also present an alternative hypothesis that COX-2 inhibition or deficiency assists in attenuating microglial activation over time, which reduces the progression of the inflammatory response and reduces the perpetuation of the vicious circle instead of inhibiting microglial activation at the early stage when initial injury occurs. This alternative hypothesis does not affect our major point: that inhibiting COX-2 reduces the progression of the inflammatory response by breaking the vicious circle of dopaminergic neuronal cell death. This study suggests that COX-2 plays an important role in the secondary activation of microglia, in the progression of the inflammatory response, and in the progressive loss of the dopaminergic neurons in MPTP-induced PD. Therefore, COX-2 may serve as a potential target for the development of therapeutic strategies to treat the progressive cell loss observed in PD.
Figure 6. Schematic flow chart depicting the role of the vicious circle in dopaminergic neurotoxicity. For this study, the initial insult MPP⁺ exerts direct dopaminergic neurodegeneration (~30% of the original numbers). Neuronal damage initiates the vicious circle with COX-2 and microglia as the key components feeding oxidative and inflammatory damage to the neurons, which in turn progresses to the secondary damage/death which is coupled to the release of factors that initiate another cycle of oxidative and inflammatory insults. The positive feedback loop may continue until the additional neuronal death (~50% of the remaining of the initial death) combined with the initial loss exceed the amount needed for normal motor control (~80% total loss); thus the PD symptoms, such as postural instability or hypokinesia, occurs. This vicious circle helps explain the chronic and prolonged nature of PD progression. It is hypothesized that the blockade of COX-2 activity by selective COX-2 inhibitors or deficiency of COX-2 would attenuate the vicious circle and alleviate dopaminergic neurotoxicity by directly reducing COX-2 free radical production as well as by indirectly decreasing microglial activation and subsequent microglia-mediated damage.

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References


Rattanavijit Vijitruth
Vita

**Date of Birth:** January 31, 1978  
**Place of Birth:** Bangkok, Thailand

**Education:**

2001-present  
**Ph.D. candidate.** Department of Anatomy and Neurobiology  
University of Kentucky College of Medicine, Lexington, KY.

1997-2001  
Washington University in St. Louis, MO.

**Research Experience:**

2002-present  
**Graduate student,** Laboratory of Dr. Guoying Bing, Department of Anatomy and Neurobiology, University of Kentucky College of Medicine.

2001-2002  
**Graduate student,** Integrate Biomedical Sciences (IBS) Program, University of Kentucky College of Medicine. Laboratory rotations with Dr. Subbu Apprasundaram, Dr. Stephen Scheff, Dr. George Smith, and Dr. Guoying Bing.

1999-2001  
**Undergraduate independent research,** Laboratory of Dr. John W. McDonald, Department of Neurology, Washington University College of Medicine.

**Teaching Experience:**

Fall 2002  
**ANA 209 Principles of Human Anatomy,** University of Kentucky, Department of Anatomy and Neurobiology. Teaching Assistant.

Spring 2003  
**ANA 209 Principles of Human Anatomy,** University of Kentucky, Department of Anatomy and Neurobiology. Teaching Assistant.

**Professional Affiliations:**

2002-present  
**Society for Neuroscience.**

**Professional Activities:**

2006  
Ad hoc reviewer: **Neurochemistry International**
Publications:

Research Papers

Rattanavijit Vijitruth, Mei Liu, Dong-Young Choi, Xuan Nguyen, Randy L Hunter, Guoying Bing (27 March 2006) *Journal of Neuroinflammation* 2006, 3:6 Cyclooxygenase-2 mediates microglial activation and secondary dopaminergic cell death in the mouse MPTP model of Parkinson's disease


Abstracts/Poster presentations


Rattanavijit Vijitruth
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