Selective Increase of Neuronal Cyclooxygenase-2 (COX-2) Expression in Vulnerable Brain Regions of Rats with Experimental Wernicke's Encephalopathy: Effects of Nimesulide

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TABLE OF CONTENTS

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ABBREVIATIONS

AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
APP	amyloid precursor protein
ASA	acetylsalicylic acid
ATP	adenosine triphosphate
ATTP	adenosine thiamine triphosphate
BBB	blood brain barrier
BDNF	brain-derived factor neurotrophic factor
CNS	central nervous system
COX	cyclooxygenase
COX-2 ir	cyclooxygenase-2 immunoreactive
EC	endothelial cells
EFA	essential fatty acids
ERK	extracellular signal-regulated protein kinase
eNOS	endothial nitric oxide synthase
KGDH	alpha-ketoglutarate dehydrogenase
GFAP	glial fibrillary acidic acid
IC 50	half-maximal inhibition
iNOS	inducible nitric oxide synthase
IL	interleukin
IFN	interferon
JNK	Janus kinase
JNKK	JNK kinase
LPS	lipopolysaccharide
LTP	long-term potentiation
MAPK	mitogen activated protein kinase
MKK	MAPK kinase
MS	multiple sclerosis
NADPH	nicotinamide adenine dinucleotide phosphate hydrogen
NFkB	nuclear factor kB
NMDA	N-methyl-D-asparate
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NSAID	nonsteroidal anti-inflammatory drugs
PDHC	pyruvate dehydrogenase complex
PG	prostaglandin
PGHS	prostaglandin endoperoxide H synthase
PLA-2	phospholipase A2
SPAK	serine protein kinase

TCA cycle	tricarboxylic acid cycle
TD	thiamine deficiency
TDP	thiamine diphosphate
TK	transketolase
TMP	thiamine monophosphate
TNF	tumor necrosis factor
TPK	thiamine pyrophosphokinase
TTP	thiamine triphosphate
TXA2	thromboxane A2
WE	Wernicke's encephalopathy
WKS	Wernicke-Korsakoff Syndrome

ABSTRACT

Wernicke's encephalopathy is a neuropsychiatric disorder resulting from thiamine deficiency (TD) and is characterized by neuronal loss, astrocytic proliferation and microglial activation. Cyclooxygenases (COX) are enzymes which catalyze the first step in the synthesis of prostanoids. COX-1 is expressed constitutively and COX-2 is the inducible isoform. Groups of TD rats and pair-fed controls were killed at presymptomatic and symptomatic stages of encephalopathy. Cresyl violet and NeuN staining showed decreased numbers of neuronal cells in vulnerable regions (medial thalamus and inferior colliculus) but not in a spared region (frontal cortex). Numbers of GFAP-positive and OX-42-positive cells were increased at symptomatic stage of encephalopathy. Expression of COX-2 mRNA and neuronal COX-2 immunoreactivity were selectively increased in vulnerable regions of TD rats at symptomatic stages of encephalopathy. Nimesulide, a highly selective COX-2 inhibitor, lowered PGE2 levels and precipitated the progression of encephalopathy suggesting that COX-2 in this model is conferring neuroprotection.

RESUMÉ

L'Encéphalopathie de Wernicke est un désordre neuropsychiatrique causé par une déficience en thiamine (TD) et qui se caractérise par une perte neuronale, une astrocytose et l'activation de la microglie. Dans la présente étude, des rats TD ont été sacrifiés aux stades pre-symptomatique et symptomatique de l'encéphalopathie. La coloration au Crésyl violet et l'immunoréactivité pour NeuN, la GFAP et OX-42 ont confirmé une perte neuronale, une astrocytose et l'activation de la microglie dans les régions vulnérables (thalamus médian et colliculus inférieur) mais non dans une région non-vulnérable (cortex frontal). Une augmentation sélective de l'ARNm codant pour COX-2 et de l'immunoreactivité de la protéine dans les neurones a été observées chez les rats TD au stade symptomatique. La nimesulide, un inhibiteur spécifique de la COX-2, a entrainée une diminution des taux de prostaglandines E2 et une précipitation de la progression de l'encéphalopathie chez les rats TD suggérant un effet neuroprotecteur de COX-2.

PREFACE

This thesis is composed of four chapters. Chapter 1 is a review of the literature concerning thiamine, thiamine deficiency, the neuropathology and mechanisms involved in the pathogenesis of Wernicke's encephalopathy, as well as definition, regulation, expression and function of cyclooxygenases (COX). Chapter 1 also describes the pathway leading to prostaglandin synthesis, the COX inhibitors, and finally, the expression of COX in other neuropathological conditions. Chapter 2 describes the experimental design including the animal model, methods, and statistical analysis. Chapter 3 contains the results of the experiments, including immunohistochemistry, RT-PCR and ELISA and chapter 4 is the discussion including potential further directions for the research.

RATIONALE AND OBJECTIVES OF THE STUDY

Cyclooxygenase-2 (COX-2) has been implicated in the pathogenesis of several brain diseases, including stroke, multiple sclerosis and neurodegenerative diseases.COX-2 inhibitors have been used successfully to prevent neuronal cell death in animal models of various neurodegenerative diseases. The role of COX-2 in the pathogenesis of Wernicke's encephalopathy, however, has not yet been investigated even though inflammation clearly plays a role in this pathology. We therefore investigated the expression of COX-2 in brain of animals with experimental Wernicke's encephalopathy at the transcriptional level, investigated the cellular localization of COX-2 using immunohistochemistry, and tested the effects of nimesulide, a highly specific COX-2 inhibitor, on the progression of encephalopathy, and investigated whether this inhibitor could offer neuroprotection in this model.

Chapter 1
INTRODUCTION

1.1 Thiamine and thiamine deficiency

1.1.1 Thiamine

Thiamine (Vitamin B1) is a water-soluble vitamin. In 1910, Suzuki discovered thiamine when doing studies of the effects of rice bran in patients with beriberi. Thiamine is found naturally in whole-grain and enriched cereals, yeast, and the aleurone layer of unpolished rice. Thiamine cannot be stored in the body, and after being absorbed is concentrated in muscle, kidney, heart and brain. (Dreyfus et al., 1959). Thiamine deficiency (TD) is prevalent in alcoholic patients, but also occurs in other conditions that include malignant disease (Shah et al., 1973; Miyajima et al., 1993), hyperemesis gravidarum (Ohkoshi et al., 1994), gastrointestinal disorders (Lindboe et al., 1989), and AIDS (Butterworth et al., 1991; Soffer et al., 1989). TD also occurs in patients with diets high in thiaminase-rich foods (raw freshwater fish, raw shellfish, and ferns) and/or foods high in anti-thiamine factors (tea, coffee, betel nuts). Thiamine deficiency in alcoholics results from inadequate dietary intake (Leevy et al., 1965) reduced gastrointestinal absorption (Tomasulo et al., 1968), and depletion of liver and brain stores of the vitamin (Baker et al., 1964). TD causes a range of neurological disorders including beriberi and Wernicke's encephalopathy, (Wernicke-Korsakoff syndrome).

1.1.2 Thiamine and thiamine phosphate esters

Pyrithiamine and thiazole moieties joined via a methylene bridge make up the thiamine molecule. There are four known natural thiamine phosphate esters: thiamine monophosphate (TMP), thiamine diphosphate (TDP), thiamine triphosphate (TTP) and adenosine thiamine triphosphate (ATTP). TDP is the major form of thiamine with biological function. Pyrophosphokinase converts thiamine to its diphosphate ester (TDP). Adenosine triphosphate (ATP) acts as the pyrophosphate donor (Gubler and Johnson, 1968). In the brain, approximately 80-85% of thiamine is in the TDP form. (Heroux et al., 1995), the remainder is in the thiamine monophosphate (TMP) or thiamine triphosphate (TTP) ester forms. While TDP is involved in carbohydrate metabolism, TTP is involved in membrane excitability and nerve conduction (Bettendorff et al., 1994).

Three important enzyme systems require TDP as cofactor, namely pyruvate dehydrogenase complex (PDHC), alpha-ketoglutarate dehydrogenase (KGDH), and transketolase (TK). Decreases of TK activity occurs in brains of TD animals. In the chronic thiamine deprivation model (without pyrithiamine antagonist injection), transketolase activities are reduced 25% in lateral vestibular nucleus and surrounding pons. Further progression of thiamine deprivation results in a generalized reduction in TK activity. In the pyrithiamine-treated model, all brain regions (vulnerable and non-vulnerable) show TK reduction before onset of neurological symptoms (Giguere et al., 1987). Moreover, the decrease of TK activities parallels the onset of

neurological symptoms of thiamine deficient animals (Dreyfus et al., 1967). TK is an important enzyme in the pentose phosphate shunt. Reduced stability of TK protein and reduction of mRNA translation of TK leads to a decrease of the activity of the enzyme (Sheu et al., 1996). However TK is unlikely to have a primary function in the neurological symptoms of TD since the activity of the pentose phosphate shunt does not change in experimental TD (McCandeless et al., 1976). However, TK levels changes can be used as a useful index to monitor TD.

1.1.3 Wernicke's encephalopathy (WE)

Wernicke's encephalopathy (WE) was first described by Wernicke in 1881. It is characterized by ataxia, ophthalmoplegia, and mental changes. It is also referred to as the Wernicke-Korsakoff syndrome (WKS). WKS symptoms include confusion, aphonia, confabulation and memory loss. WE has a treatable stage, and following thiamine administration (50 to 100mg daily), ophthalmoplegia is generally rapidly reversed (within days). Nystagmus, ataxia and mental confusion respond more gradually (weeks to months), recovery of memory loss is minimal in the majority of patients

Pyrithiamine is a thiamine antagonist; it inhibits thiamine pyrophosphokinase (TPK) that converts thiamine to TDP (Gubler and Johnson, 1968). The consequence is that TDP product decreases. Pyrithiamine also has a function of inhibiting the transport of thiamine into the brain (Spector et al.,

1976) and pyrithiamine can also replace thiamine from the vagus nerve of the rabbit (Cooper et al., 1968).

To study WE, experimental animal models are used. The most common one is the pyrithiamine-treated TD model. Both pyrithiamine and oxythiamine are thiamine analogues and have been used to create experimental thiamine deficiency animal models. While pyrithiamine creates neurological symptoms, which resemble WE, injections with oxythiamine do not lead to those symptoms. As mentioned above, symptoms include ataxia, nystagmus and finally coma. Pyrithiamine crosses the blood-brain barrier (BBB) readily (Rindi et al., 1963), and consumes brain thiamine stores (Murdock et al., 1973).

KGDH activity is decreased in brain in TD animal models (Butterworth et al., 1986). A mitochondrial enzyme KGDH converts α-ketoglutarate to succinyl CoA and is a key rate-limiting enzyme of the ATP-producing tricarboxylic acid cycle (TCA cycle). So TD disturbs the TCA cycle leading to reduced production of energy, and therefore a decrease in ATP content in vulnerable regions of the brain. Since the pyruvate can be produced from lactic acid, one outcome from KGDH dysfunction is lactic acid accumulation. Increased lactic acid can result in acidosis. Lactic acidosis may cause tissue and cellular acidification.

1.1.4 Neuropathology of WE

Neuropathological evaluation of the brains of patients with WE discloses a highly selective and reproducible neuronal cell death pattern involving primarily diencephalic and brainstem structures. Bilateral symmetrical impairments are consistently observed in thalamic nuclei, inferior colliculi, inferior olivary nuclei, mammillary bodies, and lateral vestibular nuclei. Other major structures, including cerebral cortex, caudate nuclei, and hippocampi manifest little significant neuronal cell death in WE. Animal TD models, such as the rat TD model also result in selective neuronal loss in diencephalic and brainstem structures.

Microscopically the lesions vary depending on the age of the lesion, the severity of the disease, and the brain region involved. In general, acute lesions are characterized by slight loss of neurons, axons, myelin sheaths and proliferation of pleomorphic microglia, prominence of blood vessels, including proliferation of capillaries and hypertrophy of endothelial cells. In later stages, TD produces well-defined changes in neurons, microglia, astrocytes and endothelial cells. Changes in microglia and endothelial cells are among the earliest changes reported in TD (Todd et al., 1998).

In chronic TD lesions there is a loss of parenchymal elements and the main reactive cells present are fibrous astrocytes. End stage tissue has a loose vacuolated appearance. Other neuropathological studies have also described differences in the topographic distribution and severity of lesions in acute and

chronic WE (Torvik et al., 1982). In general, acute cases show more extensive and severe lesions than the chronic cases. In acute cases lesions are seen in the mammillary bodies, thalamus, and subependymal structures along the ventricles and aqueduct, whereas in the chronic cases lesions were found mainly in the thalamus (Tovik et al., 1987).

Neuropathological examination of pyrithiamine-induced thiamine-deficient rats shows lesions that are similar to WE in humans. (Papp et al., 1981). Studies of the severest lesions reveal edema, necrosis of neurons and neuropil, and hemorrhages surrounding the capillaries. Similar to WE in humans, histological studies have also revealed that lesions of the inferior colliculus and vestibular nuclei were characterized by bulbuos spongiform appearance of the neurophil and severely damaged neurons. In thalamus, shrunken neurons appear.

Microglia, which makes a substantial fraction of all glial cells (10-20%) (Perry et al., 1994), are generally considered the resident macrophages of the CNS. Activation of immune-like glial cells such as astrocytes or microglia has been showed in numerous neuropathological conditions. (eg: chronic inflammatory diseases). Glial activation can be induced by chemicals released from neurons such as prostaglandins (PGs), nitric oxide, substance P, fractalkine, excitatory amino acids, and ATP released from the primary afferents (Watkins et al., 2001). Glial activation has been reported following

neurodegeneration in CNS and neuronal cell death in peripheral sensory nerves (Watkins and Maier, 2002). Glial activation leads to the release of plenty of inflammatory factors such as, cytokines, kinins, amines, purines and growth factors. The inflammatory factors change neuronal excitability by increasing pre- and post-synaptic signalling of excitatory neurons and by decreasing inhibitory transmission (Yamagata et al., 1993), resulting in transcriptional changes within neurons and the expression of genes such as COX-2 (Kaufmann et al., 1996).

1.1.5 Causes of neuronal cell death

There are several mechanisms proposed to explain neuronal cell death of TD, they include: impaired cerebral energy metabolism (Aikawa et al., 1984), lactic acid accumulation and subsequent pH changes (Hakim et al., 1984), damage to the BBB (Calingasan et al., 1995), glutamate excitotoxicity mediated by the N-methyl-D-aspartate (NMDA) receptor (Langlais et al., 1993), and oxidative stress (Todd et al., 1998).

1.1.5.1 Energy metabolism

Glucose is the primary energy substrate for brain and glucose oxidation is thiamine-dependent. TD will cause the decreased activities of enzymes, such as TK, PDHC, α-KGDH and decreased synthesis of high energy phosphates (such as ATP and phosphocreatine). When thiamine is deficient, reduced activities of thiamine-dependent enzymes cause mitochondrial abnormalities

and impaired cerebral energy metabolism, this is one possible cause of selective neuronal cell death.

1.1.5.2 Lactic acid

When thiamine is deficient, α-KGDH and PDHC activities are reduced, and then brain lactate level rises leading to acidosis, another possible cause of neuronal cell loss (Hakim et al., 1984). Secondly, if the rate of ATP breakdown exceeds the rate of synthesis large quantities of H+ accumulate and intracellular acidification may occur (Erecinska et al., 1989). Acidosis has been implicated in forming free radicals, which can mediate lipid peroxidation and disrupt the membranes. Acidosis can cause denaturation of proteins and nucleic acids; acidosis can also cause cell swelling and osmolysis. Moreover acidosis can inhibit mitochondrial energy metabolism and facilitate calcium release from intracellular stores.

1.1.5.3 BBB

The BBB protects the brain by maintaining ionic homeostasis (Abbott et al., 1992) and protects the brain from chemicals in the blood while still allowing essential metabolic functions. It is composed of endothelial cells, which are jointed by tight junctions in brain capillaries. This restricts passage of substances from the blood stream much more than endothelial cells in capillaries elsewhere in the body. Processes from astrocytes called astrocytic end feet around the endothelial cells of BBB provide biochemical support to

those cells. The BBB is distinct from the similar blood-cerebrospinal fluid barrier, a function of the choroid plexus.

In human WE, blood vessels are changed. Such changes include luminal dilatation and endothelial swelling so that increased permeability of the BBB may play a role in the pathogenesis of selective neuronal loss seen in TD. Several studies have revealed breakdown of BBB in selective brain regions of TD mice and rats (Warnock et al., 1968, Robertson et al., 1971). In early stages of TD, before the appearance of the major histological lesions, permeability of the BBB was seen (Calingasan et al., 1995). Other studies utilizing immunohistological approaches in TD models reveal a disruption of the BBB to large molecules such as IgG and albumin prior to and during the symptomatic stage of the disorder (Harata et al., 1995).

Disruption of the BBB in TD may occur as a result of physical processes. Mechanical opening of endothelial tight junctions are rare in TD animals' brains. In other experiments, the vesicular transport across brain endothelial cells increased (Manz et al., 1972). BBB changes have been assessed quantitatively by studying the effect of TD on the unidirectional blood to brain transfer constant Ki for the low molecular weight species α-aminoisobutyric acid (Hazell et al., 1997). Increased BBB permeability was detected as early as day 10 of TD, with more brain regions showing increased BBB disruption at the symptomatic stage.

Chemical-mediated processes may occur at the opening of the BBB. Amyloid precursor protein (APP) is found in several vulnerable structures, including the thalamus and inferior colliculus (Calingasan et al., 1995). The fragment of APP, amyloid-β peptide increases neuronal membrane fluidity and lipid peroxidation (Avdulov et al., 1997). Such alterations in the membrane lipid environment may result in BBB changes in TD models.

1.2 Cyclooxygenase (COX)

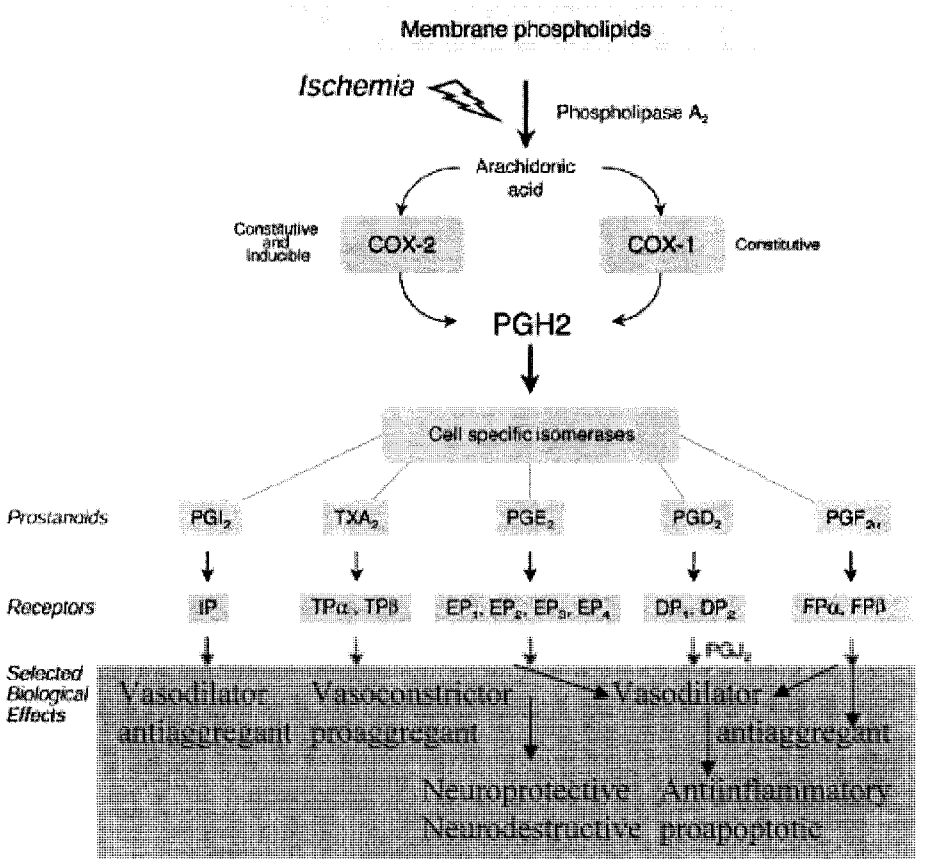
1.2.1 Introduction

Cyclooxygenase (COX) also named prostaglandin endoperoxide H synthase (PGHS) catalyzes the committed step in prostanoid synthesis (Smith and Dewitt, 1996). The importance of COXs resides in their function, which is to convert arachidonic acid to prostanoids As shown below, the biosynthesis of prostanoids involves a three step sequence:

i-) Phospholipase A2 (PLA2) is involved in the first step that initiates hydrolysis of arachidonate and releases it from esterified membrane phospholipid. In cPLA2 knockout mouse, mRNA level and protein level of COX-2 are reduced, and rate of formation of prostaglandin E2 (PGE2) is reduced 50-60% (Bosetti et al., 2003).

ii-) COXs have functions in the second and third steps in the biosynthesis of prostanoids. In the second step, COXs convert arachidonic acid to prostaglandin endoperoxide G2 through oxygenation.

iii-) In the third step, COXs convert PGG2 to PGH2 through peroxidation. It is believed that the cyclooxygenase and peroxidase activities of COX occur at distinct enzymatic sites on the protein.



Modified from Iadecola and Gorelick , Stroke 32, 2005

In the latter process, PGH2 will switch to different biologically active end products, PGD2 PGE2, PGF2, PGI2 (prostacyclin), or TXA2

(thromboxane A2). Specific synthases have functions to form those biologically active end products (Hara et al., 1994). Different prostanoids interact with their respective receptors that each has specific biological activities.

1.2.2 Similarities and differences between COX-1 and COX-2

There are 3 isoforms, namely COX-1, COX-2 and COX-3. COX-1 and COX-2 are encoded by 2 different genes located on human chromosome 9 (murine chromosome 2) and 1, respectively.

While COX-1 is constitutively expressed in most tissues and responds to hormonal stimuli during physiological processes, COX-2 is inducible and typically produces prostanoids that respond to stress, such as infection and inflammation (Masferrer et al., 1994,), hypotonicity (Lundgren et al., 1997), hyperpolarization in nerve cells (Kaufmann et al., 1996; Yamagata et al., 1993), and mechanical or shear stress (Shimizu et al., 1998; Tanabe et al., 1997).

1.2.3 Location of expression of COX-2 in brain.

Western blot and immunohistochemistry is used to study the biomedical characterization and anatomical distribution of COX expression in rat brain. COX-2-like immunoreactive staining is found in dendrites and cell bodies of neurons. COX-2-ir neurons were primarily observed in the cortex and allocortical structures, such as the hippocampal formation and amygdala.

In the diencephalon, COX-2 was expressed in the paraventricular nucleus of the hypothalamus and in the nuclei of the anteroventral region surrounding the third ventricle. COX-2 expression was also observed in the subparafascicular nucleus, the medial zona incerta. In the brainstem, COX-2 expressing neurons were observed in the dorsal raphe nucleus, the nucleus of the brachium of the inferior colliculus, and in the region of the subcoeruleus (Breder et al., 1995)

Also COX protein was also found to be increased in a number of non-neuronal cell types such as macrophages, human monocytes, and synoviocytes, including microglia in CNS inflammation (Bauer et al., 1997; Minghetti et al., 1999). It is well established that activated macrophages commonly express COX-2 (Luo et al., 2002).

In rat brain, while COX-1 immunoreactivities are shown in cerebral cortex and hippocampus, it also prevails in other regions, such as midbrain, pons and medulla (Breder et al., 1995). COX-1 message and immunoreactivity are shown to be localized to neurons, microglial cells in gray and white matter of brains. COX-1 appeared to be expressed in microglial cells regardless of their activation status as determined by HLA-DR immunostainning (Yermakova et al., 1999)

1.2.4 COX-2 triggers

A variety of agonists, including growth factors, phorbol esters, bacterial endotoxin and platelet activating factor can elicit COX-2 expression (Herschman et al., 1996). Besides these, changes of calcium concentrations, NO and inflammatory factors also trigger production of COX-2.

1.2.4.1 NMDA receptor-mediated events

Neuronal COX-2 expression is rapidly and transiently induced by NMDA-dependent synaptic activity or seizures (Yamagata et al., 1993). In ischemia, the impaired area of brain tissue becomes hypoxic and hypoglycemic. Quick release of glutamate from presynaptic nerve terminals causes overstimulation of NMDA and other glutamate receptors (Endres et al., 2002). This excitotoxicity results in influx of calcium. Upregulated calcium mediates activation of PLC/A2, COX-2, followed by signal transduction intermediates (MAPK), nitric oxide, and lipid peroxidation products, respectively, causing tissue damage and neuronal necrosis. Calcium can trigger the inflammatory process (Iadecola et al., 2001). Rapid induction of transcription factor occurs in damaged astroglia, microglia, endothelial cells (EC), and leukocytes and peripheral-derived immune cells causing upregulation of inflammatory cytokines and chemokines.

In TD animals, glutamate concentrations are changed (Butterworth and Heroux, 1989). Raised extracellular glutamate concentrations in brains of TD rats could lead to uncontrolled calcium influx. Glutamate can cause

depolarization, which will activate the NMDA receptor resulting in excessive entry of calcium in neurons. Loss of calcium homeostasis is a major cause of excitotoxic cell death (Siesjo and Bengtsson, 1989). The NMDA receptor antagonist MK-801 can reduce the neuronal cell death caused by TD (Todd and Butterworth, 1999).

Substrates, which increase intracellular calcium, e.g., glutamate and Aβ peptides can activate phospholipase A2 (Murakami et al., 1997), an enzyme that esterifies phospholipid, causing the release of arachidonate which is the primary substrate for COX; the increase of arachidonate possibly causes the increase of COX.

1.2.4.2 NO and free radicals

Nitric Oxide (NO) is an inorganic free radical gas, generated from L-arginine through a complex enzymatic reaction catalyzed by nitric oxide synthase and using nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) as co-substrates and tetrahydrobiopterin, flavin adenine nucleotide and flavin mononucleotide as cofactors. Nitric oxide (NO) has toxic effects in the central nervous system (CNS) (Iadecola et al., 1997). NO promotes oxidative damage by reacting with superoxide anions to form peroxynitrite (ONOO), a strong oxidant anion which easily decomposes to hydroxyl radical OH which is a highly reactive oxidant that reacts indiscriminately (Merill and Murphy, 1996). The oxidative insult can lead to lipid peroxidation, DNA

breakage, and enzyme inactivation, including free radical scavenging enzymes. Superoxide radicals are generated from metabolism of arachidonic acid, mitochondria, xanthine oxidase and autoxidation. Superoxide anions are converted to hydrogen peroxide either spontaneously or by the actions of superoxide dismutase. Reactive oxygen species have been found in the brains of animal models of TD. (Langlais et al., 1997). Rats treated with the free radical scavenger deprenyl showed increased neuronal survival (Todd and Butterworth., 1998)

There are three kinds of isoforms of NOS, neuronal NOS (type I or nNOS), endothelial NOS (type III or eNOS) and induced NOS (type II or iNOS) (Iadecola et al., 1997). nNOS and eNOS isoforms are constitutively expressed whereas iNOS is not normally expressed but can be induced in several cell types including neutrophils, endothelial and muscle cells, macrophages and microglia, by selected stimuli. For example, products of gram positive or negative bacteria and proinflammatory cytokines, such as IL-1 TNFα, and IFN-γ. eNOS expression increases in blood vessels in brains of TD animals (Calingasan et al., 1998; Kruse et al., 2004).

NO can also modify cysteine residues on proteins and produce S-nitrosylated derivatives. S-nitrosylation has been shown to inhibit the activity of various enzymes such as glyceraldehyde-3-phosphate dehydrogenase and glutathione reductase. S-nitrosylation has been shown to trigger apoptotic

pathways through activation of matrix metalloproteinases and therefore likely contributes to neuronal cell death associated with extracellular matrix disruption and BBB breakdown in TD animal models. Similar mechanisms have been confirmed in ischemia models (Gu et al., 2002). Free radical mediated lipid peroxidation can activate COX-2 expression (Halliwell et al., 1989).

Modulation of the COX pathway by NO has been observed in several systems, both in vitro and in vivo. Inhibitory activity of NO was reported in several kinds of cells, including vascular endothelial cells, and macrophages (Habib et al., 1997). Stimulatory activity was also reported in different cells, such as in mesangial cells (Tetsuka et al., 1994), and astrocytes (Molina-Holgado et al., 1995). In some cases, NO has no effect on the COX pathway (Curtis et al., 1996). Distinct mechanisms may account for the reported opposite effects of NO on prostanoid synthesis. In most studies, the stimulatory effect of NO was not dependent on cGMP formation and it was suggested to involve a direct interaction of NO with the heme of COX, which in turn would facilitate COX enzymatic activity. On the other hand, the main target of NO inhibitory action seems to be the expression rather than the activity of COX-2 (Minghetti et al., 1996). Thus, NO is likely to interfere with the COX pathway at distinct levels, possibly by using different signal transduction pathways. The final outcome on prostanoid synthesis in a specific cell type may depend on several factors, including cell redox state and the NO concentration. In a study

on microglia and macrophage cell lines, PGE2 production was prevented through inhibition of COX-2 expression by NO (Swierkosz et al., 1995).

High concentrations of the NOS inhibitors L-NAME and L-NMMA can change the activity of other enzymes, including COX (Southan et al., 1996). Other factors should be considered, for example, during acute inflammation NO alters vascular permeability and causes plasma extravasation, which can promote prostanoid formation (Appleton et al., 1996). Consequently, abrogation of NO production will cause prostanoid inhibition indirectly. It should be considered also that changes in cytokine levels may favour the NOS or the COX-2 pathway. COX-2 and prostaglandin expression can also affect or feedback regulate NO. Oxygen free radicals and inducible nitric oxide synthase (iNOS) can trigger the inflammatory process.

1.2.4.3 Inflammation and IL-1

Many factors can cause inflammation and the increase of expression of inflammatory cytokines or chemokines will trigger inflammation. Peripheral LPS injection induces rapid COX-2 mRNA expression in endothelial cells of the blood vessels of the brain. COX-2 mRNA is induced in the close vicinity of blood vessels when LPS is administrated peripherally regardless of the dose and route of the LPS administration (iv or ip) (Quan et al, 1998). Nuclear factor kB (NFkB), a major protagonist, activates tumor necrosis factor-alpha (TNF-α), interleukin-1alpha(IL-1 α), interleukin-1beta(IL-1 β), and inteleukin-

6 (IL-6). Up-regulation of inflammatory cytokines induces expression of adhesion molecules including intracellular adhesion molecule-1 (ICAM-1).

IL-1 β stimulates expression of COX-2 showing that the JNK/SAPK and p38/RK/Mpk2 are the relevant MAPK pathways activated by this cytokine (Guan et al., 1997). The p38 kinase inhibitor SC68376 prevents IL-1 β - dependent induction of COX-2 in mesangial cells (Guan et al., 1998). In mesangial cells, overexpression of dominant negative mutants of JNKK/MKK4, which stopped activation of JNK/SAPK, also attenuated IL-1-dependent COX-2 expression (Guan et al., 1998) suggesting that both the JNK and P38 signaling pathways are involved.

1.2.5 COX in other models

Indirect evidence of COX-2 involvement in synaptic plasticity has been shown in the recent years by using COX inhibitors in vivo and in vitro in experimental models of synaptic plasticity. COX-2 inhibitors, but not COX-1 inhibitors, administered systemically shortly after training Morris water maze (a hippocampus dependent learning task) was shown to impair spatial memory in rats (Teather et al., 2002). Moreover, pre-training infusions of COX-2 specific inhibitor in the hippocampus of adult rats damaged acquisition of Morris water maze, suggesting that COX-2 activity in the hippocampus of adult rats is necessary for both memory and learning of a spatial task (Rall et al., 2003). The COX-2 activity plays a permissive role in synaptic plasticity

and spatial learning via brain-derived neuronal growth factor neurotrophic factor (BDNF)-associated mechanisms (Shaw et al., 2003). COX inhibitor ibuprofen stopped the increase in PGE2 and BDNF levels following LTP and spatial learning. PGE2, but not PGD2, reversed the suppression of LTP induced by COX-2 inhibitor in hippocampal dentate granule neurons in vitro (Chen et al., 2002). PGE2, which is preferentially formed during the enzymatic activity of COX-2, rather than COX-1, could take part in synaptic plasticity through several mechanisms, including regulation of adrenergic, noradrenergic and glutamatergic neurotransmission, reforming of actin in the cytoskeleton thus influencing the shape of spines and dendrites, and modulation of membrane excitability (Bazan et al., 2003).

1.2.5.1 Ischemic stroke

In rodents and in humans, COX-2 expression in neurons is unregulated in cerebral ischemia. Induction of COX-2 expression is found in glia, vascular cells and inflammatory cells in the ischemic brain (Nogawa et al., 1997; Nakayama et al., 1998; Miettinen et al., 1997; Iadecola et al., 1999). Inhibition of COX-2 decreases ischemic injury after middle cerebral artery occlusion (Nogawa et al., 1997; Sugimoto et al., 2003). In addition, ischemic injury is decreased in COX-2 deficient mice and is increased in transgenic mice overexpressing COX-2 (Dore et al., 2003). COX-2 contributes to the damaged effects of the inflammatory reaction involving the ischemic brain. The free radicals and prostanoids are also the mediators of deleterious effects of COX-2

in ischemic brain injury. Prostanoids, mainly PGE2 is considered to have a pivotal one. PGE2 activates 4 receptors; named EP1 through EP4. There is evidence suggesting that EP2 receptors have cytoprotective function (McCullough et al., 2004). Therefore, EP2 receptors are unlikely to mediate the neurotoxicity of COX-2. EP1 receptor activation is deleterious in models of excitotoxicity and oxygen-glucose deprivation (Kawano et al., 2004).

1.2.5.2 Alzhemeir's Disease (AD)

AD brains lack the classical inflammation signs, such as neutrophil infiltration and perivascular mononuclear cuffing. However, like other neurodegenerative diseases, a local inflammatory reaction is formed by activated microglia and reactive astrocytes, as shown by the existence of antigens associated with microglia/macrophage activation and inflammatory mediators, such as elements of complement system, cytokines and free radicals (Perry et al., 2003).

Although quantitative in situ hybridization revealed no differences between COX-1 mRNA levels in control and AD CA3 hippocampal neurons, COX-1 immunopositive microglia were found in association with Aβ plaques, and the density of COX-1 immunopositive microglia in AD fusiform cortex was upregulated (Yermakova et al., 1999). Elevation of neuronal COX-2 but not COX-1 levels in AD brain was also found. Since Aβ can form free radicals in hippocampal neurons in vitro (Pike et al., 1997), COX-2 can be activated by

activated by the increase of free radicals. In AD frontal cortex (Brodmann area 6), COX-2 immunostainning is mainly localized in neurons (Pasinetti and Aisen, 1998). COX-2 immunostainning was also found selectively localized in neurofibrillary tangle positive neurons with damaged axons (Oka and Takashima, 1997). Since oxidative stress is likely to be involved in the pathogenesis of AD, COX-2 activity may contribute to neurodegeration in AD via oxidative mechanisms.

Hoozemans et al (2004) also showed a colocalization and a significant correlation of neuronal COX-2 expression with cell cycle regulators controlling the G0/G1 phase like cyclin D1 and E and the retinoblastoma protein. Loss of cell cycle control due to changed expression of cell cycle proteins has been proposed as a primary mechanism by which post-mitotic neurons undergo apoptotic death in AD (Nagy et al., 1998)

The moderate increase in COX-2 expression and activity at early stages of AD could explain the protective effect of NSAIDs, which may be blocking early steps leading to neurodegeneration. Upregulated susceptibility to excitotoxicity in COX-2 overexpressing neurons and neuroprotection by COX-2 inhibition has been shown in several experimental models (Aisen et al., 2002). Still, upregulation of COX-2 expression could be an adaptive reaction to pathological conditions, such as early inflammatory processes or oxidative stress, in an attempt to regain lost physiological mechanisms.

1.2.5.3 Multiple sclerosis (MS)

Several inflammatory mediators, including proinflammatory cytokines and free radicals, are thought to contribute to cell damage in MS (Martino et al., 2002). COX-2 immunoreactivity has been observed in experimental autoimmune encephalomyelitis (EAE), a commonly used animal model of MS (Alosi et al., 1999). In certain EAE animal models, COX-2 immunoreactivity is exclusively observed associated within neurons and endothelial cells. The numbers of COX-2 positive endothelial cells increase with the development of the disease, mostly in areas of cellular infiltration. In brain tissue of MS patients, COX-2 positive cells of chronic active lesions are generally located on the border of myelinated regions (Rose et al., 2004).

COX-2 may have a protective role in MS since COX-2 producing PGE2 level was increased during the recovery stage in murine model of MS. (Khoury et al., 1992).

1.2.6 Prostaglandins induced by COXs.

Prostaglandins are synthesized in the cell from essential fatty acids (EFAs) and bind to a subfamily of cell surface seven transmembrane G-protein coupled receptors. There are currently nine known receptors of prostaglandins from various cell types. The activation of the prostanoid cascade is often accompanied by the generation of a broad range of other active molecules, including cytokines and platelet activating factor which may cause neuronal

cause neuronal damage (Chen et al., 1995). On the other hand, a neuroprotective function of prostanoids is evident. The cytoprotective role of prostagladins is shown in various organs besides brain; for example, during the stomach injury and kidney injury (Konturek et al., 1986). PGE2 and PGI2 are reported to protect cultured neurons from several kinds of noxious conditions, including hypoxia/reoxygenation, glutamate-induced injury (Akaike et al., 1994). The neuroprotective effects of PGE2 and PGI2 are mediated by cAMP elevation. Moreover, prostaglandins, in particular PGE2 could have an indirect protective role through the regulation of inflammatory and immune responses occurring in many brain pathologies. In fact, PGE2, besides its well-known proinflammatory activity, it can limit the activation of macrophages and microglia, and regulate immune T, B cells (Weissmann et al., 1993).

Most of the central effects of prostanoids have been attributed to their actions on neuronal receptors of neurons, but glial cells and microglia are also involved in the process. In addition to membrane receptors, nuclear receptors for the 15d-PGJ2 support the hypothesis that prostanoids could also function as physiological intracellular messengers, and directly regulate gene expression (Forman et al., 1995).

1.2.7 COX inhibitors

About 35 years ago, aspirin and nonsteroidal anti-inflammatory drugs (NSAIDS) were shown to block prostaglandin production via the suppression

of COX. (Vane et al., 1971). NSAIDs (i.e., ibuprofen and diclofenac) are widely used analgesics. The agents reversibly suppress platelet COX-1 by about 70-90% at conventional analgesic doses. Besides the effect of reducing inflammation and pain, ASA and NSAIDs have severe side effects such as gastrointestinal complications.

COX-1 is thought to provide physiologic maintenance, while COX-2 is related with inflammation, mitogenesis, and specialized signal transductions. All COX-2 specific inhibitors induce time-dependent inhibition of COX-2 but not COX-1. In other words, COX-2 selective NSAIDs have little effect on COX-1. The inhibition of COX-2 is via a time-dependent, pseudoirreversible mechanism while COX-1 inhibition is due to a very rapid, competitive, and reversible mechanism. When the blood concentration of COX-2 specific NSAIDs is less than that required for half-maximal inhibition (IC50) of COX-1, the activity of COX-1 will be slightly affected while COX-2 will have become functionally inactivated.

Prophylactic injections of the preferential COX-2 inhibitor, nimesulide cause an obvious delay in the onset of amyotrophic lateral sclerosis (ALS) type motor impairment. (Pompl et al., 2003). In other experiments, injection of nimesulide every day, in chronic cerebral hypoperfusion model, decreases white matter damage in different brain location (Wakita et al., 1999). In AD, the effects of modulating PGs levels with COX-2 inhibitors are unclear; for

example, Iwamoto et al (1989) reported an elevation of PGD2 in cerebral cortex of AD while Wong et al (1992) reported a reduction of PGD2 and PGE2 (among other prostanoids) in AD brain. Aspirin (acetylsalicylic acid, ASA) and other widely used NSAIDs abrogate prostanoid formation by suppressing the COX activity, but do not affect the peroxidase activity of the enzyme (Smith et al., 1991).

Chapter 2

EXPERIMENTAL PROCEDURES

2.1 Animal model

Adult male Sprague Dawley rats weighing 200–225 g (Charles River, St. Constant, Quebec, Canada) were housed individually under constant conditions of temperature, humidity and 12 h light/dark cycles and had free access to water at all times. Rats were allowed to adapt to their environment for 3 days prior to the initiation of treatments. Animals were randomly assigned to either thiamine-deficient groups or pair-fed controls. All animal treatment procedures were approved by the Animal Ethics Committee of Saint-Luc Hospital and the University of Montreal.

Rats in the thiamine deficient group (TD) were fed a thiamine-deficient diet (ICN Nutritional biochemicals, Cleveland, OH) and pyrithiamine

hydrobromide (Sigma Aldrich, St-Louis, MO) (0.5 mg/kg body weight; i.p.) was administered daily. Control rats were pair-fed to equal food consumption with the TD rats using the same thiamine-deficient food and were supplemented with daily injections of thiamine (0.1 mg per kg body weight; i.p.). For COX-2 inhibition, rats were injected daily with nimesulide (10mg/kg; i.p.) After 12 days of treatment, rats were randomly assessed for neurological abnormalities and selected to establish a pre-symptomatic stage without any seizures or loss of righting reflex (Hazell et al., 1998). Treatments were continued in the rest of the groups until rats displayed specific behaviors such as loss of righting reflex (where the animal is no longer able to right itself when placed on its back). This stage was considered as the acute symptomatic stage. Any rats exhibiting spontaneous seizures were eliminated from the protocols. The animals were sacrificed by decapitation. The brains were promptly removed and flash frozen in isopentane on dry ice and stored at −80°C until use. They were then dissected on ice into three different regions (i.e. frontal cortex, medial thalamus and inferior colliculus) according to the rat brain atlas of Paxinos and Watson (1986).

2.2 RNA extraction

Total RNA was extracted from medial thalamus and frontal cortex using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. RNA samples were resuspended in diethylpyrocarbonate-treated water then kept at –70°C until use.

2.3 Semi-quantitative RT-PCR analysis

Expression of COX-1 and COX-2 were investigated by reverse transcription-polymerase chain reaction. β-Actin was used as an internal standard to monitor loading variations. Total RNA (1 μg) was mixed with 10 mM Tris-HCl (pH 8.3), 1.5mM $MgCl_2$, 50 mM KCl, 0.01% (w/v) bovine serum albumin, 200μM dNTPs, primers at 1 μM each, AMV reverse transcriptase (80 U/ml), *Taq* DNA polymerase (20 U/ml) and 50 μCi/ml [α ^{32}P]dCTP (3000 Ci/mmol), for a total reaction volume of 50μL. The reactions were initially heated at 50°C for 15 min followed by PCR at 95°C for 30 sec, 60°C for 45 sec and 72°C for 1 min. Amplification efficiency conditions were determined after a kinetic study to ensure all experiments were performed in the exponential phase of amplification and PCR products remain proportional to initial template concentration (data not shown). β-Actin, COX-1 and COX-2 were amplified for 16, 26, 24 cycles respectively. After amplification, samples were electrophoresed onto 9% polyacrylamide gels, dried, autoradiographed at –70°C with an intensifying screen. Each band was excised and Cerenkov radiation was quantified using a β-counter.

Oligonucleotide primers were designed using the PRIME program (genetic Computer Group, Wisconsin) and synthesized by Invitrogen based on the following GeneBank accession numbers: V01217 (β-actin, Nudel et al., 1983), U03388 (COX-1, Feng et al., 1993) and L25925 (COX-2, Kennedy et

al., 1993). The forward and reverse primer sequences were as follows: 5'-CAT CCCCCAAAGTTCTAC-3' and 5'-CCAAAGCCTTCATACATC-3' (β-actin, 347 bp); 5'-CTTTTCAACCAGCAGTTCCAGT-3', and 5'-TTCATCTCTCT GCTCTGGTCAA-3' (COX-2, 281bp); 5'-TGCCCTCTGTACCCAAAGAC-3', and 5'-CAGGACCCATCTTTCCAGAG-3' (COX-1, 201bp). The specificity of the oligonucleotide primers was verified using the program BLASTN (National Center for Biotechnology Information, Bethesda, MD).

2.4 Immunohistochemistry

Rats were anaesthetized with pentobarbital (80 mg/kg) and perfused transcardially with 150 ml of saline followed by 150 ml of neutral-buffered formalin (Fisher Scientific, Pitsburgh, PA) containing 4% formaldehyde in sodium phosphate buffer (PBS) The brains were then removed and post-fixed overnight in the same solution. Sections of 40 μm thickness were cut on a vibratome. Free-floating sections were incubated in 0.3% H_2O_2 in methanol for 30 min at room temperature and then washed with PBS. The non-specific binding sites were blocked using 10% goat serum or 10% rabbit serum in 0.5% Triton X-100/PBS for 30 min. The sections were incubated overnight at 4 °C with either rabbit anti-COX-2 antibody (1:250, Cayman Chemicals, Ann Arbor, MI), mouse anti-NeuN (1/1000, Chemicon, Temecula, CA), rabbit anti-GFAP (1/500, DAKO, Carpinteria, CA), mouse anti-CD11b (OX-42) (1/1000, Serotec, Raleigh, NC). After washing in PBS, the sections were incubated for 1 hour at room temperature with biotinylated goat anti-rabbit or rabbit anti-mouse IgGs secondary antibody (1:500, Vector Laboratories), followed by

incubation with ABC reagent (Vector Laboratories, Burlingame, CA). COX-2 immunoreactivity was subsequently detected by incubation with 3,3′-diaminobenzidine containing urea hydrogen peroxide (Sigma-Aldrich). Negative control sections were treated identically except that the primary antibodies were omitted.

2.5 Prostaglandin E2 ELISA

Medial thalamus, frontal cortex, and inferior colliculus tissue samples were homogenized at 4 °C in 50 mM Tris–HCl buffer pH 7.4, containing a protease inhibitor cocktail (Sigma-Aldrich) using a Potter-Elvehjem tissue homogenizer. After centrifugation of samples at 12,000×g for 45 min, the cytosolic fraction was collected and stored at −80°C. Protein concentrations were estimated using protein assay kit (Bio Rad Laboratory, Hercules, CA) using bovine serum albumin as standard. Prostaglandin E2 levels were measured using commercially available ELISA kits (Cayman Chemicals) according to the manufacturer instructions.

2.6 Transketolase assay

In order to confirm the TD status of experimental animals, the activity of the thiamine-dependent enzyme transketolase was measured as previously described (Giguere et al., 1987). Briefly, the medial thalamus, inferior colliculus and frontal cortex were freshly dissected from the brains of symptomatic TD rats and their pair-fed controls, and the tissues harvested in

50mM potassium phosphate buffer (pH 7.6), homogenized in 50 volume of glycylglycine buffer 40mM, pH 7.6. Homogenate (0.5ml) was added to 0.3ml ribose-5-phosphate and the mixture incubated for 30 min at 37 °C. The reaction was stopped by the addition of 20% TCA and the samples centrifuged at 2500g for 15 min. Aliquots of the supernatant were added to concentrated H2SO4 and boiled for 4 minutes. Addition of cysteine (3%) was followed by measurement of sedoheptulose-7-phosphate values 15h later from the difference in absorbance at 510 and 540 nm using standard curve. significance between comparisons. The cell counts were expressed as means +/-S.D. A probability of $P < 0.05$ was chosen to establish significance between the groups.

2.7 Statistical analysis

Data were expressed as mean +/- SEM values. Statistical analysis was performed using two-way analysis of variance (ANOVA) with Tukey post hoc analysis to establish significance between comparisons. A probability of $P<0.05$ was chosen to establish significance between the groups. Data were analyzed by using Prism 4.0 software (Prism 4.0, San Diego, CA).

Chapter 3

RESULTS

3.1 Progression of encephalopathy

In agreement with previous reports using the pyrithiamine-treated rat model of thiamine deficiency (Gibson et al., 1984; Butterworth and Héroux, 1989), daily administration of pyrithiamine to rats fed a thiamine-deficient diet resulted within 14 days in neurological symptoms including loss of righting reflex and opisthotonus (symptomatic stage). Neither pair-fed controls at any time point nor thiamine-deficient rats killed at day 12 of treatment (presymptomatic stage) showed any such neurological symptoms.

3.2 Transketolase activity

As shown in Table 1, brain transketolase (TK) activity was significantly reduced at symptomatic stage of encephalopathy in both vulnerable (medial thalamus and inferior colliculus) and spared regions (frontal cortex). TK activity was reduced by 56% ($p<0.01$) in medial thalamus, 27% in inferior colliculus ($p<0.01$), and 18% in frontal cortex ($p<0.01$) in symptomatic TD rats.

Brain regions	Pair-fed controls	Pre-symptomatic	Symptomatic
Medial thalamus	7.5 ± 0.22	6.32 ± 0.13*	3.26 ± 0.30*
Inferior colliculus	8.62 ± 0.36	8.28 ± 0.13	6.30 ± 0.16*
Frontal cortex	10.26 ± 0.21	9.3 ± 0.16**	8.40 ± 0.10*

Table 1. Transketolase activity in the brain of TD rats (nmoles/min/mg protein).*$p<0.01$ v.s. pair-fed; **$p<0.05$ vs pair-fed

3.3 Nissl staining

Cresyl violet staining was performed to investigate changes in cell morphology in the frontal cortex, inferior colliculus and medial thalamus of TD rats. Extensive neuronal damage can be seen in the medial thalamus and inferior colliculus, but not in the frontal cortex, of symptomatic rats compared with pair-fed controls (Figure 1). No changes were observed in the vulnerable regions of TD rats at presymptomatic stages of encephalopathy.

3.4 NeuN immunolabeling

Immunohistochemical staining was performed to investigate changes in the expression of the neuronal marker NeuN in the frontal cortex, inferior colliculus and medial thalamus of TD rats. Decreased NeuN immunoreactivity was observed in neurons of the medial thalamus and inferior colliculus of symptomatic rats compared with basal level of expression in the pair-fed control group (Figures 2, 3). NeuN immunoreactivity was unchanged in the frontal cortex or in vulnerable regions of rats at presymptomatic stage of encephalopathy.

3.5 Glial Fibrillary Acidic Protein (GFAP) immunolabeling

Immunohistochemical staining was performed to investigate GFAP expression in the frontal cortex, inferior colliculus and medial thalamus of TD

rats. Increased GFAP immunoreactivity resulting from reactive gliosis was observed in the medial thalamus and inferior colliculus of symptomatic rats compared with basal level of expression in the pair-fed control rats (Figure 4). No increase of GFAP immunoreactivity was observed in the frontal cortex or in vulnerable regions of rats at presymptomatic stage of encephalopathy.

3.6 OX-42 immunolabeling

Immunohistochemical staining was performed to investigate OX-42 expression in the frontal cortex, inferior colliculus and medial thalamus of TD rats. Increased OX-42 immunoreactivity resulting from microglial activation was observed in the medial thalamus (1.5 fold, $p<0.001$) and inferior colliculus (1.4 fold, $p<0.001$) of both pre-symptomatic and symptomatic rats compared with basal level of expression in the pair-fed control rats (Figures 5, 6). No increase of OX-42 immunoreactivity was observed in the frontal cortex or in the vulnerable regions of rats at presymptomatic stage of encephalopathy.

3.7 COX-2 immunolabeling

Immunohistochemical staining was performed to investigate COX-2 expression in the frontal cortex, inferior colliculus and medial thalamus of TD rats. Significant increases of COX-2 immunoreactivity were observed in neurons of the medial thalamus (2.4 fold, $p<0.001$) and inferior colliculus (2.2 fold, $p<0.001$) of TD rats at symptomatic stage of encephalopathy compared

with basal level of expression in the pair-fed control rats (Figures 7, 8). No increase of COX-2 immunoreactivity was observed in the frontal cortex or in vulnerable regions of rats at presymptomatic stage of encephalopathy.

3.8 PGE2 assay

Brain PGE-2 levels as measured by ELISA were increased concomitantly to COX-2 mRNA in vulnerable regions. Significant increases were observed in medial thalamus (1.9 fold, $p<0.001$) and in inferior colliculus (2.9 fold, $p<0.001$) of TD rats at symptomatic stages of encephalopathy compared with basal PGE2 levels in pair-fed control. However, PGE-2 levels were unchanged in frontal cortex as well as in the medial thalamus and inferior colliculus of TD rats at pre-symptomatic stage of encephalopathy (Figure 9).

3.9 RT-PCR analysis

After normalization to β-Actin, expression of COX-2 mRNA was significantly increased in the medial thalamus of symptomatic thiamine deficient rats (2 fold, $p<0.001$, Figure 10a) compared to pair-fed controls. Significant increase of COX-2 mRNA was also observed in the inferior colliculus of symptomatic rats (2 fold, $p<0.001$, Figure 10b). No significant alterations of COX-2 expression were observed in medial thalamus or inferior colliculus at presymptomatic stage. There was no significant variation of COX-2 mRNA in the frontal cortex (Figure 10c). Expression of COX-1 mRNA was unchanged in all three regions.

3.10 Effect of COX inhibitors on progression of encephalopathy

As shown in Table 2, nimesulide significantly ($p<0.05$) precipitated the onset of symptoms in TD rats from 12 days and 27.4±2.7 hours to 12 days and 22.4±1.7 hours.

Drug	Time to symptomatic stage (hours)
Saline	27.4 ± 2.7
Nimesulide	22.4 ± 1.7*

Table 2. Progression of encephalopathy (after day 12) in TD rats treated with COX inhibitors nimesulide (*$p<0.05$ v.s. saline)

3.11 Effect of COX inhibitors on PGE2 levels

The effects of nimesulide on PGE2 levels were investigated in the frontal cortex, inferior colliculus and medial thalamus of TD rats at symptomatic stage of encephalopathy. Nimesulide significantly ($p<0.001$) reduced PGE2 levels in the medial thalamus (1.4 fold) and inferior colliculus (2.0 fold) compared to saline-injected symptomatic TD rats (Figure 9). Nimesulide, however,had no effect on PGE2 levels in the frontal cortex.

3.12 Effect of COX inhibitors on neuronal cell death

The effects of nimesulide on neuronal cell death were investigated using NeuN immunostaining. Nimesulide-treated animals killed at symptomatic stage of encephalopathy showed significant less NeuN-positive cells in medial thalamus (11.8%; $p<0.01$) and inferior colliculus (23.7%, $p<0.001$) compared to pair-killed saline-injected TD rats (Figures 11, 12, 13).

CHAPTER 4

DISCUSSION

We have investigated cyclooxygenase-2 (COX-2) expression in vulnerable (medial thalamus, inferior colliculus) and spared (frontal cortex) regions of rats with thiamine deficiency. Expression of COX-2 mRNA was significantly increased in vulnerable regions but not in spared regions of TD rats at symptomatic stage (14 days) compared to pair-fed controls or pre-symptomatic rats (days 12). Induction of COX-2 expression was accompanied by a significant increase in PGE2 synthesis in vulnerable regions at symptomatic stages of TD.

COX-2 immunoreactivity in the brain of TD rats was observed in neurons, a cell type that constitutively expresses COX-2. It is well established that COX-2 activity is closely linked to activation of N-methyl-D-aspartate (NMDA) receptors and that induction of COX-2 expression occurs mainly through activation of these receptors (Marcheselli and Bazan, 1996). Evidence for NMDA receptor-mediated excitotoxicity mechanism of neuronal cell death in TD includes the findings of decreased glutamate transporter expression and increased extracellular glutamate concentrations (Hazell et al., 1993). These findings suggest that overstimulation of NMDA receptors could account for upregulation of COX-2 expression in this model.

Administration of nimesulide, a highly specific COX-2 inhibitor, significantly reduced PGE-2 levels in vulnerable regions, precipitated progression of encephalopathy and aggravated neuronal cell death mediated by TD, suggesting that endogenous prostaglandins have neuroprotective functions in this model. Although nimesulide has been shown to prevent neuronal cell death in many neurological models such as ischemia (Candelario-Jalil et al., 2002), detrimental effects of other COX-2 inhibitors on neuronal survival have also been observed in other pathological conditions. The selective COX-2 inhibitor NS-398, as well as the non-selective COX inhibitor indomethacin, has been shown to aggravate kainic acid-induced seizure and neuronal cell death in the hippocampus (Baik et al., 1999). NS-398 as well as nimesulide has also been shown to aggravate neuronal cell death in an in vitro model of ischemia (Gendron et al., 2004). Furthermore, the PGE2 receptors have been shown to exert neuroprotective effects in cerebral ischemia (McCullough et al., 2004) and to rescue motor neurons in a model of amyotrophic lateral sclerosis (Bilak et al., 2004).

Although COX-2 has been implicated in neuronal cell death in many human neurological diseases such as such as multiple sclerosis, amyotrophic lateral sclerosis, Parkinson disease, Creutzfeldt-Jakob disease, and Alzheimer disease (Minghetti, 2004), results of the present study suggest that, in contrast, COX-2 plays a beneficial role in TD. However, considering the opposing effects that selective COX-2 inhibitors have given in some

models (Gendron et al., 2004) and the dual effects of prostaglandin receptor signaling on neuronal survival (Wu et al., 2007), further studies using transgenic mice lacking COX-2 gene will be necessary to corroborate the neuroprotective function of COX-2 in TD.

SUMMARY AND CONCLUSION

In summary, our results confirm that TD causes selective neuronal cell death in vulnerable regions as shown by cresyl violet coloration and NeuN immunoreactivity; causes microglial activation and astrocytosis in vulnerable regions as shown by increases OX-42 and GFAP immunoreactivities. Our results also demonstrate, for the first time, that TD leads to selective increases in COX-2 expression in vulnerable regions as shown by immunohistochemistry and RT-PCR analysis, and that nimesulide, a highly selective COX-2 inhibitor, accelerates the progression of encephalopathy.

In conclusion, our data suggest that upregulation of neuronal COX-2 in thiamine deficiency results from overstimulation of NDMA receptor and plays neuroprotective role. The opposing effects of COX-2 inhibitors on neuronal survival observed in different models of neurological diseases may be explained by difference in cellular compartmentation of the isomerases requiered for prostanoids synthesis, as well as region-specific expression of

different receptor subtypes, therefore leading to different biological activities in different brain regions.

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LEGENDS TO FIGURES

Figure 1. Cresyl violet stained sections of the medial thalamus, inferior colliculus, and frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed controls. Fig 1 (a) shows no extensive neuronal damage in the frontal cortex. .Fig 1(b) show loss of total cells (neurons,microglia and astrocytes) in medial thalamus. Fig 1(c) shows loss of total cells (neurons, microglia and astrocytes) in inferior colliculus

Figure 2. NeuN immunostained sections of the medial thalamus, inferior colliculus, and frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed controls. Fig 2(a) shows no neuronal damage in the frontal cortex. Fig 2(b) shows loss of neurons in medial thalamus as a function of severity of symptoms of TD. Fig 2(c) shows loss of neurons in inferior colliculus as a function of severity of symptoms of TD.

Figure 3. Histogram showing number of NeuN-positive cells in the medial thalamus, inferior colliculus, and frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed controls.

Figure 4. GFAP immunostained sections of the medial thalamus, inferior colliculus, and frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed controls. Fig 4(a) shows no reactive gliosis in the frontal cortex. Fig 4(b) show increased GFAP immunoreactivity in medial thalamus of

symptomatic rats due to reactive gliosis. Fig 4(c) show increased GFAP immunoreactivity in inferior colliculus of symptomatic rats due to microglial activation.

Figure 5. OX-42 immunostained sections of the medial thalamus, inferior colliculus, and frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed controls. Fig 5(a) shows no reactive gliosis in the frontal cortex. Fig 5(b) show increased OX-42 immunoreactivity in medial thalamus of symptomatic rats due to microglial activation. Fig 5(c) show increased OX-42 immunoreactivity in inferior colliculus of symptomatic rats due to microglial activation.

Figure 6. Histogram showing numbers of OX-42-positive cells in the medial thalamus, inferior colliculus, and frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed controls.

Figure 7. COX-2 immunostained sections of the medial thalamus, inferior colliculus, and frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed controls. Fig 7(a) shows no COX-2 immunoreactivity increases in the frontal cortex. Fig 7(b) show increased COX-2 immunoreactivity in medial thalamus of symptomatic rats. Fig 7(c) show increased COX-2 immunoreactivity in inferior colliculus of symptomatic rats.

Figure 8. Histogram showing numbers of COX-2-positive cells in the medial thalamus, inferior colliculus, and frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed controls

Figure 9. Histogram showing PGE2 levels in the medial thalamus, inferior colliculus and frontal cortex of TD rats at presymptomatic and symptomatic stage of encephalopathy following injection of nimesulide, and in pair-fed controls.

Figure 10. RT-PCR analysis of COX-1 and COX-2 mRNA in a-medial thalamus, b-inferior colliculus, and c-frontal cortex of TD rats at different stages of encephalopathy, and in pair-fed control.

Figure 11. NeuN immunostained sections of the medial thalamus from nimesulide-treated TD rats at symptomatic stage of encephalopathy, saline-treated TD rats (pair-killed), saline-treated TD rats at symptomatic stage of encephalopathy, and pair-fed controls.

Figure 12. NeuN immunostained sections of the inferior colliculus from nimesulide-treated TD rats at symptomatic stage of encephalopathy, saline-treated TD rats (pair-killed), saline-treated TD rats at symptomatic stage of encephalopathy, and pair-fed controls.

Figure 13. Histogram showing the numbers of NeuN-positive cells in the medial thalamus and inferior colliculus of nimesulide-treated TD rats at symptomatic stage of encephalopathy, saline-treated TD rats (pair-killed), and saline-treated TD rats at symptomatic stage of encephalopathy, and pair-fed controls.

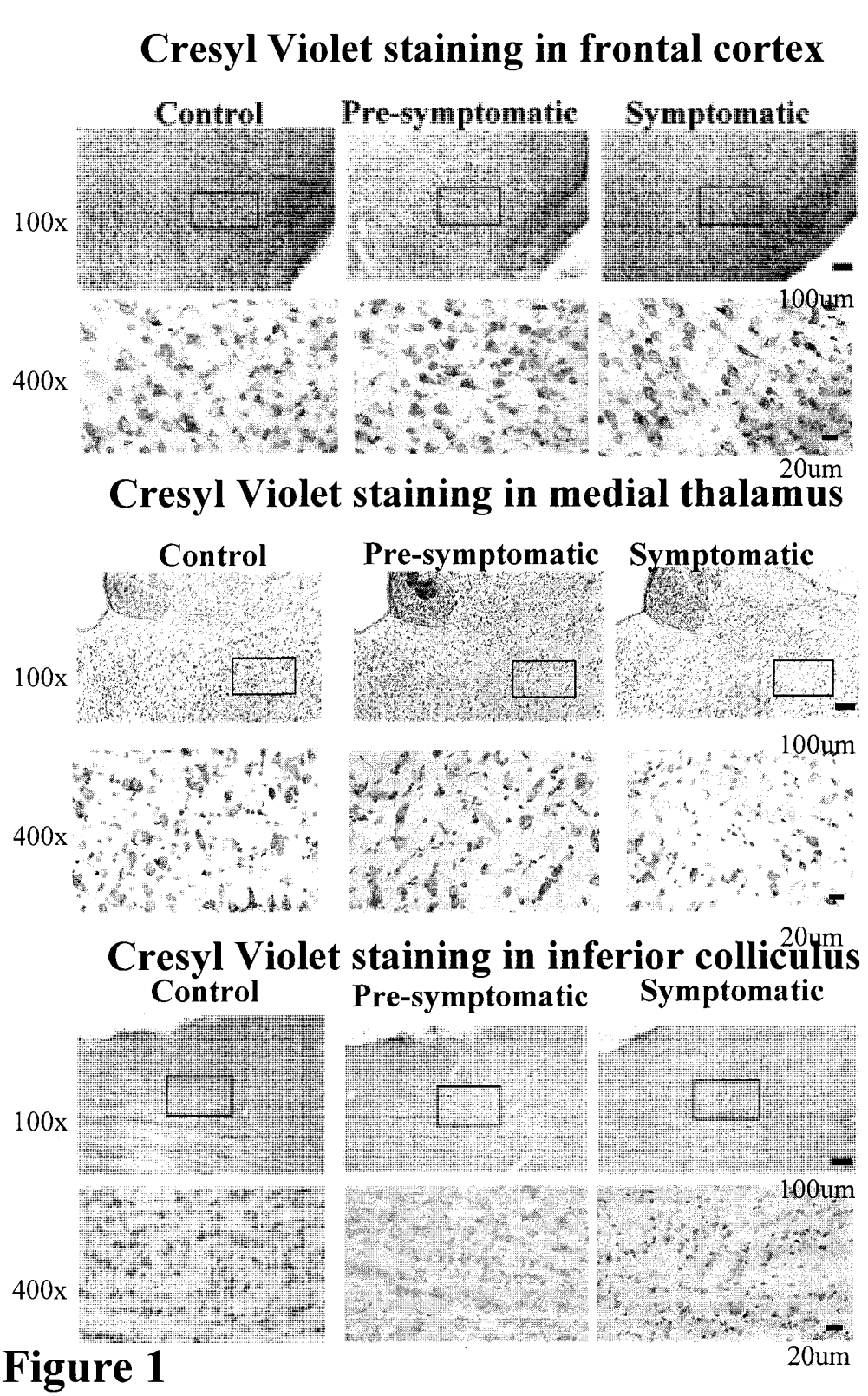

Cresyl Violet staining in frontal cortex
Control
Pre-symptomatic
Symptomatic
100x
100um
400x
20um
Cresyl Violet staining in medial thalamus
Control
Pre-symptomatic
Symptomatic
100x
100um
400x
20um
Cresyl Violet staining in inferior colliculus
Control
Pre-symptomatic
Symptomatic
100x
100um
400x
20um

Figure 1

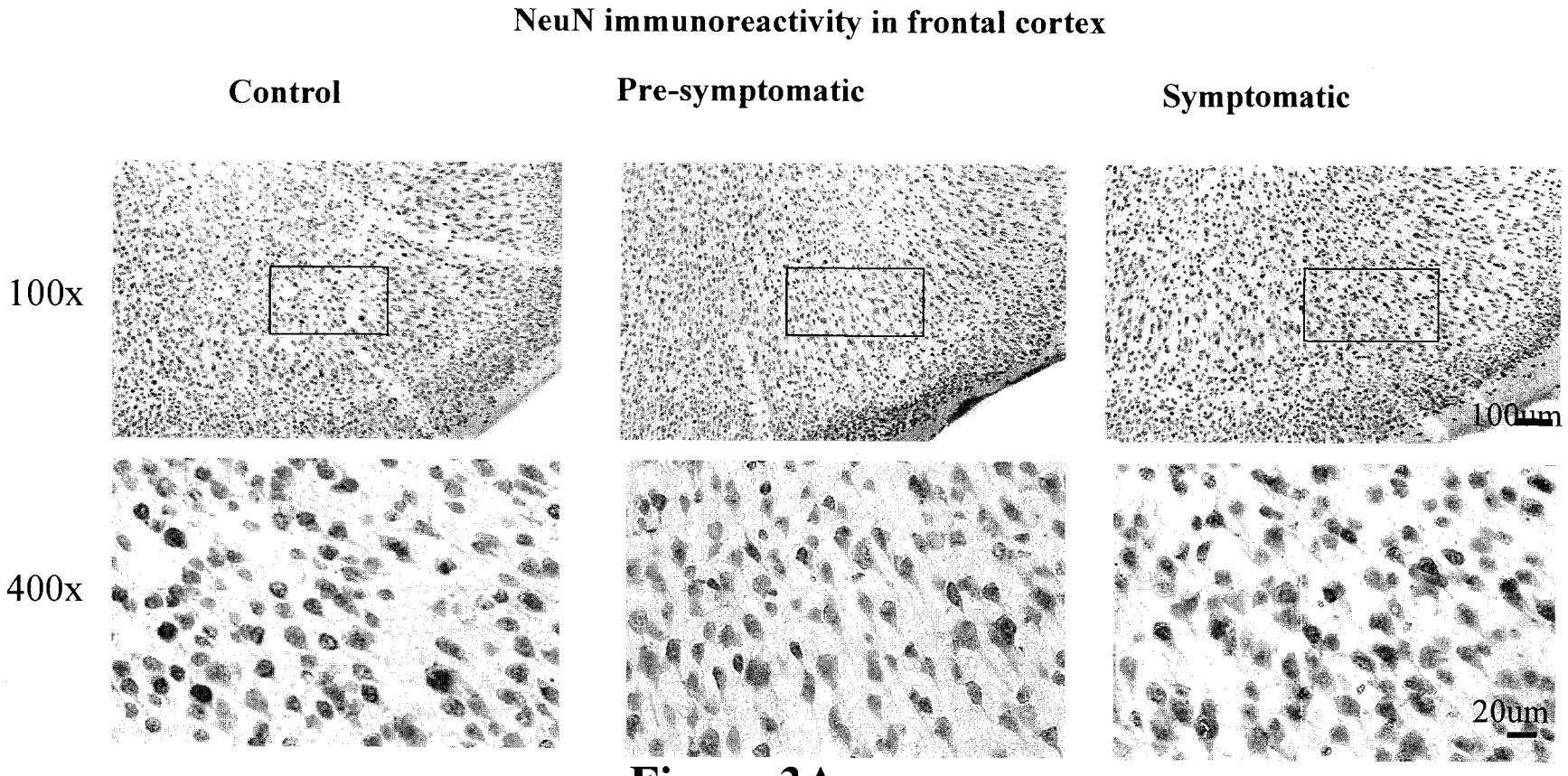

NeuN immunoreactivity in frontal cortex
Control
Pre-symptomatic
Symptomatic
100x
400x
100um
20um

Figure 3A

NeuN immunoreactivity in medial thalamus

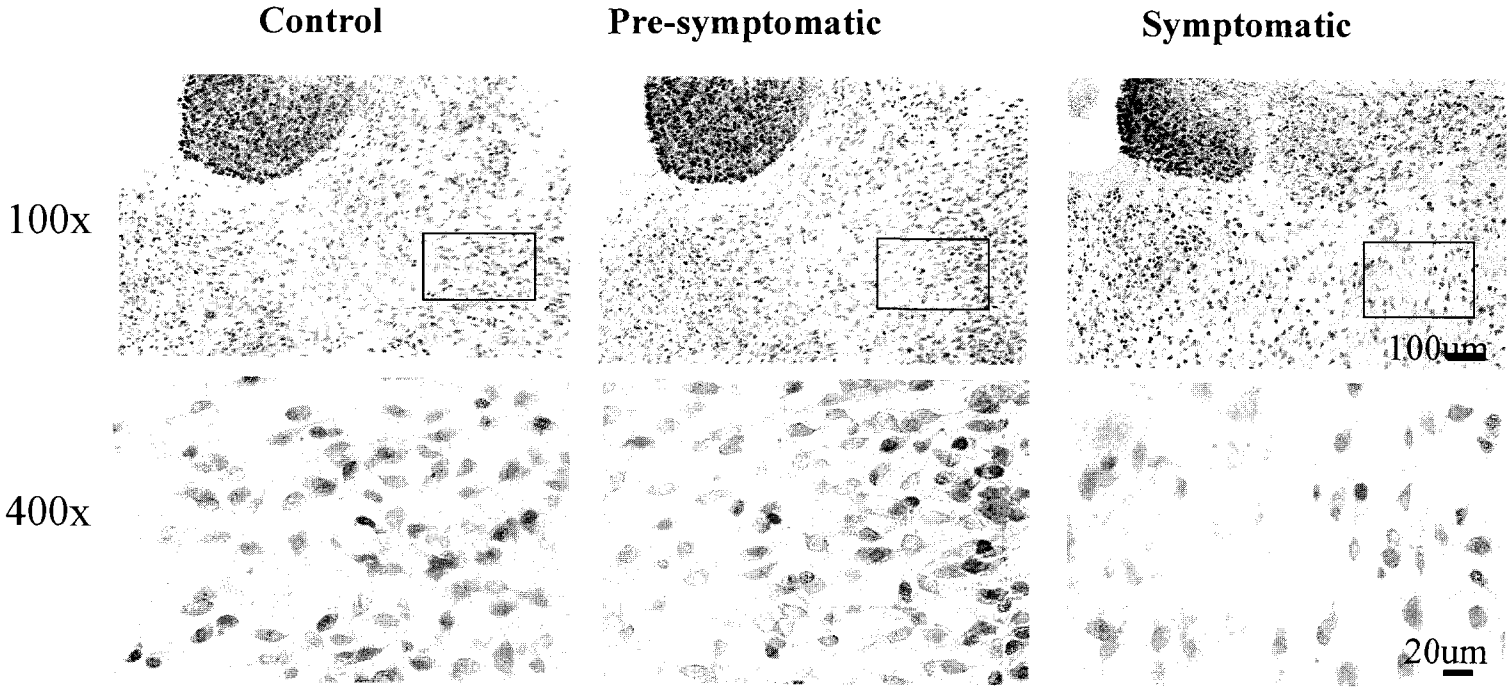


Figure 3B

NeuN immunoreactivity in inferior colliculus

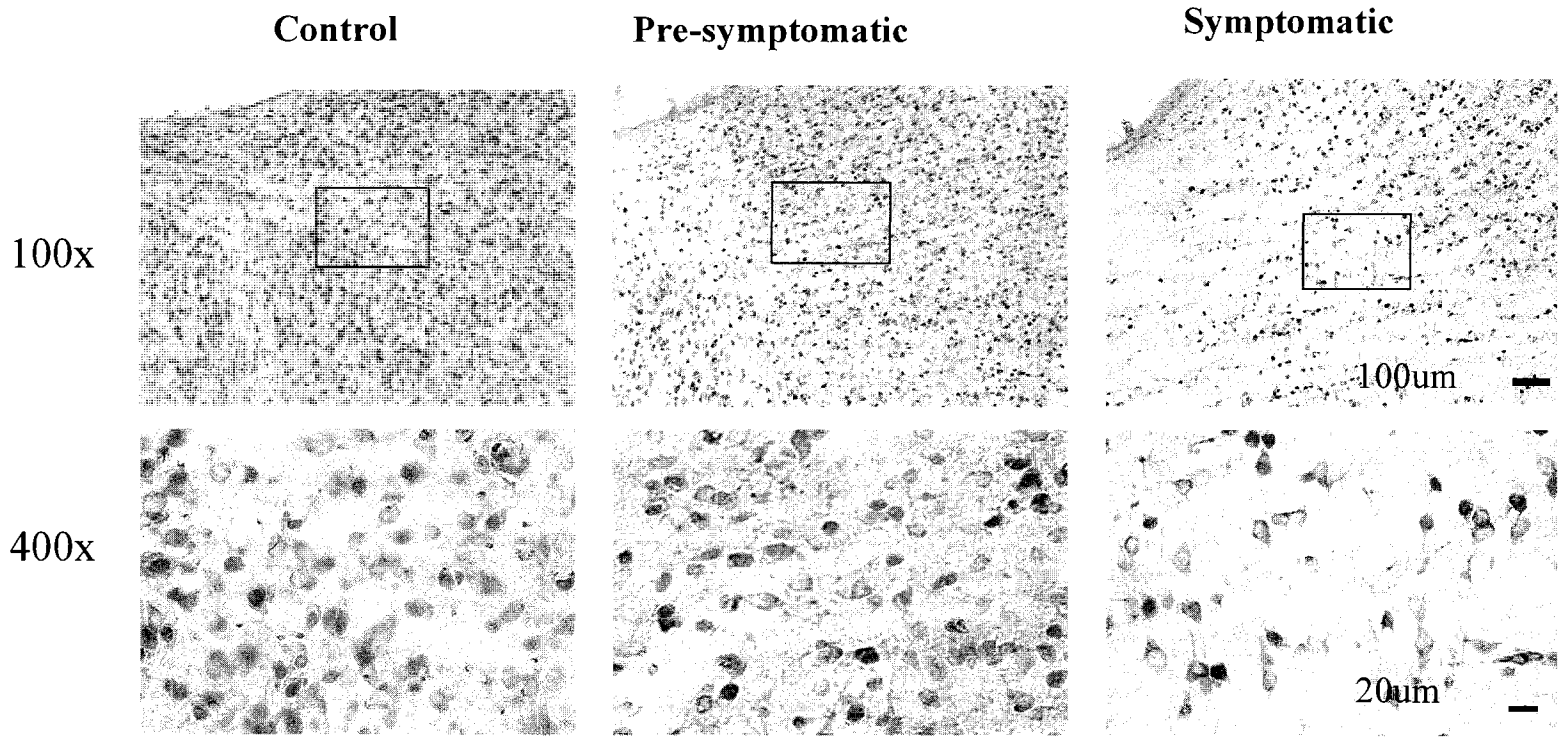


Figure 3C

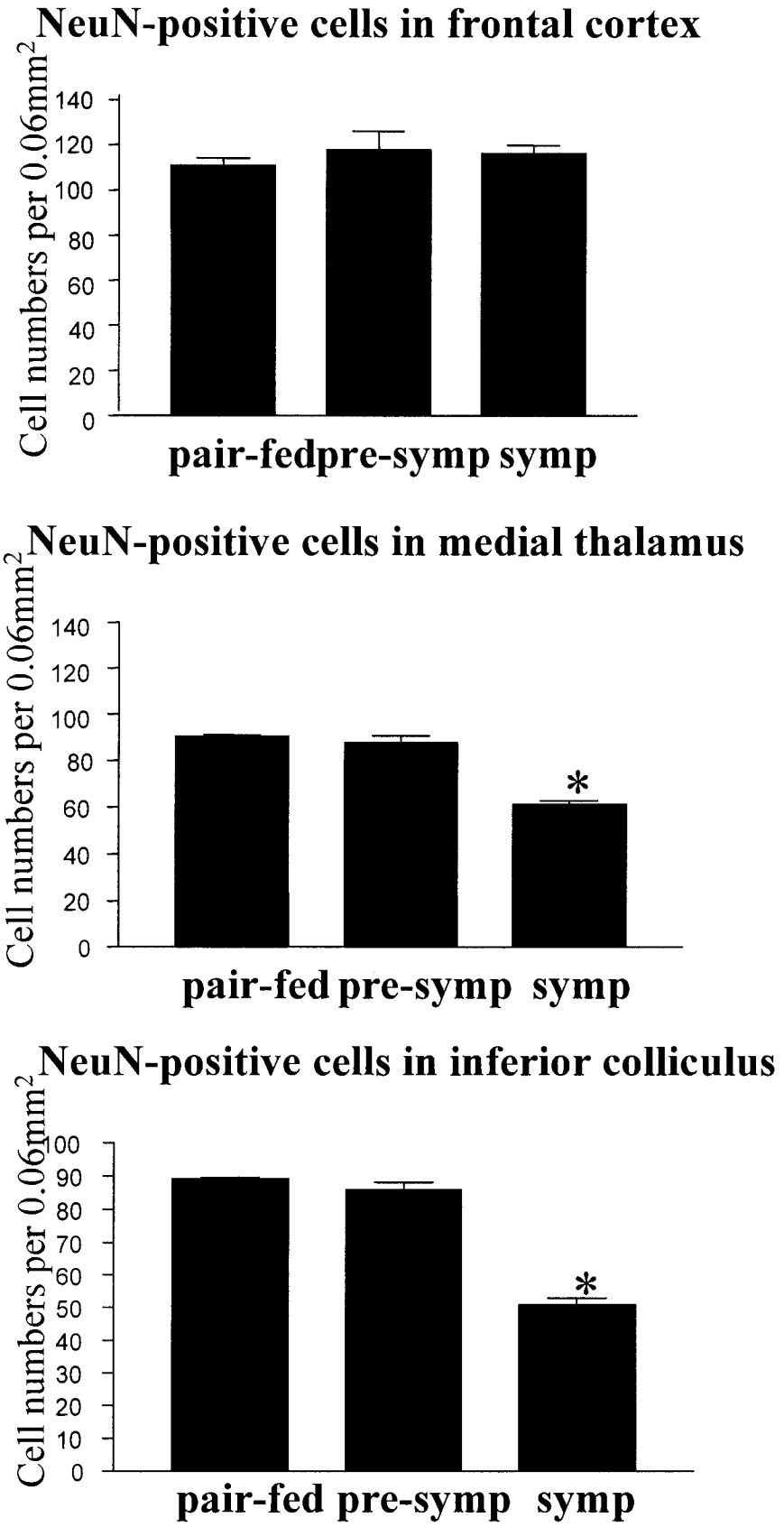


Figure 3

***p<0.001 vs. Pair-fed**

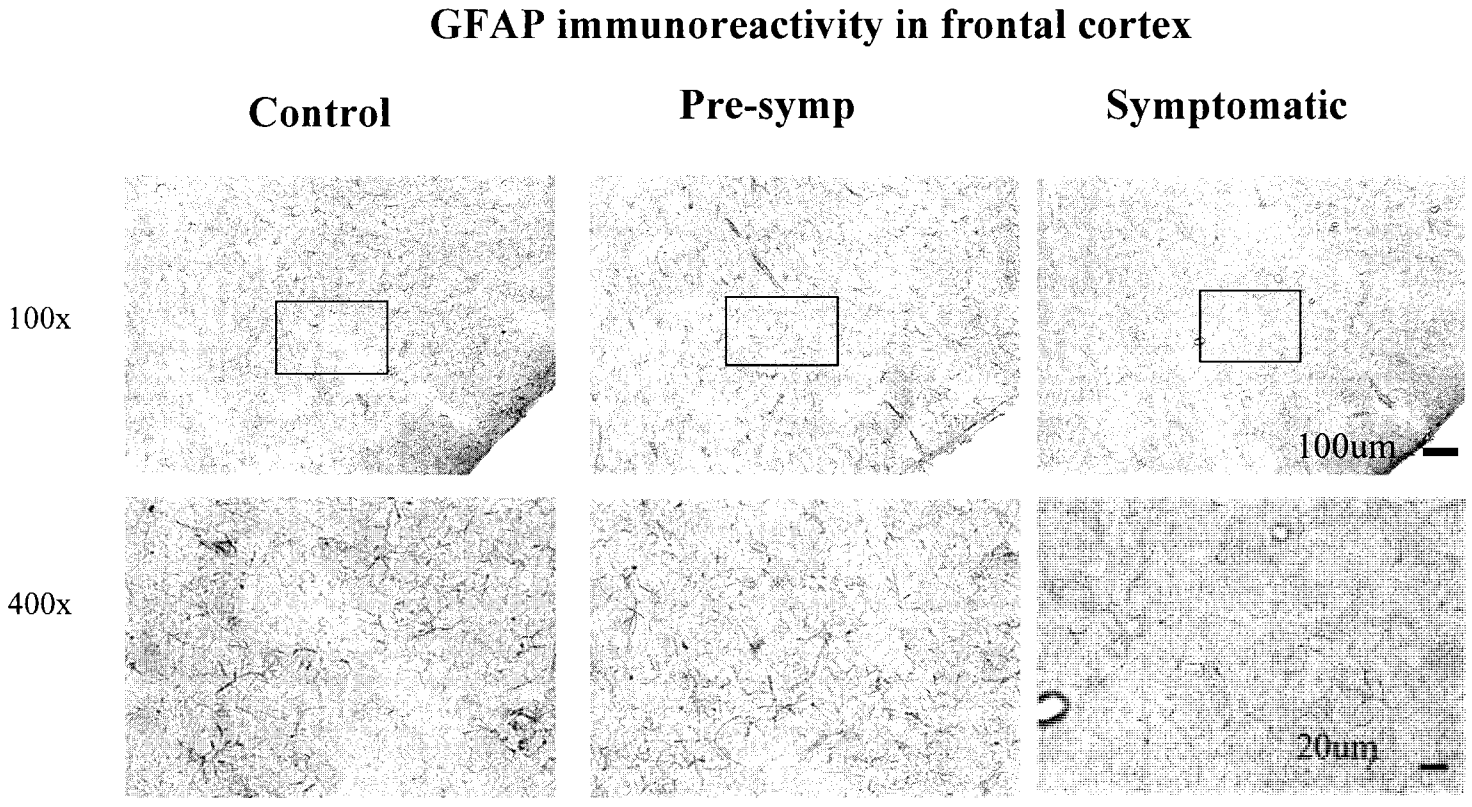

GFAP immunoreactivity in frontal cortex
Control
Pre-symp
Symptomatic
100x
400x
100um
20um

Figure 4A

GFAP immunoreactivity in medial thalamus

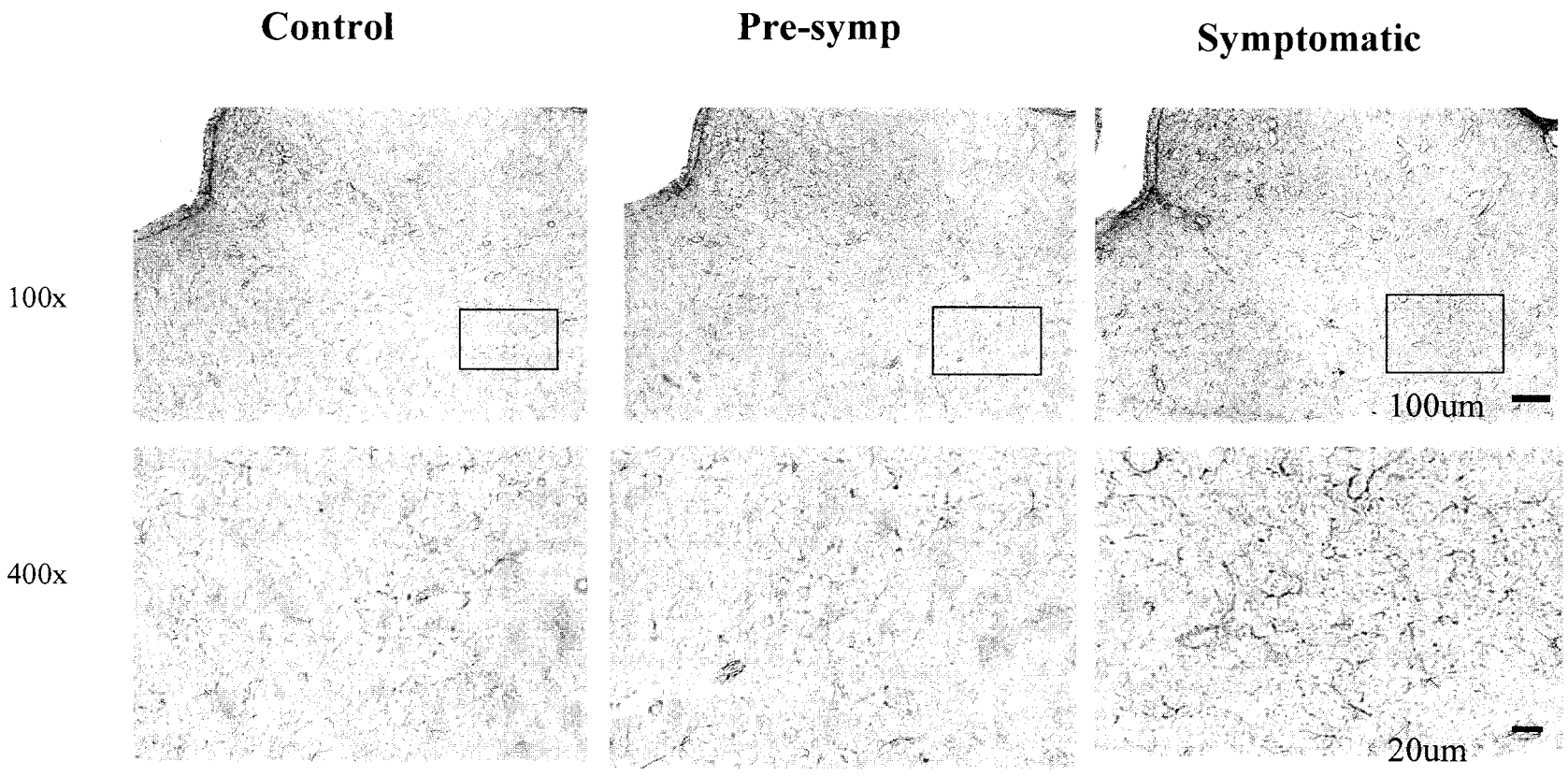


Figure 4B

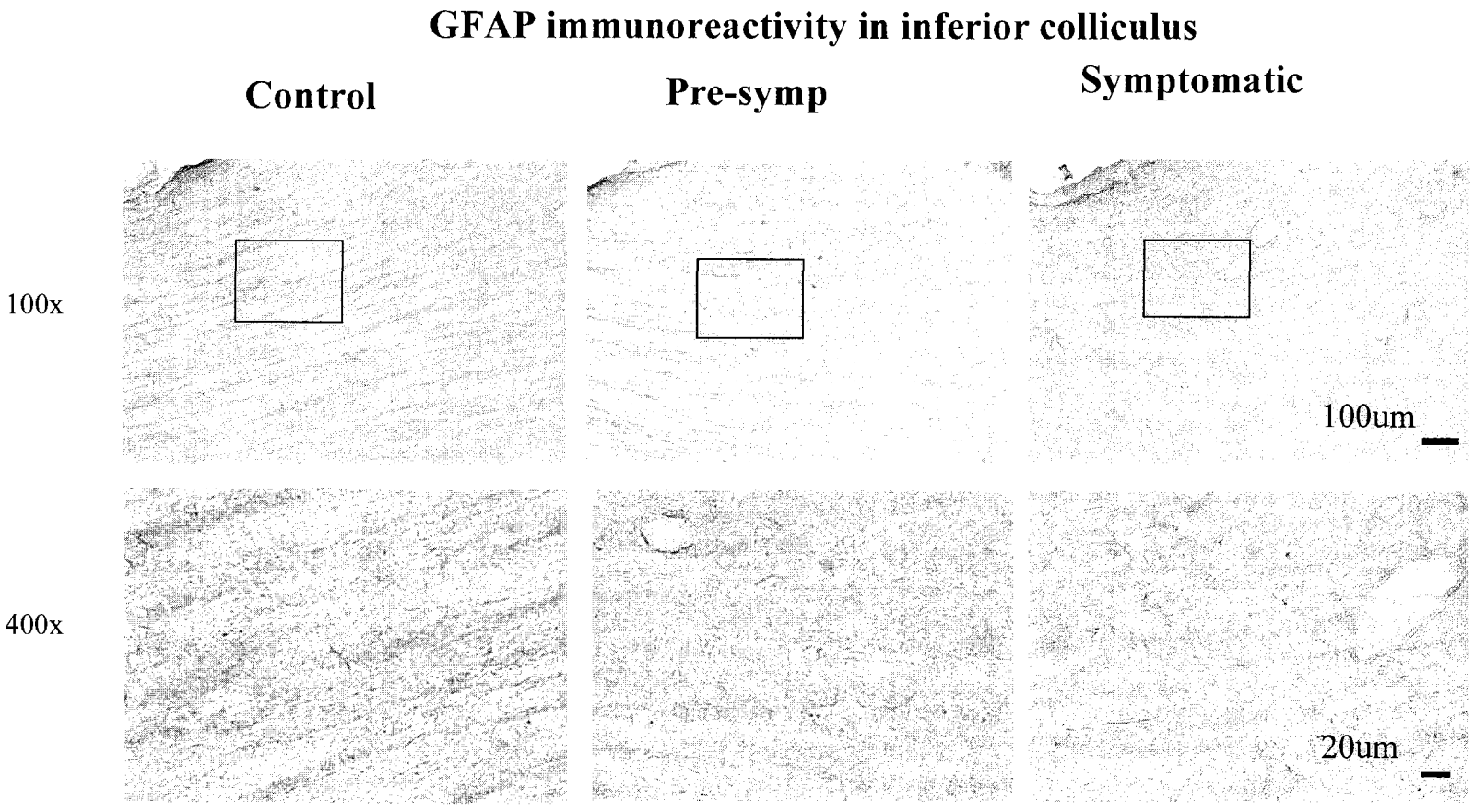

GFAP immunoreactivity in inferior colliculus
Control
Pre-symp
Symptomatic
100x
400x
100um
20um

Figure 4C

OX42 immunoreactivity in frontal cortex

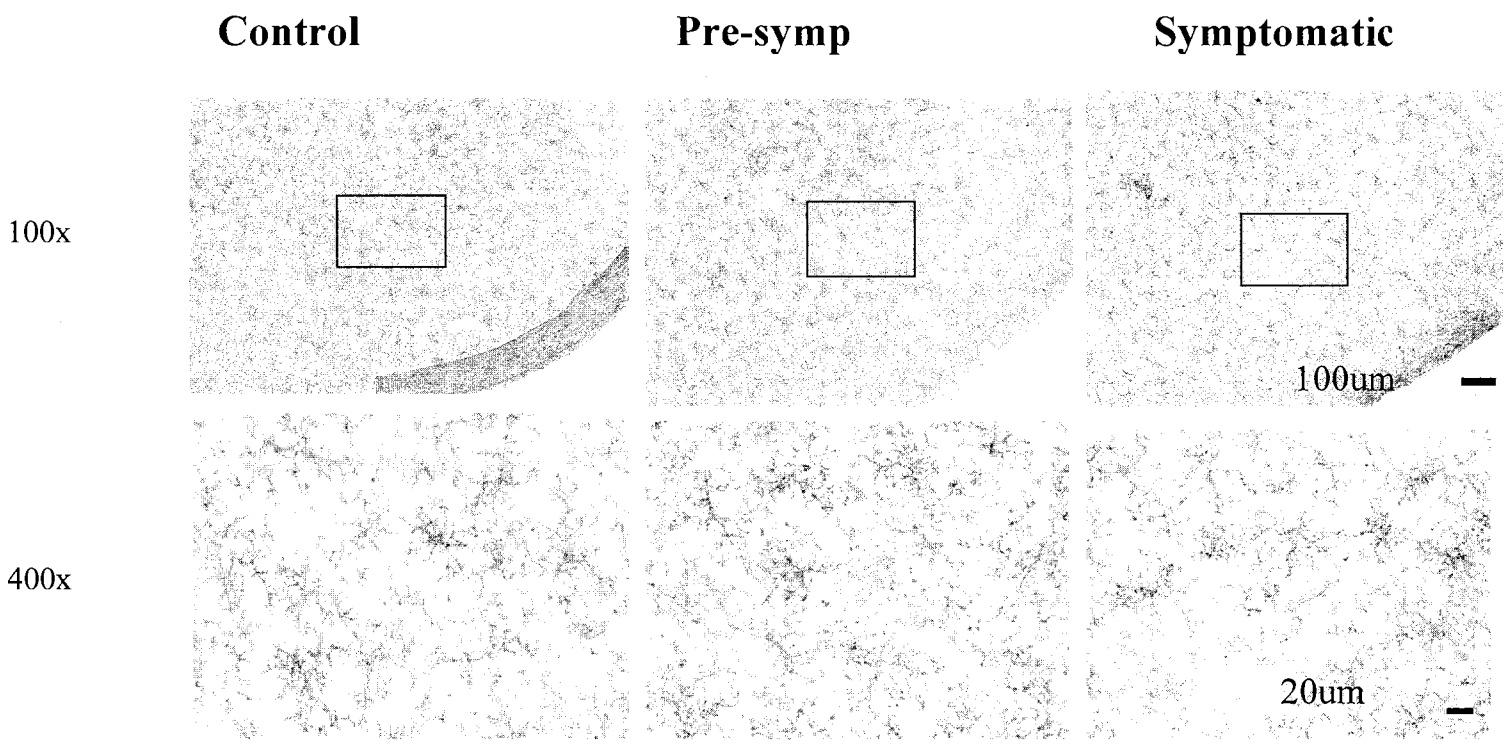


Figure 5A

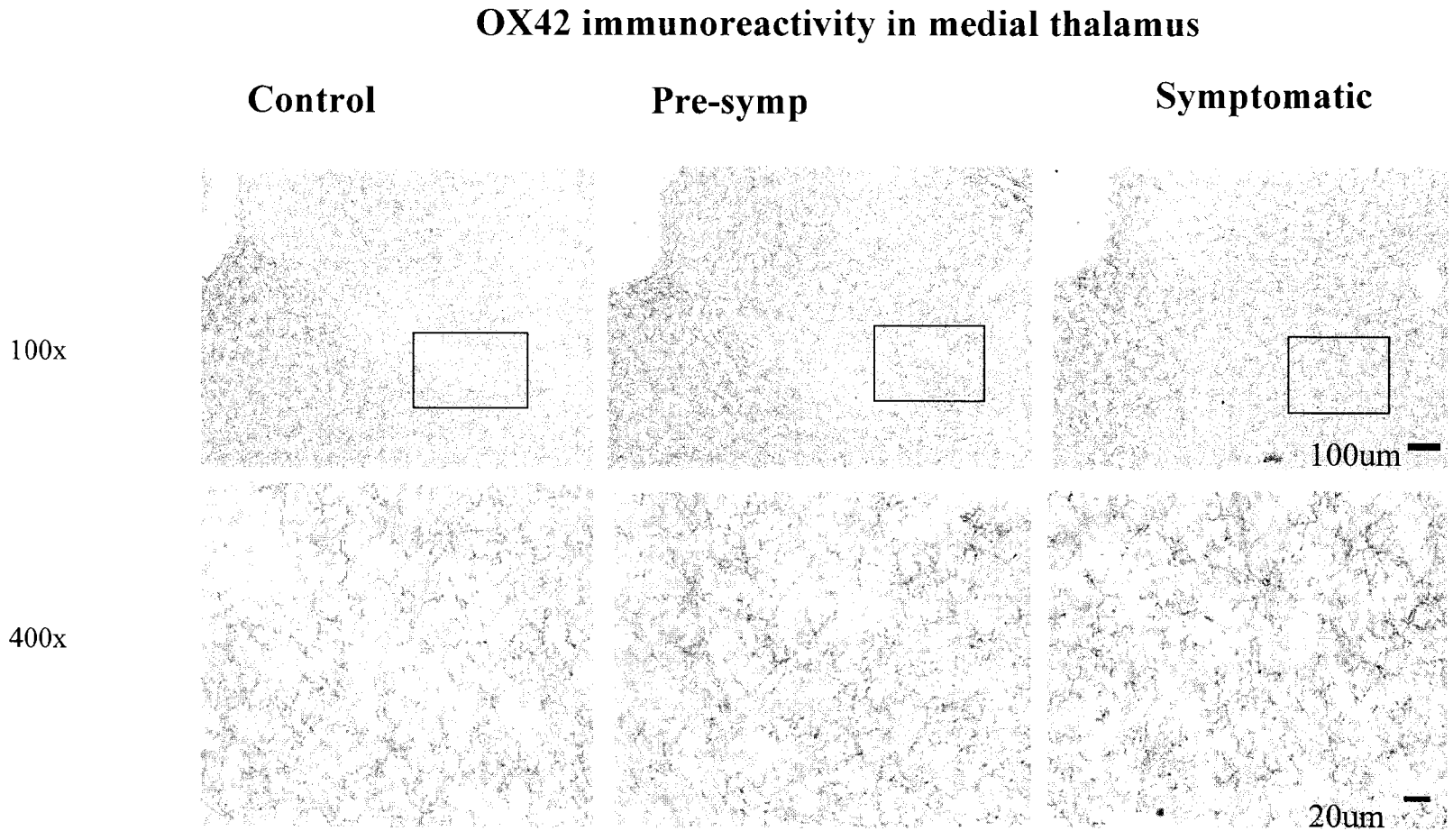

OX42 immunoreactivity in medial thalamus
Control
Pre-symp
Symptomatic
100x
400x
100um
20um

Figure 5B

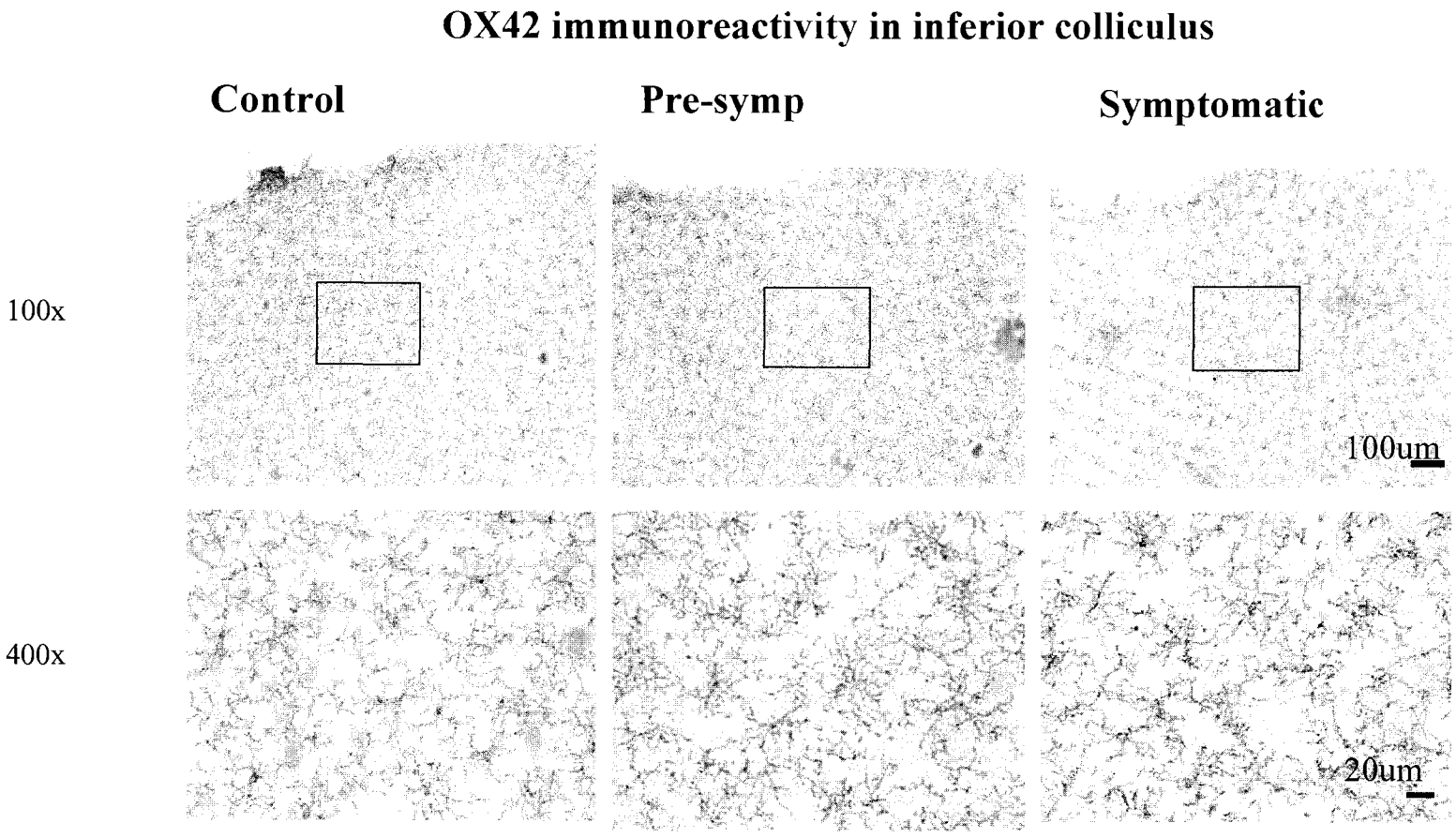
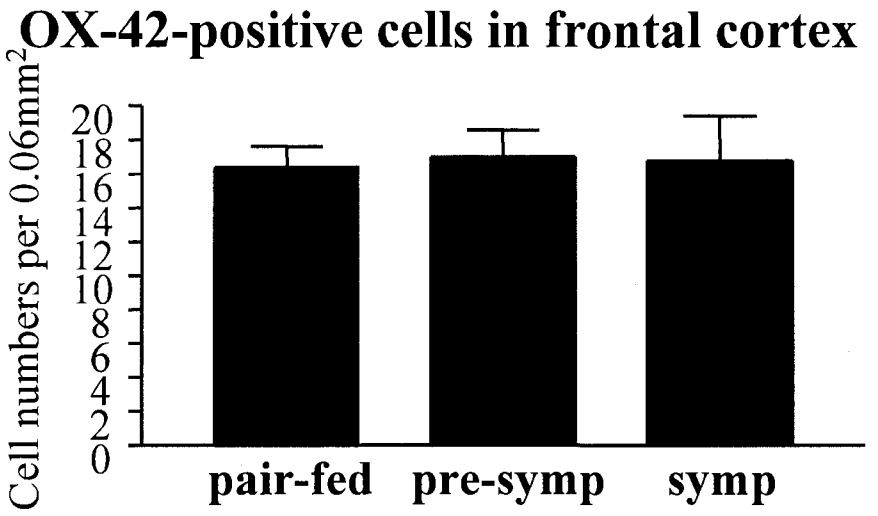
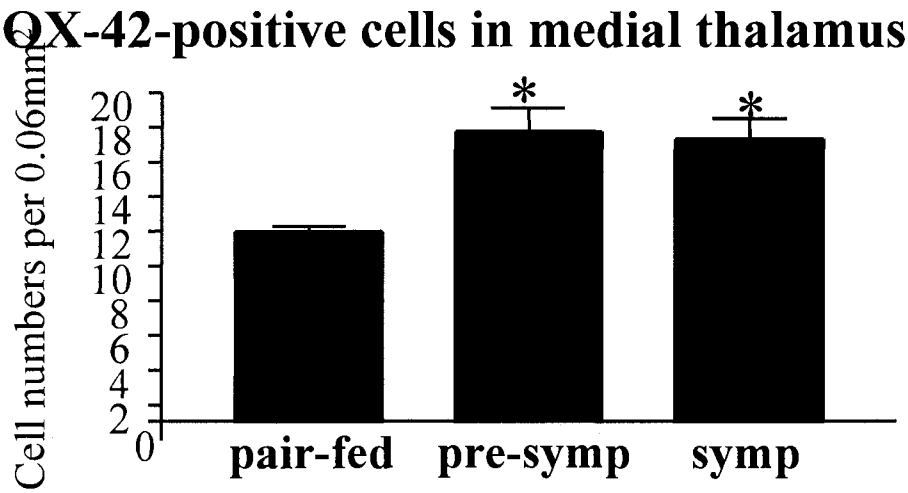

OX42 immunoreactivity in inferior colliculus
Control
Pre-symp
Symptomatic
100x
400x
100um
20um

Figure 5C





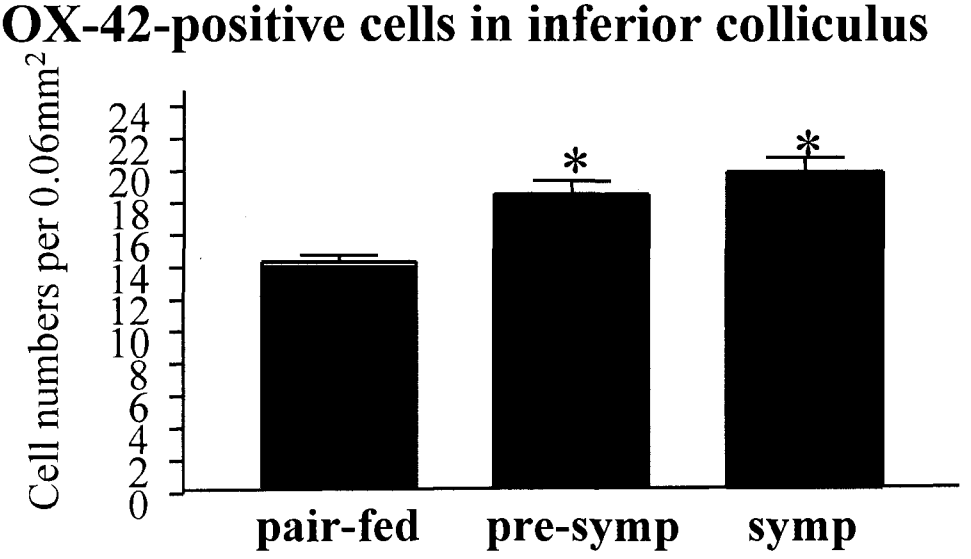


Figure 6 ***p<0.001 vs. Pair-fed**

COX-2 immunoreactivity in frontal cortex

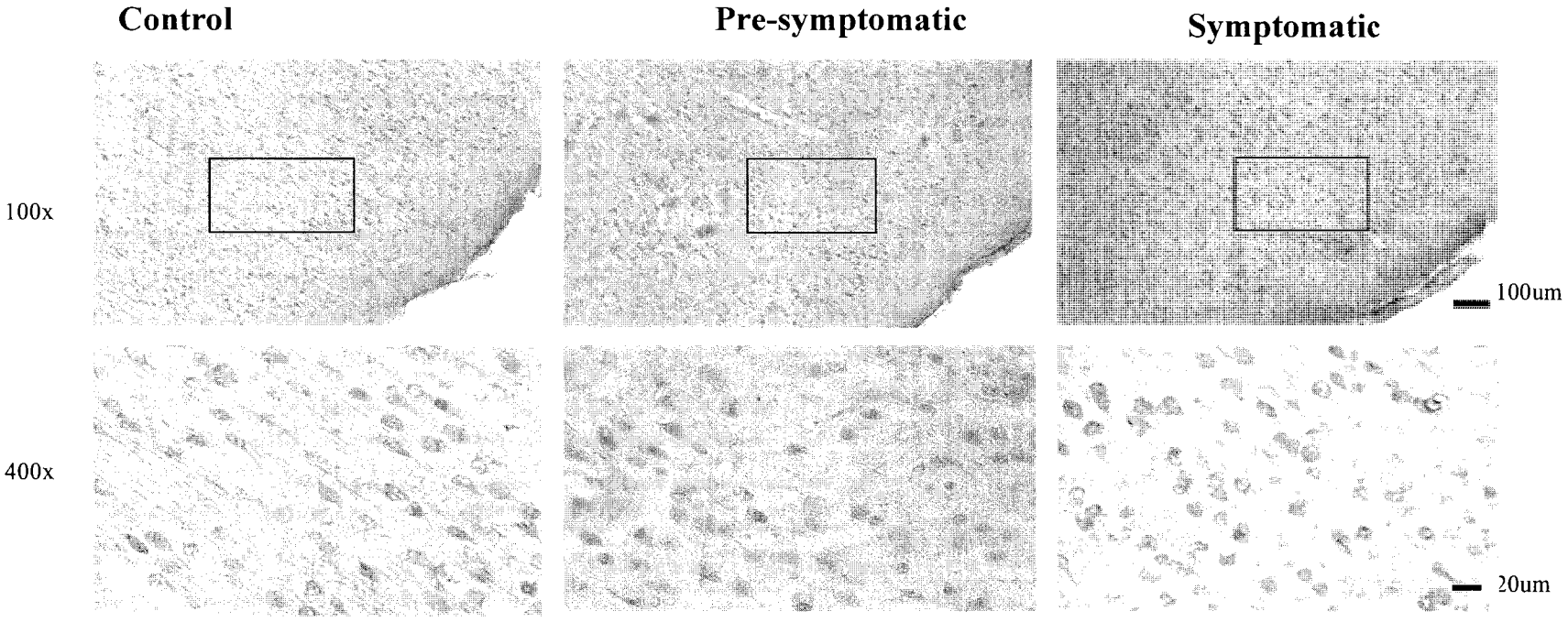


Figure 7A

COX-2 immunoreactivity in medial thalamus

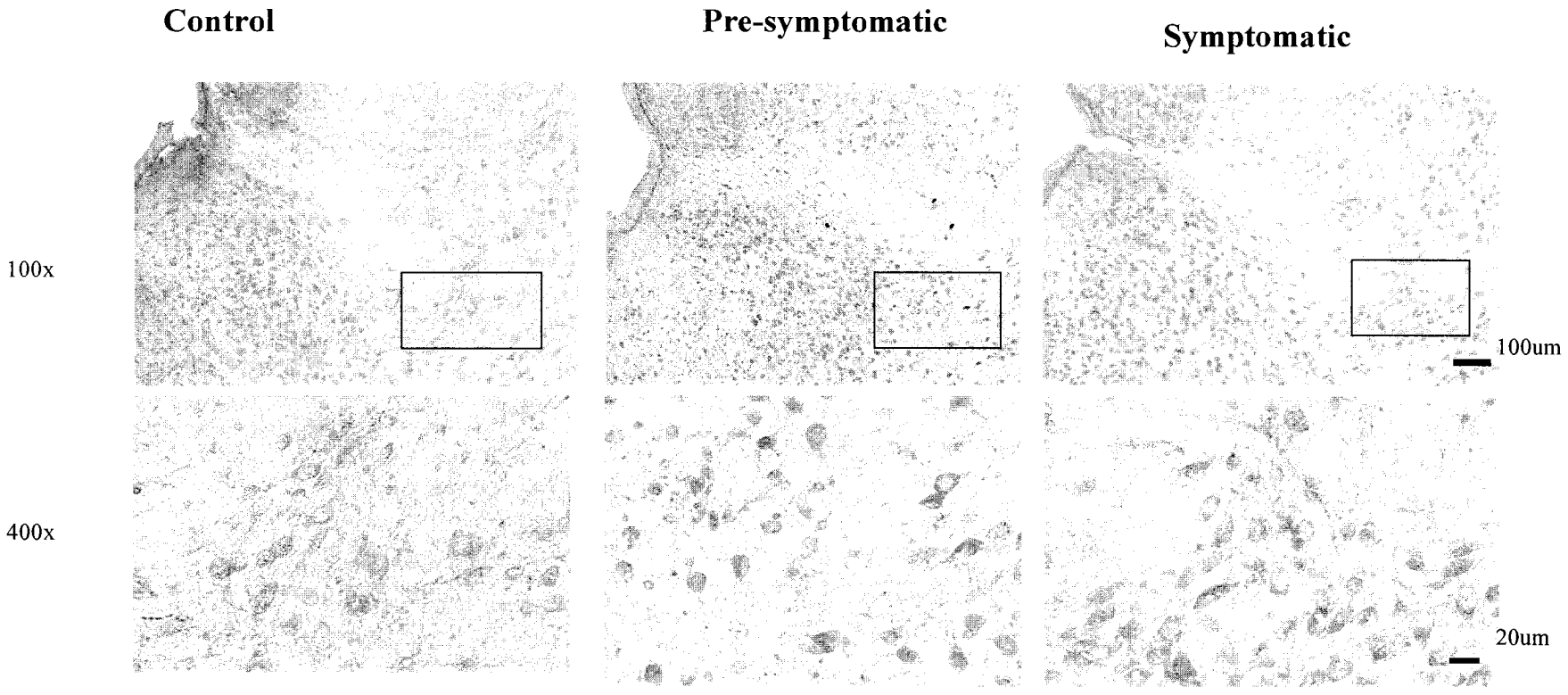


Figure 7B

COX-2 immunoreactivity in inferior collicullus

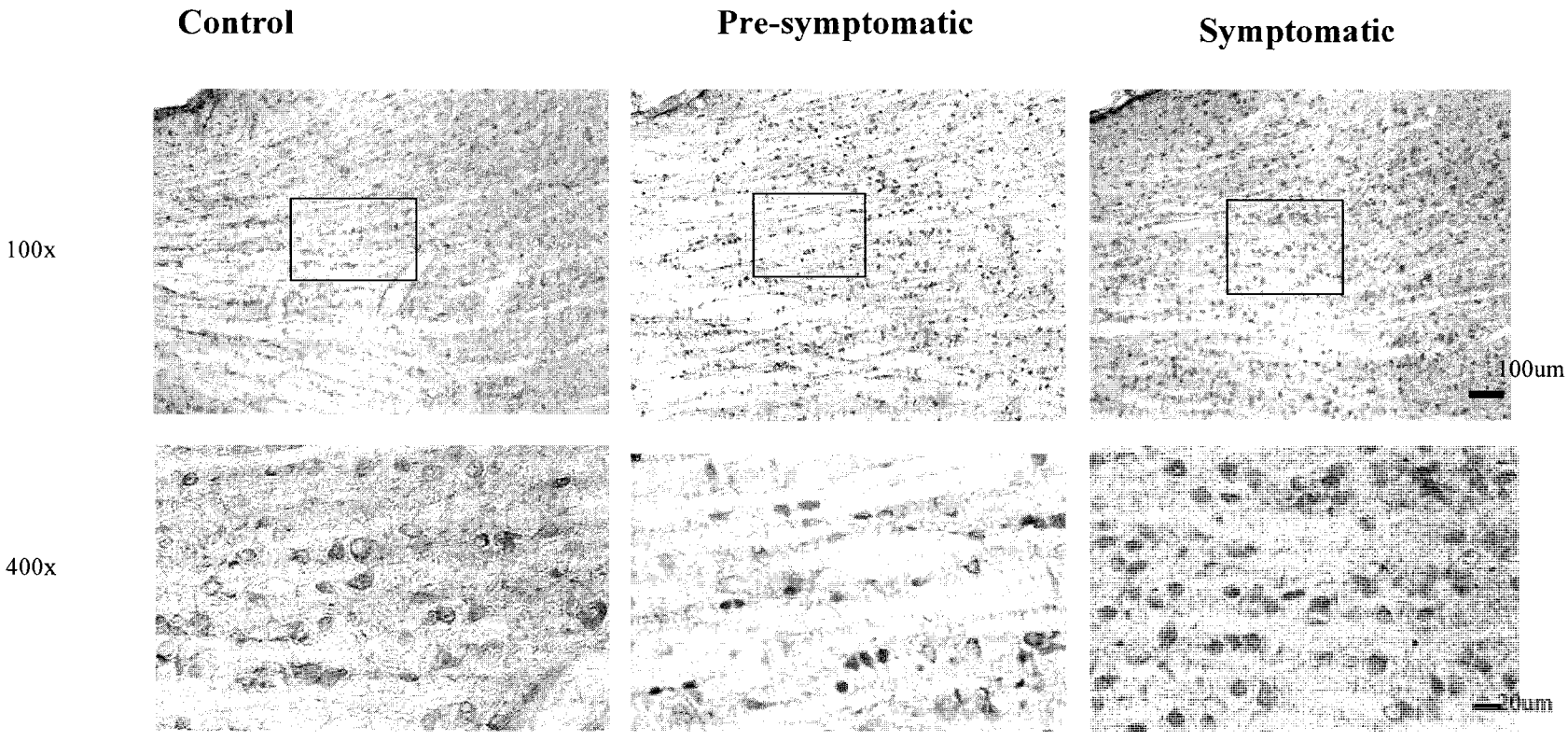


Figure 7C

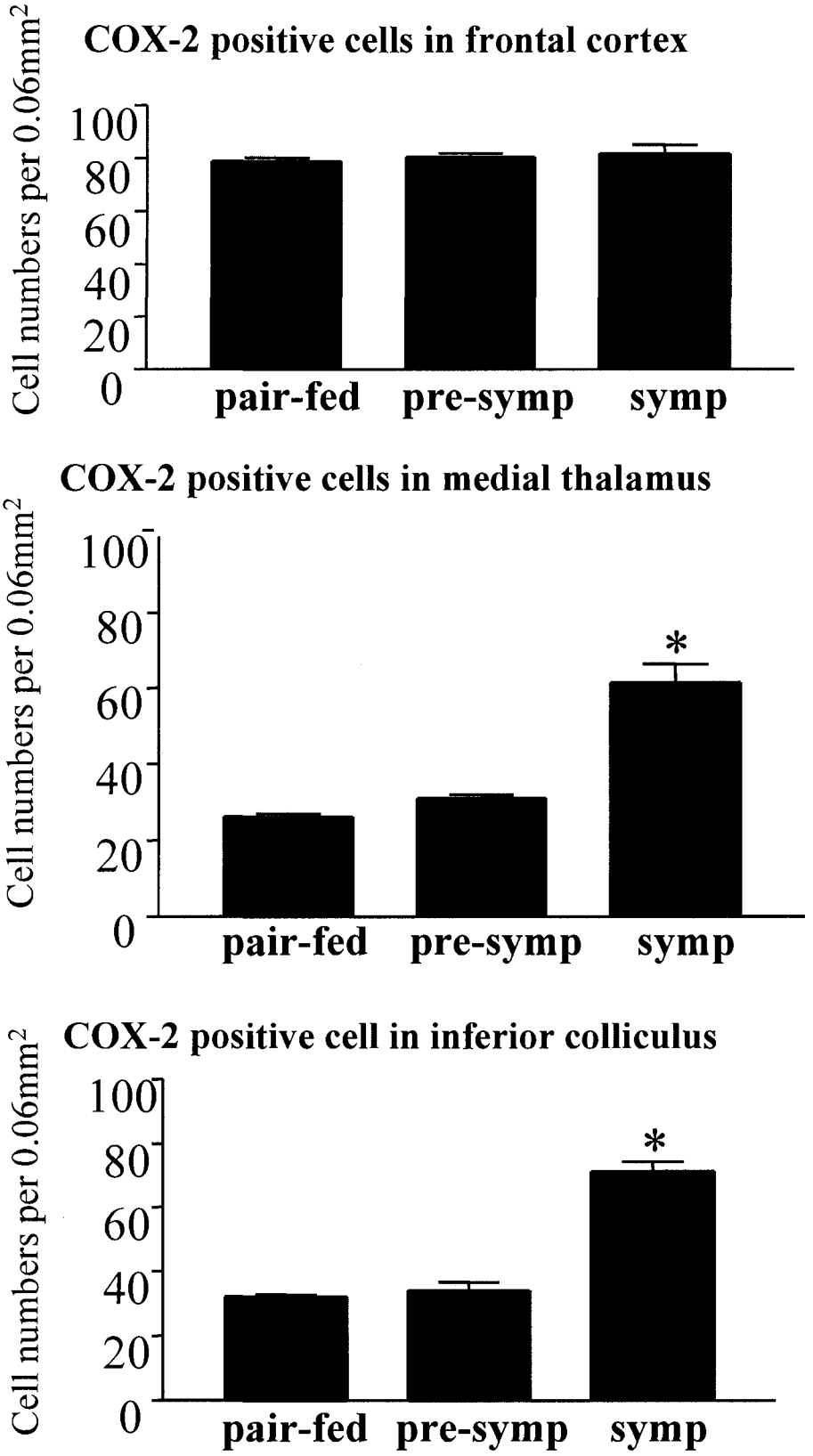
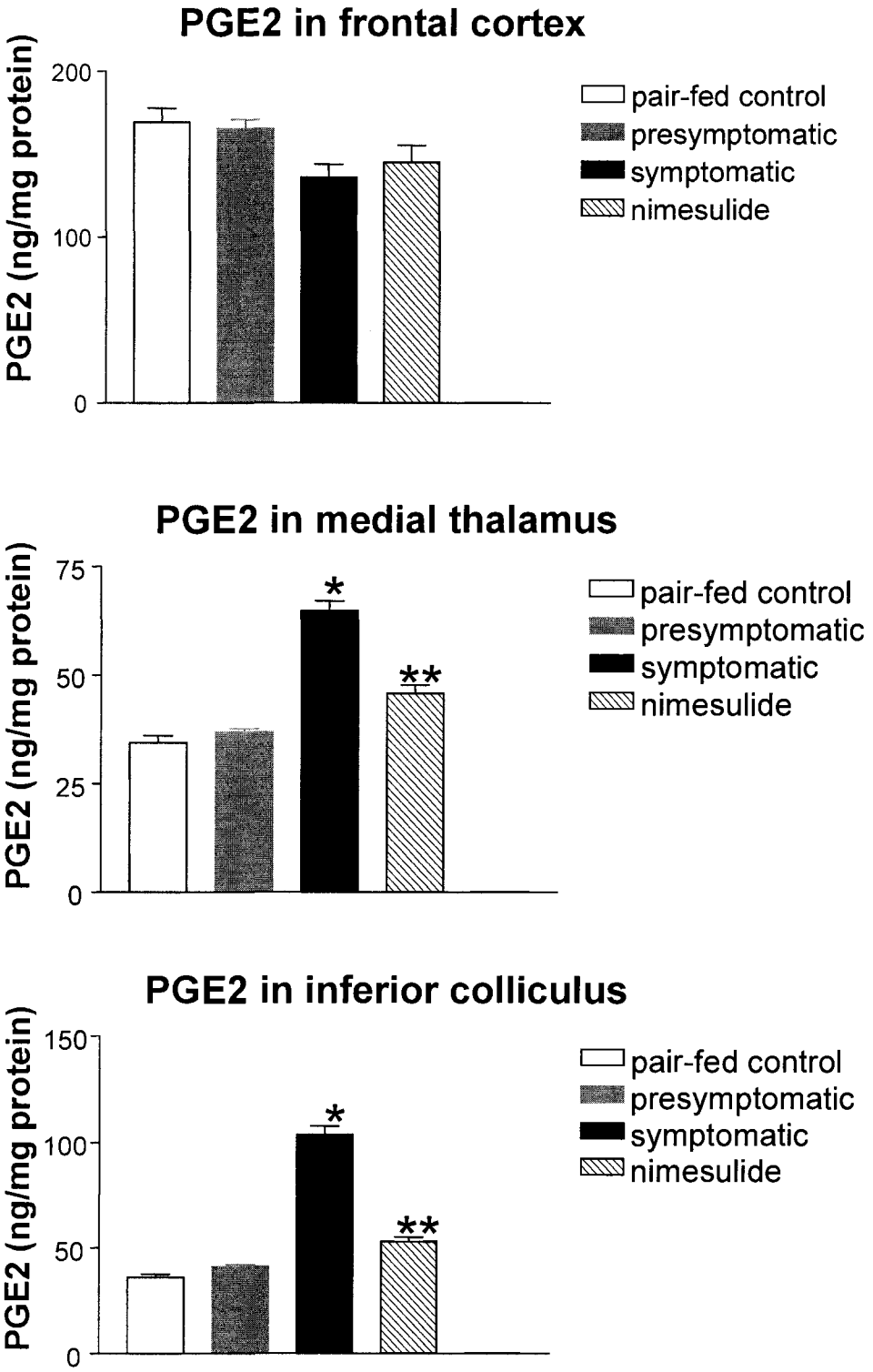


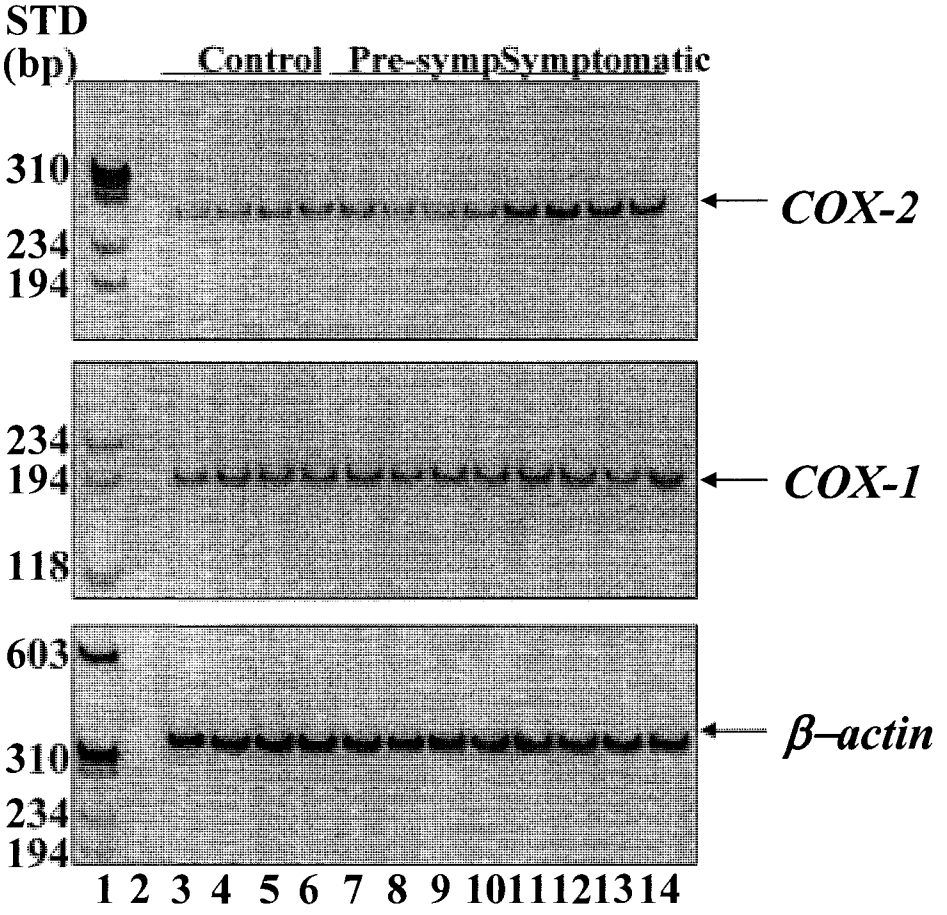
Figure 8 ***p<0.001 vs. Pair-fed**



*p<0.001 v.s. control **p<0.001 vs symptomatic

Figure 9

Expression of COX-1 and COX-2 mRNAs in medial thalamus



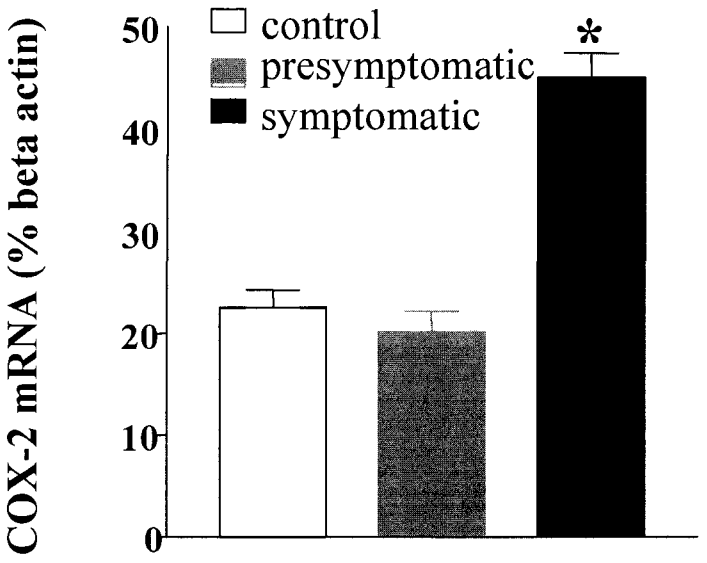
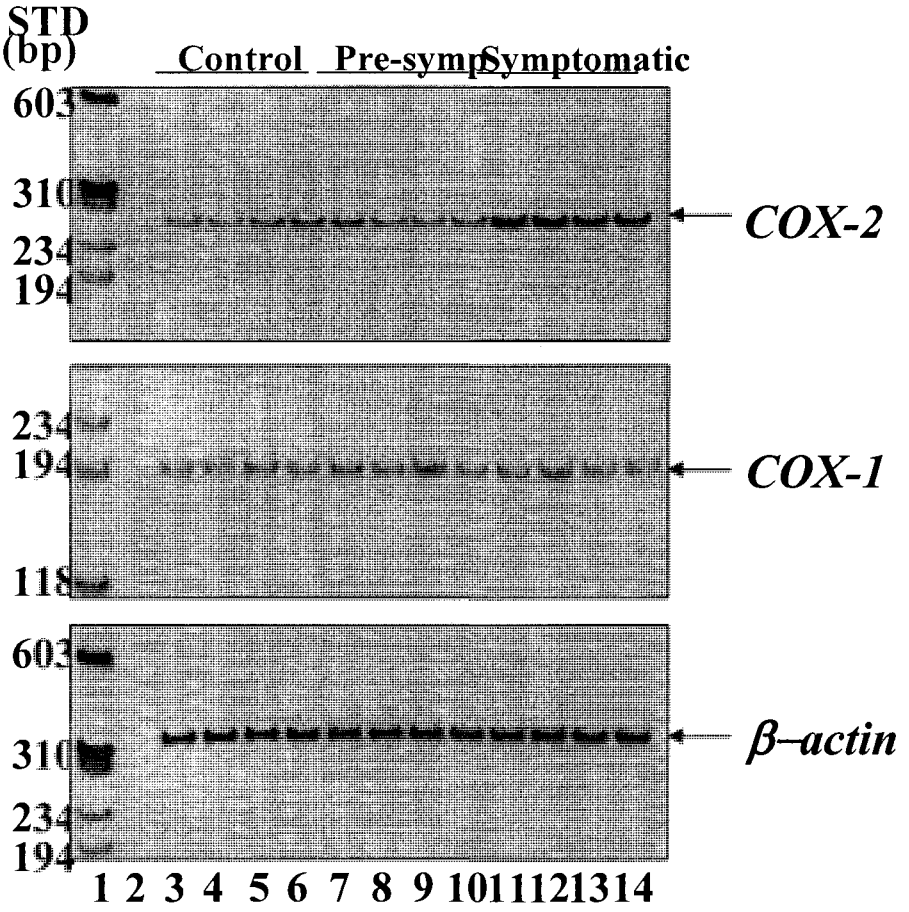


Figure 10a ***p<0.001 vs control**

Expression of COX-1 and COX-2 mRNAs in inferior colliculus



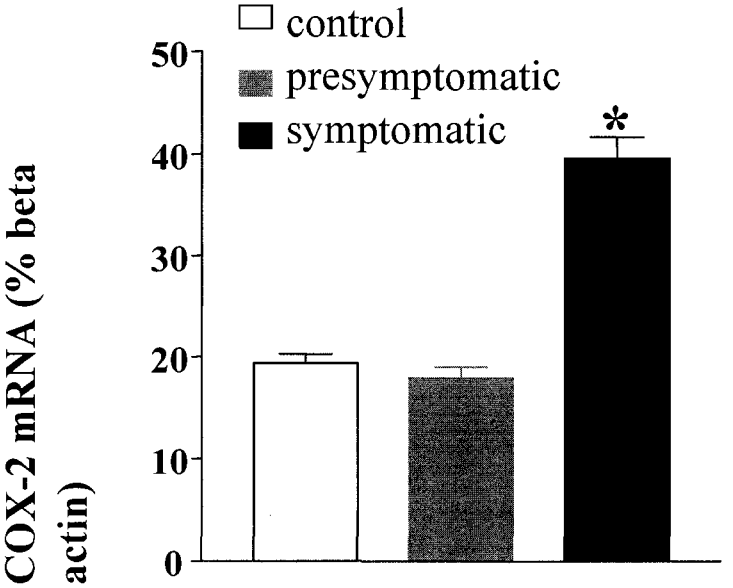


Figure 10b ***p<0.001 vs control**

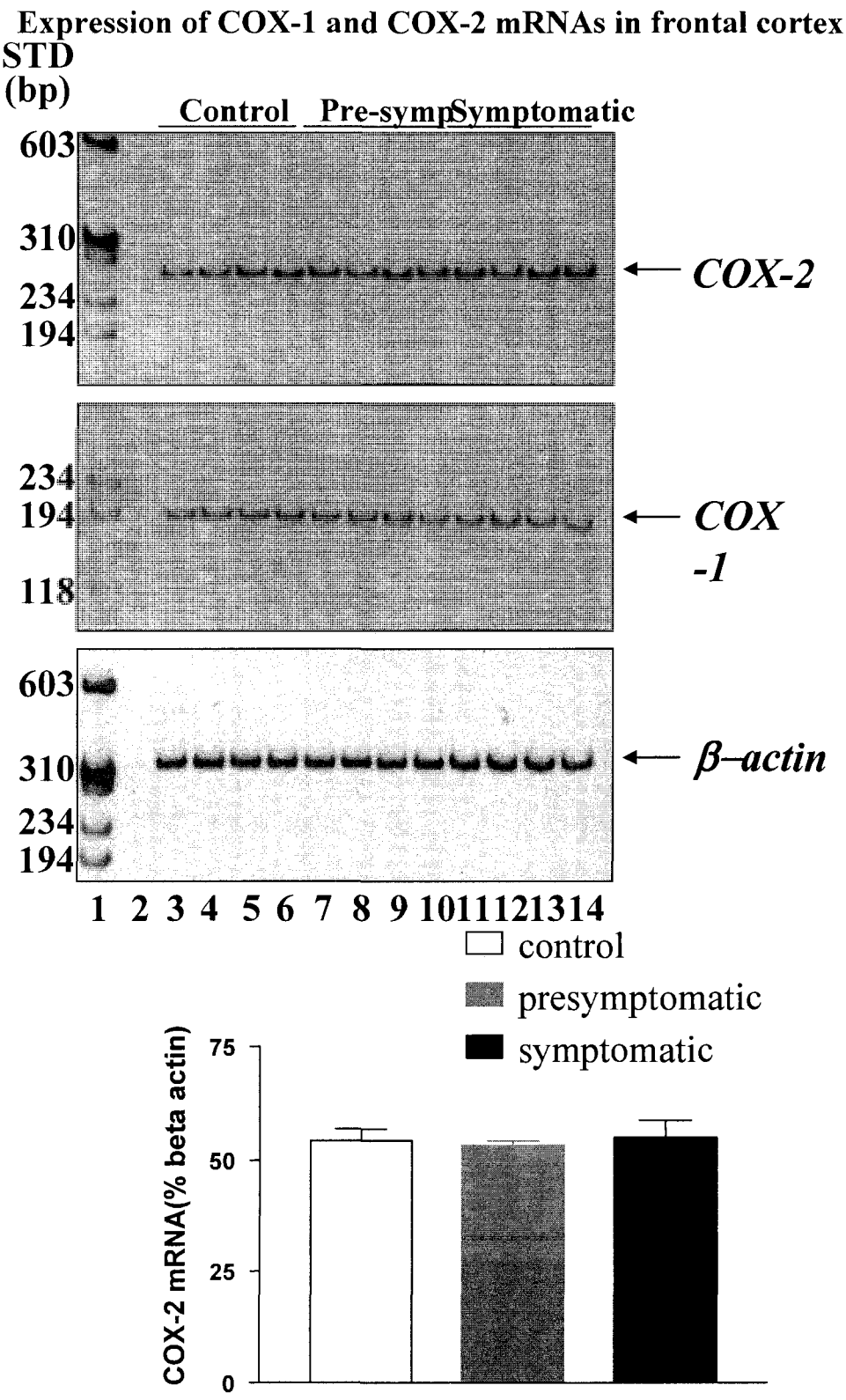

Expression of COX-1 and COX-2 mRNAs in frontal cortex
STD
(bp)
Control
Pre-symp
Symptomatic
603
310
234
194
COX-2
234
194
118
COX
-1
603
310
234
194
β-actin
1 2 3 4 5 6 7 8 9 10 11 12 13 14
control
presymptomatic
symptomatic
COX-2 mRNA(% beta actin)
75
50
25
0

Figure 10c

NeuN immunoreactivity in medial thalamus

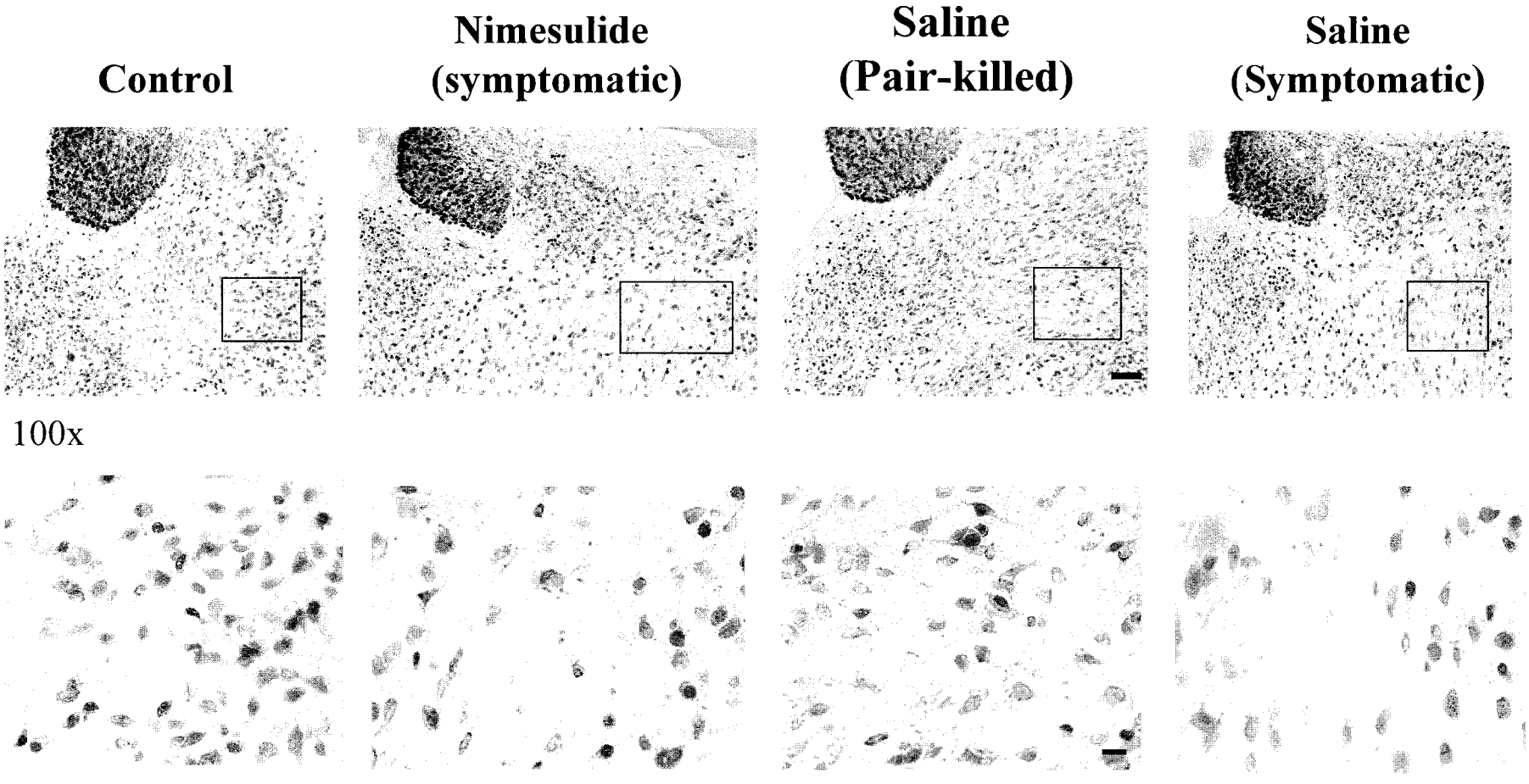


Figure 11

NeuN immunoreactivity in inferior colliculus

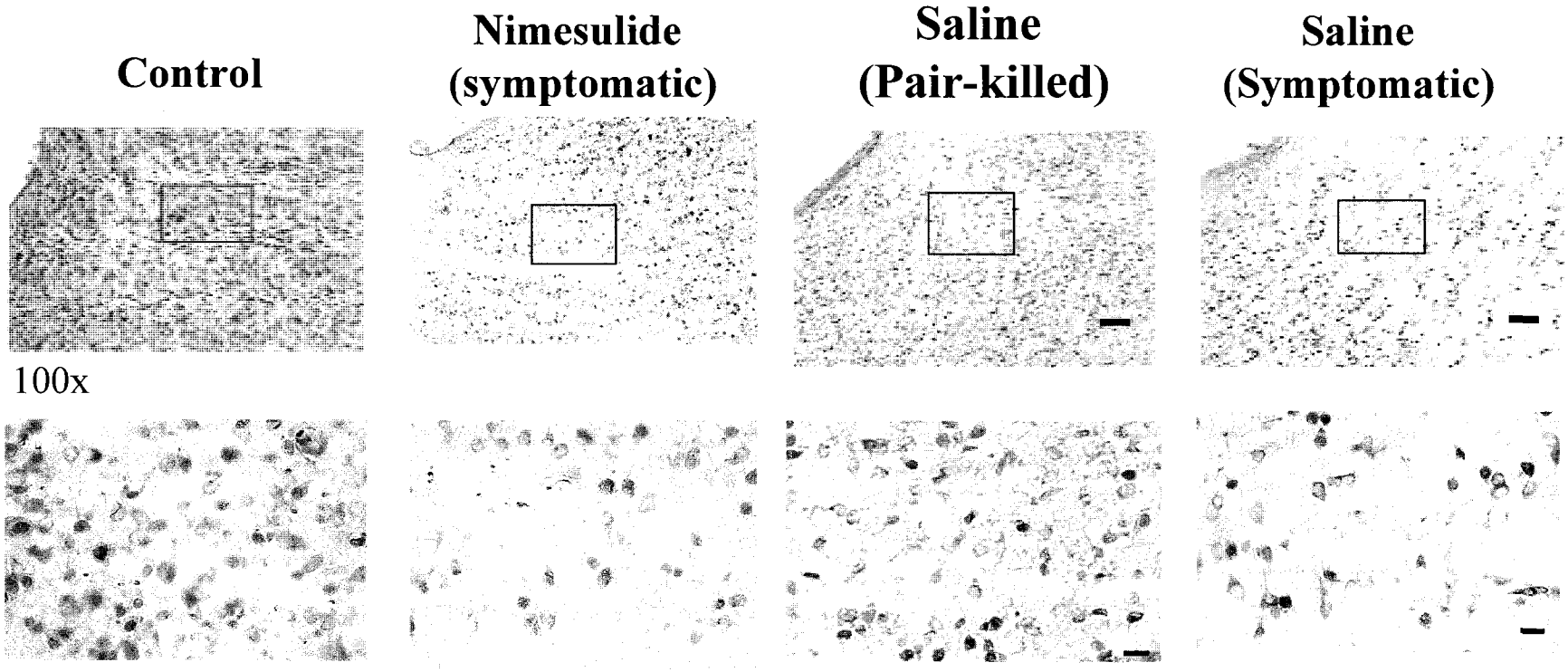
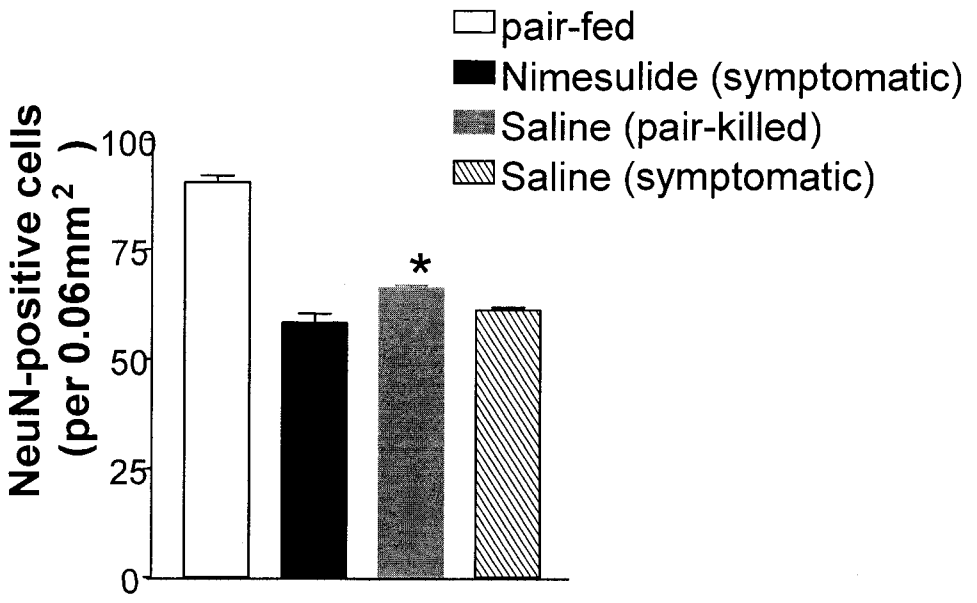
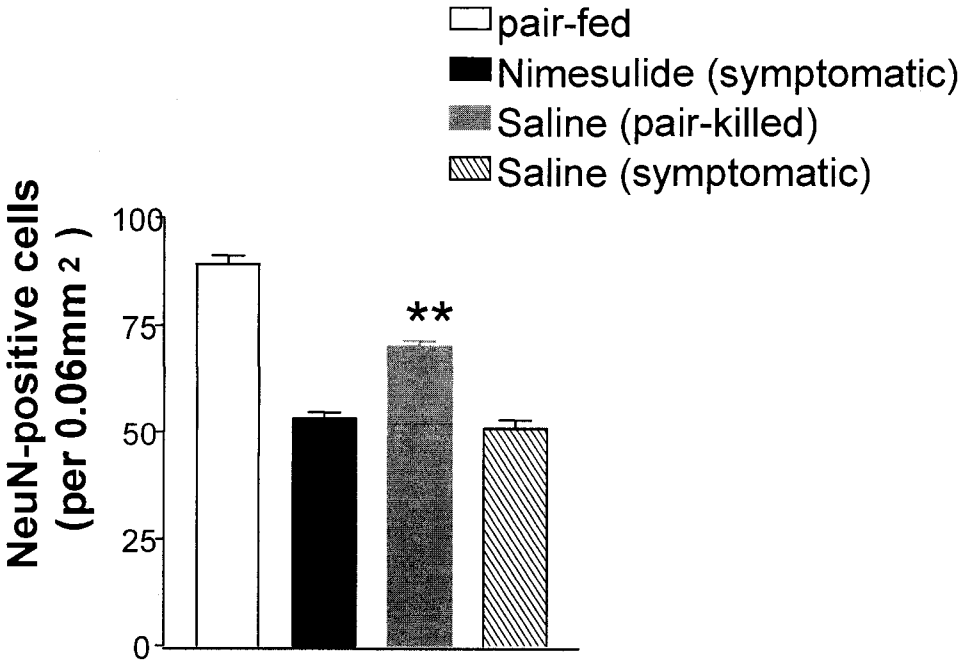


Figure 12

NeuN-positive cells in medial thalamus



NeuN-positive cells in inferior colliculus



***p<0.01 v.s nimesulide **p<0.001 v.s. nimesulide**

Figure 13

APPENDIX