Winfred Stacey, Mechanisms by which 17β-Estradiol (E2) suppress neuronal *cox-2* expression. Doctor of Philosophy (Biomedical Sciences), September 2015. 101 pages, 32 titles, 16 illustrations

Data from animal models indicate that 17β-estradiol (E2) deprivation increases susceptibility to neurodegenerative diseases. E2 attenuates inflammatory response by suppressing expression of pro-inflammatory genes; however, the mechanisms by which E2 suppress neuronal pro-inflammatory genes are not well established. Histological analyses of postmortem human brains suggest that neuronal cyclooxygenase-2 (COX-2) is upregulated in early stages of Alzheimer's disease (AD) and in Parkinson's disease (PD).

Given that COX-2 is selectively expressed in a subset of neurons in the hippocampus, cerebral cortex, and amygdala, we investigated mechanisms by which E2 could down-regulate *cox-2* expression in a neuronal system. To characterize the effect of E2 on *cox 2* in a neuronal system, we used the AR-5 and N27 rat neuronal cell line models. Our data indicate that E2 and ERβ agonist diarylpropionitrile (DPN) suppress COX-2 pre-mRNA and mRNA levels to the same extent in AR-5 but not in N27. Furthermore, PHTPP, a selective ERβ antagonist, reversed the effect of both E2 and DPN in AR-5.

Because the *cox-2* promoter lacks palindromic estrogen response elements (EREs), we targeted a proximal promoter region with a nuclear factor- κB (NF-κB) response element implicated in *cox-2* regulation. E2 and DPN failed to increase ERβ occupancy at the *cox-2* promoter. Rather, DPN decreased promoter occupancy of p65 NF-κB subunit and acetylation of histone 4 (Ac-H4). Treatment with the non-specific HDAC inhibitor Trichostatin A (TSA) counteracted DPN's repressive effects on *cox-2* expression.

In keeping with the effect of TSA, E2 and DPN increase HDAC1 promoter occupancy; however recruitment of HDAC3 was unchanged. HDAC1 is known to form a complex with Swi-*in*dependent A (Sin3A); E2 and DPN increased Sin3A occupancy. The recruitment of HDAC1 seems to correlate with decreased acetylation of histone 4 (H4) and not histone 3 (H3). Furthermore E2 alone increased methylation status in the *cox-2* proximal promoter. Taken together, these data suggest that E2 suppresses neuronal *cox-2* expression through ERβ-mediated recruitment of HDAC1, Sin3A and a concomitant reduction of p65 and H4 levels.

Here we conclude that E2 suppresses neuronal *cox-2* expression through a mechanism that involves a combination of decreasing activator and increasing repressor recruitment to the *cox-2* promoter.

MECHANISMS BY WHICH 17β-ESTRADIOL (E2) SUPPRESS NEURONAL *cox-2* EXPRESSION

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DISSERTATION

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LIST OF ABBREVIATIONS

AD	Alzheimer's disease
ANOVA	One-way analysis of variance
AP-1	Activator protein 1
cAMP	cyclic adenosine monophosphate.
C/EBPβ	CCAAT/enhancer binding protein β
ChIP	Chromatin immunoprecipitation
CNS	Central nervous system
COX	Cyclooxygenase
CpG	Cytosine-phosphate-guanine
CRE	cAMP response element
CREB	CRE binding protein (CREB)
CSF	Cerebrospinal fluid
DMSO	Dimethyl sulfoxide
DNMTs	DNA methyl transferases
DPN	Diarylpropionitrile

GPER/GPR30 G-protein-coupled estrogen receptor 1

E2	17β-Estradiol
ER	Estrogen receptors
ERE	Estrogen response element
ERK	Extracellular signal-regulated Kinases
FBS	Fetal bovine serum
FOS	FBJ murine osteosarcoma viral oncogene homolog
H3/H4	Histone 3/ Histone 4
HDAC	Histone deacetylase
ICC	Immunocytochemistry
IgG	Immunoglobulin G
IL	Interleukin
iNOS	inducible nitric oxide synthase
JUN	Jun proto-oncogene
LDL	low-density lipoprotein
MAPK	Mitogen-activated protein kinase
MMP-9	Matrix metalloproteinase-9
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA	Messenger RNA
NCoR	Nuclear receptor co-repressor
NCS	Newborn calf serum
NF-κB	Nuclear factor-kappa B
OVX	Ovariectomized
PBS	phosphate buffered saline
PD	Parkinson's disease
PGs	Prostaglandins
PHTPP	4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein Kinase A
PMA	Phorbol 12-myristate 13-acetate
PPT	4,4',4"-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)*tris*phenol
RT-qPCR	Reverse Transcription – quantitative real-time polymerase chain reaction
SEM	Standard error of the mean
Sin3A	Swi-*in*dependent 3A
SN	Substantia nigra
SN-pc	Substantia nigra pars compacta

SP-1	Stimulating protein 1
TNF-α	Tumor necrosis factor-α
TSA	Trichostatin A
VEH	Vehicle
WB	Western blot

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INTRODUCTION

A. Neuroinflammation

Inflammation is a complex defensive response to harmful stimuli such as pathogens, damaged cells or irritants [1]. Due to collateral effects of inflammatory processes, the response should be rapid, specific and self-limited. Initially, the central nervous system (CNS) was thought to be “immune privileged” neither susceptible nor contributing to inflammatory processes. However, accumulation of evidence from the last 20 years indicate that the CNS is immune competent and actively responsive to harmful stimuli and injury [2]. The term ‘neuroinflammation’ is used to discriminate inflammatory reactions in the CNS from those in the periphery.

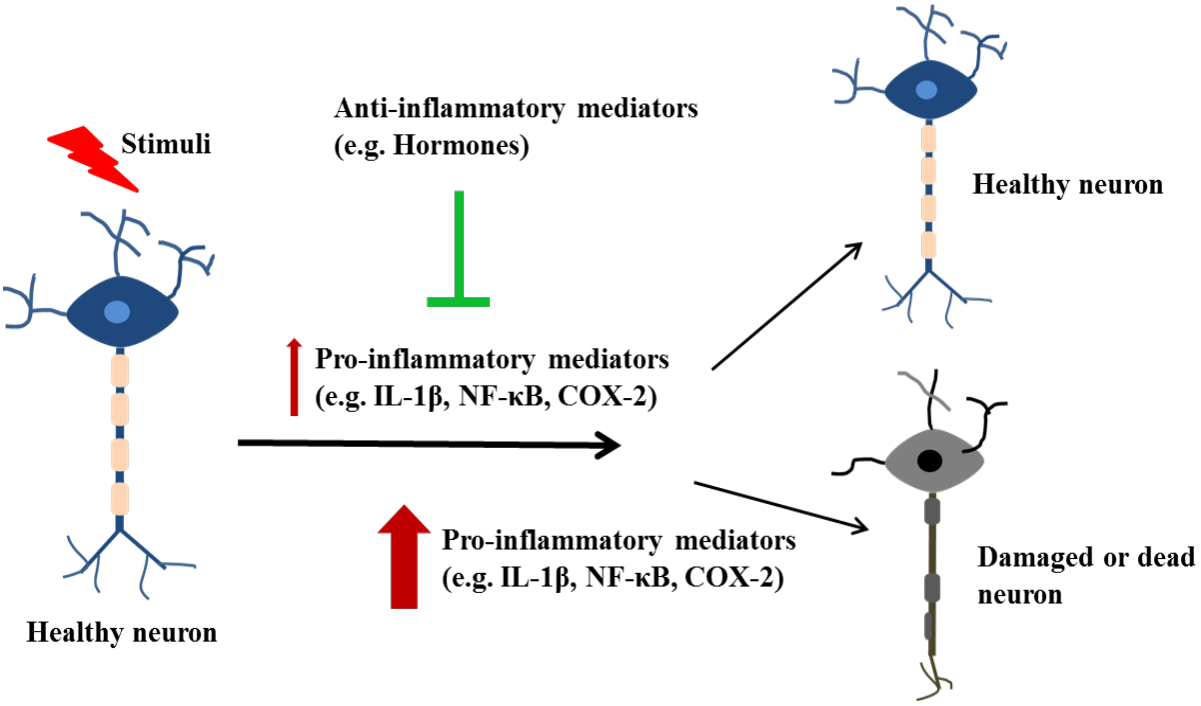
Neuroinflammation is defined as an inflammatory process that occurs in the CNS. This highly controlled process can be triggered by classic factors such as pathogens and autoimmunity, or neurogenic activity such as psychological stress and epileptic seizure [3]. In response to a stimuli, a cascade of pro-inflammatory mediators such as interleukin-1 β (IL-1β), cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), prostaglandins (PGs), and tumor necrosis factor-α (TNF-α) are produced [4]. Subsequently, anti-inflammatory mediators are

elicited to suppress exaggerated pro-inflammatory processes. This balance between pro- and anti-inflammatory responses is necessary to prevent unregulated inflammatory processes and detrimental effects.

The view that neurons play an important role in neuroinflammation was not adopted until recently. Recent data indicate that neurons are not merely bystanders but are active participants and source of inflammatory mediators [2,5,6]. As illustrated in Figure 1, in response to a stimuli, neurons take concerted and finely tuned defensive measures by releasing pro-inflammatory mediators such as IL-1β, COX-2, nuclear factor- κB (NF-κB) [3,7,8]. This activity triggers anti-inflammatory factors such as hormones, interleukin-10 (IL-10) that tightly regulate and suppress further pro-inflammatory responses [9,10]. Unchecked, inflammatory responses can result in dysregulated neuroinflammatory processes, neuronal damage and death.

Neuroinflammation is associated with pathogenesis and progression of numerous neuropathologic processes. These range from neurodegenerative processes such as in Alzheimer's disease (AD) and Parkinson's disease (PD) [8,11-13] to neuropsychiatric disorders such as depression, bipolar and schizophrenia [14-16], and other neurological disorders such as ischemic brain damage [17,18]. The common feature in these disorders is the overexpression of pro-inflammatory mediators that contribute to neuronal damage and subsequently neuronal death [19,20]. While there is overwhelming evidence of neuroinflammation in various neurological disorders, this project focuses on in vitro models of AD and PD.

Figure 1. Neuroinflammatory processes in neurons. The figure depicts a neuron's response to pathogens. Studies have indicated that neurons express and release pro-inflammatory mediators such as COX-2 and cytokines. Anti-inflammatory mediators suppress an overexpression of pro-inflammatory factors to bring about homeostasis. In the event that the regulatory arm of anti-inflammatory factors is not present, the consequences are an exaggerated response, dysregulation of inflammatory processes, damage or death to neurons and subsequent neurological disorders.


Stimuli
Anti-inflammatory mediators
(e.g. Hormones)
Healthy neuron
Pro-inflammatory mediators
(e.g. IL-1β, NF-κB, COX-2)
Healthy neuron
Pro-inflammatory mediators
(e.g. IL-1β, NF-κB, COX-2)
Damaged or dead
neuron

Neurodegenerative Diseases

Alzheimer's disease (AD)

AD is the most common neurodegenerative disease affecting millions in the modern world [21,22]. AD is an age-associated and progressive neurodegenerative disorder that causes debilitating dementia. Although the etiology of AD is unknown, mounting evidence indicates that neuroinflammation contributes to its pathology and progression [14]. One key aspect of the neuroinflammatory milieu is the dysregulated production of pro-inflammatory mediators such as cytokines and COX-2 [23]. Cytokines, microglia and astrocytes activate and enhance each other, establishing a self-propagating cycle of neuroinflammation evident in AD [13,23]. Postmortem brain specimens of AD patients indicate elevated neuroinflammatory markers, microglia hyperactivity and overexpression of pro-inflammatory genes [23-26]. Consequently, elevated neuroinflammatory markers and loss of synaptic proteins results in damage and death of neurons [26,26-28]

Extensive studies have focused on abnormalities in cerebral cortex, entorhinal cortex, hippocampal formation and how they correlate to clinical impairment [29-31]. The amygdala is less appreciated even though shrinkage and neuronal loss has been observed in early stages of AD [32,33]. Magnetic resonance imaging data of mild AD dementia patients observed that the magnitude of amygdaloid atrophy correlated to the severity of cognitive impairment [32] and emotional memory impairment [34].

Even though in both genders an increased risk of AD has been indicated with increased age, there are sex differences both in prevalence and severity. Clinical studies show that postmenopausal women have a higher risk of developing AD, and greater cognitive deterioration than age-matched men [35] . Furthermore, the sex-differences in AD are corroborated by

neuroimaging and postmortem human studies. These studies report that women with AD manifest more pathology than male AD patients. The estrogen-deprived state in postmenopausal women may contribute to the prevalence and severity of AD [36].

Parkinson's disease (PD)

PD is the second most common neurodegenerative disease characterized by selective degeneration of dopaminergic neurons in substantia nigra pars compacta (SNpc) [37]. The etiology of PD is not well understood. However, the contributing role of neuroinflammation to the pathology of PD has been suggested. Neuroinflammatory processes characterized by highly activated microglia and increased levels of pro-inflammatory genes such as *COX-2, TNF-α* and *IL-1β* are evident in cerebrospinal fluid (CSF), post-mortem brain tissue as well as *in vivo* [12]. This sustained neuroinflammatory process leads to slow and irreversible death of substantia nigra (SN) dopaminergic neurons eventually contributing to the PD pathology.

Gender differences have been observed in PD; it is more prevalent in men than in women [38]. However, the risks go up in postmenopausal women, suggesting that estrogen may delay and prevent PD onset. It is still unclear how estrogen protects the SN dopaminergic neurons. Animal studies have suggested that estrogen modulates the nigrostriatal dopamine metabolism, release, reuptake and receptor binding [38]. When estradiol (E2) is injected into adult ovariectomized (OVX) rats, it increases striatal dopamine metabolites thereby modulating the nigrostriatal dopaminergic system. Even though it is evident that E2 has profound effects on dopaminergic neurons of SN, the exact mechanisms are not known.

B. Cyclooxygenase

Cyclooxygenase (COX) is a key rate-limiting enzyme that converts arachidonic acid to prostaglandins (PGs) (Figure 2). PGs are lipid mediators that regulate diverse physiological functions such as pain, inflammation, [39] synaptic transmission and plasticity [40]. Two important isoforms have thus been identified, COX-1 and COX-2. Structurally, the amino acid sequence homology between them is about 60%. COX-3, a recent novel splice variant of COX-1 has been reported in canine cerebral cortex; however, not much is known about its role in regulation of physiological processes [21]. In most tissue, COX-1 is constitutively expressed and considered an important player in maintaining physiology and homeostasis. COX-2 expression in most tissue is generally restricted to low levels except when induced by stress and insults [41]. The present study focuses on regulation of neuronal *cox-2* expression. It should be noted that *COX-2* denotes the human gene, while *cox-2* denotes the rodent gene.

COX-2

COX-2 is expressed at high levels in specific regions of the brain. Its expression appears to be exclusively in neurons, and not in glia or microglia [42]. Yamagata et al. identified high levels of COX-2 mRNA and immunoreactivity in discrete populations of neurons of hippocampus, cerebral cortex and amygdala. Lower levels were reported in the basal ganglia and hypothalamus [43]. COX-2 is localized in dendritic branches and spines suggesting that production of PGs may modulate synaptic signaling associated with neural plasticity [42]. Thus, the physiological role of neuronal COX-2 and PGs far exceed inflammatory response functions. In the brain, PGs are mediators not only of inflammatory response but also of pain induction [44] and cytotoxic processes [45]. Furthermore, COX-2 derived PGs participate in the coupling of

synaptic activity to neocortical blood flow [46]. Mutant mice lacking *cox-2* have an impaired hyperemic response. The role of COX-2 in learning and memory has been demonstrated. Studies indicate that COX-2 inhibitor (Celecoxib) pre-training infusion in the hippocampus of adult rats impairs memory acquisition [41]. Clearly, the high levels of COX-2 are necessary for physiological functions in these neurons; however, this expression is highly regulated. Left unchecked, COX-2 expression is associated with elevated levels of PGs in the brain [47].

Overexpression of COX-2 and PGs have been implicated in a cascade of deleterious effects that promote neuronal injury and dysfunction [18,48]. Studies of human postmortem brains report increased COX-2 immunostaining in a subset of neurons of the hippocampal formation that correlate to early stages of clinical AD [30]. Additional postmortem brain studies of AD patients confirm that COX-2 protein and mRNA are elevated in the frontal cortex [24,31]. Histological analysis of postmortem AD brains has produced conflicting reports. Several studies report increased neuronal COX-2 immunoreactivity while other studies report decreased expression in end stage AD. These studies suggest that the levels of COX-2 expression vary with AD stage hence the controversial findings [41].

Increased COX-2 expression and PGs levels have been reported in the SN and CSF of idiopathic PD patients compared to normal controls [41]. Postmortem SNpc samples from PD patients showed increased COX-2 protein and PGs compared to their age matched controls. Though COX-2 immunoreactivity was also identified within dopaminergic neurons of PD patients, no immunoreactivity was seen in normal controls [49]. Moreover, in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model, COX-2 is induced in SN dopaminergic neurons and PGs levels are increased. In this mouse model, COX-2 mRNA and protein as well as

PGs are undetectable in control mice but detected in the MPTP-treated mice [39,49]. The evidence suggests that COX-2 and PGs play a role in the pathology of PD.

When studying neuronal COX-2 expression, two aspects should be borne in mind. First, the constitutive COX-2 expression in the brain is essential for neuronal functions such as synaptic activity, memory consolidation and inflammatory response. Second, COX-2 expression is tightly regulated to prevent detrimental and pathological effects. It is still unclear how neuronal COX-2 expression is regulated. Steroid hormones such as glucocorticoids have been shown to repress neuronal COX-2 expression [43]. However, there is limited literature on E2 regulation of neuronal COX-2.

Regulation of COX-2 expression

COX-2 expression is regulated by specific transcription factors that bind to their corresponding response elements in the promoter initiating gene transcription [50]. The transcription factors involved in COX-2 regulation are highly cell-type specific, a fact that underscores the need to delineate its regulation in the neuronal context. A number of transcriptional elements including the cyclic adenosine monophosphate (cAMP) response element (CRE), the CCAAT/enhancer binding protein (C/EBP), NF-κB and the E-box are known to be involved in the regulation of *COX-2* (human) and *cox-2* (rodent) genes [50-52]. Of these elements, NF-κB is a key transcription factor that regulates pro-inflammatory genes including *COX-2* [52]. In fact, neuronal *COX-2* expression has been reported to be regulated by NF-κB activity [53]. In their study using hippocampal slice cultures, Kaltschmidt et al. inhibited NF-κB activation using aspirin, which resulted in substantial suppression of *COX-2* expression. Intriguingly, constitutive NF-κB activity and nuclear localization within a subset of neurons of

the amygdala, hippocampus and cortex has been identified [54-56]. Its presence in synaptic terminals suggests it plays a role in synaptic modulation [57,58]

NF-κB is a transcription factor of five Rel family members that can associate to form homo- and heterodimeric complexes [59,60]. The widely studied heterodimer consists of p50 and p65 subunits. Generally, in unstimulated cells, inactive NF-κB is retained in the cytoplasm bound to regulatory proteins known as inhibitors of κB (IκB) [56]. In response to stimuli, IκB is phosphorylated by IκB kinases (IKK) and consequently marked for ubiquitination and degradation. Subsequently, degradation of IκB allows NF-κB to translocate to the nucleus where p65-p50 heterodimers binds to a consensus site in a given promoter. The p65 subunit unlike the p50 contains a transactivation domain that is necessary for NF-κB transcriptional activity and recruitment of histone acetyltransferases [58-61].

C. Estrogen

Estrogens are steroid hormones whose functions far exceed regulation of female reproductive functions. Studies have indicated that estrogens are involved in non-reproductive functions such as regulating lipid and carbohydrate metabolism, skeletal homeostasis, cardiovascular and central nervous systems [35,62]. The brain can synthesize estrogen *de novo* from cholesterol, underscoring its significance in the brain. There are reports of the presence of all enzymes needed for synthesis and metabolism of estrogen in various regions of the human brain [35]. A key enzyme in the last step of estrogen synthesis is aromatase. Significant levels of aromatase have been found in neurons of hippocampus [63], amygdala, thalamus, the bed nucleus of the stria terminalis and basal ganglia [64]. This evidence underscores the significance of estrogen in these neurons.

Estrogen Receptors (ER)

Three major estrogens exist in humans and rodents; 17β-estradiol (E2), estrone (E1) and estriol (E3) [65]. E2 is the most potent and dominant estrogen in mammals. Classically, E2 exerts its physiological effects through two nuclear estrogen receptors (ER), ERα and ERβ [66]. ERα and ERβ sequence homology is high in the DNA binding domain (more than 95%). Homology in the ligand binding domain is ~55% while the N-terminal domain is ~15% [67]. Both receptors are widely expressed in neurons although the spatiotemporal distribution and expression levels are divergent. Beyond the classic steroid receptors, E2 can also mediate rapid signaling through an orphan transmembrane bound receptor, the G-protein-coupled estrogen receptor 1 (GPER or GPR30) [68]. GPR30 immunoreactivity has been detected in the hippocampus, cortex, hypothalamus, substantia nigra and amygdala of rodent brain [69]. It is unclear if E2 initiates responses through GPR30 alone or in concert with the ER. The focus for this study was limited to the ER mode of action. Roughly, this activity encompasses the binding of E2 ligand to ER, promoting their dimerization and initiation of downstream signaling cascades.

Mode of Action

Genomic pathway

In this classic mechanism of ER action, the binding of E2 to ER residing either in the cytoplasm or nucleus induces a conformational change in the receptors [62]. Activated ERs form homodimers or heterodimers and are ligand-activated transcription factors in which case they can directly bind consensus palindromic estrogen response elements (EREs) or ERE half-sites in E2 responsive gene promoters (Figure 3) [70,71]. An ER-ligand complex can recruit coregulators to the chromatin and consequently alter gene expression, thereby acting as a

ligand-dependent transcriptional factor. However, a number of genes regulated by E2 lack the ERE-like sequences in their promoters. ER can regulate gene expression without direct binding to DNA. Through protein-protein interactions with other transcription factors bound to their response elements, ER can modulate gene expression [72]. ER have been shown to directly interact with FBJ murine osteosarcoma viral oncogene homolog (FOS) and jun proto-oncogene (JUN) proteins at the activator protein-1 (AP-1) binding site [65,70,72]. Direct physical interaction between ER and other transcriptional factors such as nuclear factor κβ (NF-κβ) and CCAAT/enhancer binding protein β (C/EBPβ) to repress interleukin -6 (IL-6) has been shown. [73,74]. Furthermore, an interaction with stimulating protein-1 (SP-1) mediates ER-DNA binding to induce genes such as low-density lipoprotein (LDL) receptor gene [70].

Nongenomic pathway

There is evidence that E2 can exert actions that are too rapid to be accounted for by the genomic signaling pathway. The ER-dependent nongenomic pathway is characterized by rapid E2 action. This rapid response is associated with a subset of membrane-associated-ERs [72,75,76]. Induction of these receptors by E2 directly activates a number of signaling pathways such as the cAMP-protein kinase A-cAMP response element-binding protein (cAMP-PKA-CREB), the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3K) pathways (Figure 3) [77-79]. These cell type-specific intracellular pathways can regulate transcription factors via phosphorylation. MAPK signaling pathway has been shown to phosphorylate C/EBPβ and CREB [72]. Phosphorylation of these transcription factors can initiate signaling cascades that result in binding of factors to their response elements in a target gene promoter. Furthermore, ER can repress genes by directly or indirectly inhibiting the binding of transcription factors to their response elements and recruitment of repressor proteins to the

promoter region (Figure 3) [80]. Thus, a possible convergence of genomic and nongenomic activities at multiple response elements in the promoter of target genes provides an extremely fine and complex degree of control for ER-dependent gene regulation.

ER-Independent Pathway

This pathway involves the E2-mediated GPR30-dependent signaling cascade. E2 binds GPR30 and initiates activation of intracellular signaling cascades such as MAPK and PI3K pathway [66,68]. Additional studies are needed to elucidate the contribution of GPR30 in E2-mediated gene regulation in the brain. Furthermore, studies are needed to see if ER and GPR30 mechanisms and functions overlap or interact to regulate genes.

Anti-inflammatory properties of E2

There is substantial evidence that E2 deprivation has profound direct effects on brain structure and function. E2 provides a wide array of neuroprotective effects such as neurotrophic, neurogenerative, antioxidative and anti-inflammatory effects [81,82]. The focus here will be on anti-inflammatory effects of E2. The anti-inflammatory potential of E2 are well described in animal and cellular models of neurodegeneration. For example, *in vivo* mouse models indicate that E2 suppression of inflammation is through ER-mediated inhibition of inflammatory response in microglia [83], suppression of pro-inflammatory cytokines such as IL-1β, IL-6 and brain chemokine levels [84]. *In vivo* rat model of burn injury showed that E2 decreases burn-induced brain inflammation by decreasing levels of TNF-α, IL-1β, IL-6 [85]. Moreover, E2 activates the extracellular signal-regulated kinases (ERK) pathway which promotes cell survival and growth. In astrocytes, E2 is able to suppress induction of inflammatory factors such as IL-1β, TNF-α and matrix metalloproteinase-9 (MMP-9) [86]. In the MPTP mouse model of PD, E2

decreased glial activation in the SN and as a result prevented further loss of dopaminergic neurons [87]. Taken together, the evidence indicates that E2 exhibits potent anti-inflammatory properties that protect the brain.

E2 can suppress brain inflammatory processes by repressing pro-inflammatory genes and preventing their induction. The mechanisms by which E2 represses these genes are not well understood. Interestingly pro-inflammatory genes such as *TNF-α* and *IL-6* lack the canonical EREs in their promoters but nonetheless are suppressed by E2 [73,74,88] . Studies have reported that E2 treatment suppresses COX-2, iNOS and IL-6 induction by inhibiting NF-κβ DNA binding [89-91]. Furthermore, these studies indicate that E2 prevents induction of inflammatory genes by inhibiting NF-κB intracellular transportation through an ER-mediated activation of the PI3K pathway. It is likely that a multitude of complex mechanisms are employed by E2 in suppressing pro-inflammatory genes in the brain. The diverse cellular environment in the brain further contributes to the complexity of E2 anti-inflammatory action. This study focused on elucidating molecular mechanisms by which E2 suppresses the inflammatory gene *cox-2* in neurons.

The rapid and marked drop in E2 levels that characterizes menopause is attributed to deleterious consequences. These include altered inflammatory processes [92], cognitive and mood impairment and increased risk of neurodegenerative diseases [93-97]. Extensive evidence from studies using OVX animals and cell cultures that are deprived of E2 reveal marked increase in expression of inflammatory genes such as *TNF-α, IL-1β* [11,19] and inflammatory mediators such as chemokines [84]. Furthermore, microarray data analysis of cortical regions of postmenopausal women shows altered expression of macrophage-associated, regulatory and pro-inflammatory genes [92]. Substantial evidence of immunomodulatory effects of E2 in OVX rats

has been reported in the frontal cortex [98] and striatum [84] and hippocampus [99] and SN [19]. Cell culture studies report E2 suppression of inflammatory mediators such as cytokines and their respective receptors [84,86,96,100,101].

E2 mediated epigenetic repression of genes

In addition to directly inhibiting activity of transcriptional factors such as NF-κB, recruitment of a variety of coregulators in large complexes is inherent to E2 signaling. Coregulators such as repressor proteins interact with members of nuclear receptors and are involved in chromatin remodeling, histone modifications resulting in gene repression [62]. *In vitro* and *in vivo* studies indicate that ligand-bound ER recruits and interacts with corepressor such as nuclear receptor co-repressor (NCoR), ligand-dependent corepressor (LCOR) and silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) [102,103]. ER direct interaction with multiprotein complexes containing histone deacetylase (HDACs) to repress genes has been reported in E2 repressed genes [104,105]. HDACs can exist as components of large complexes with other repressor proteins such as Sin3A (Swi-*in*dependent 3A), SMRT and DNA methyl transferases (DNMTs) [106-108]. Sin3A is a master scaffold protein that provides a platform for the assembly of numerous transcription factors and repressor proteins such as HDACS and DNMTs. DNA methylation by DNMTs and removal of acetyl groups from lysine residues of core histones particularly H3 and H4 by HDACs result in a more compact nucleosome structure and consequently transcription repression [109,110].

Studies have indicated that E2 can increase DNA methylation and epigenetically silence target genes [62,111]. DNA methylation occurs when a methyl group is added to the cytosine (C) by a DNMT. The C base is located next to a guanine (G) base and high frequency of CpG

dinucleotides cluster to form CpG islands which are often located in or near the promoter of a gene [112]. A study using human gastric carcinoma cell lines suggested that the transcriptional silencing of *COX-2* was strongly related to the aberrant methylation status of the CpG island in the proximal promoter region spanning −590 to +186 [113,114]. Furthermore, the increased expression of COX-2 seen in frontal cortex of AD brains correlates to the hypomethylated state of *COX-2* promoter CpG clusters [115] . Taken together, the evidence indicates that E2 suppresses neuronal *COX-2* expression by various mechanisms including recruitment of repressor proteins and methylation of CpGs in the *COX-2* promoter. This study will focus on the methylation status of CpG clusters in the proximal region of the rat *cox-2* promoter identified bioinformatically [116].

D. Goals of the current research

Gaps in the literature

Most studies on AD have focused on the hippocampus and frontal cortex; however, the there are few reported studies of the amygdala. In early stages of AD, post-mortem studies and magnetic resonance imaging data shows that the amygdala undergoes substantial atrophy and neuronal loss [32]. This may partly account for the emotional memory impairments and behavioral deficits associated with AD. One study using a transgenic mouse model overexpressing human COX-2 in the amygdala, hippocampus and cortex indicated that these mice developed cognitive deficits [47]. Even though studies have shown that COX-2 is constitutively expressed in the amygdala, there is limited literature on how its expression is regulated. It is likely that overexpression of COX-2 in the amygdala contributes to neuronal loss

associated with early AD. The question arises of how neuronal COX-2 is regulated in the amygdala.

Mounting evidence indicates that COX-2 contributes to the pathology of PD [41,49]. Furthermore, epidemiological and clinical evidence indicate that E2 protects dopaminergic neurons and appears to delay the onset of PD [87]. However, there are gaps in the literature on whether E2 suppresses the expression of both *COX-2* (human gene) and *cox-2* (rodent gene) in amygdala and dopaminergic neurons of SN.

Based on the gaps identified in the literature, the focus of this research was to elucidate the mechanisms by which E2 suppresses neuronal *cox-2* expression. The working hypothesis of this study is: **E2 suppresses *cox-2* expression in a neuronal cell line by an ER-mediated mechanism.**

Figure 2. Schematic of Arachidonic Acid pathway. Cyclooxygenases metabolize arachidonic acid to prostaglandins. Prostaglandins mediate various physiological processes such as inflammation and synaptic transmission.

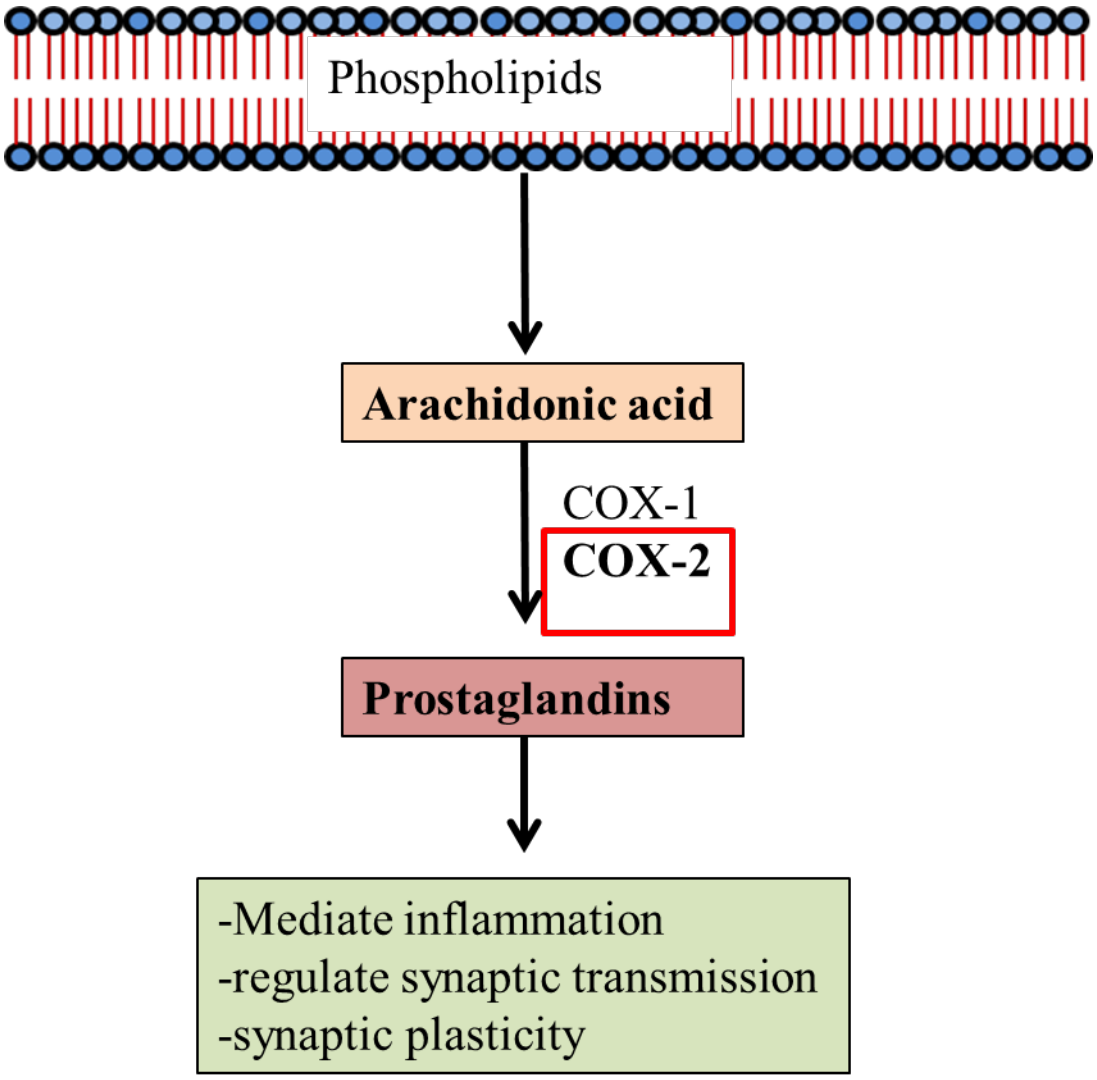
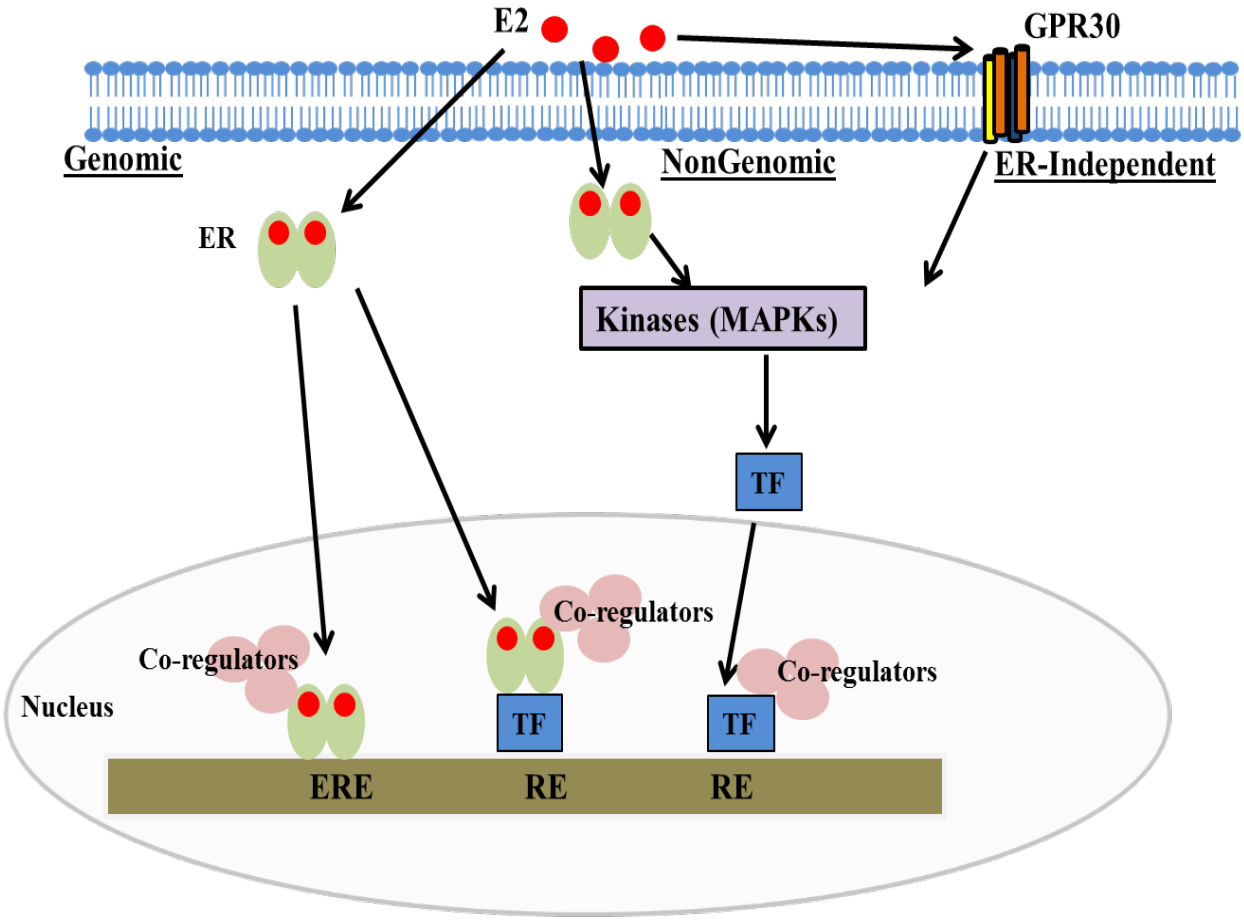

Phospholipids
Arachidonic acid
COX-1
COX-2
Prostaglandins
-Mediate inflammation
-regulate synaptic transmission
-synaptic plasticity

Figure 3. Illustration of E2 mechanism of action. The genomic pathway consists of direct DNA- binding of ligand activated estrogen receptors (ER) to estrogen response elements (ERE). For promoters without EREs, ER modulate transcription factors (TF) in a protein-protein or "tethering" mechanism. The nongenomic pathway involves rapid activation of second messengers by ligand activated ER. The ER-independent pathway involves E2 binding G-protein-coupled estrogen receptor 1 (GPER or GPR30) and downstream signaling cascades.


E2
GPR30
Genomic
NonGenomic
ER-Independent
ER
Kinases (MAPKs)
TF
Co-regulators
Co-regulators
Co-regulators
Nucleus
TF
TF
ERE
RE
RE

CHAPTER 2

MATERIALS AND METHODS

A. Cell Culture

AR-5 cell line

AR-5 is an immortalized neuronal cell line derived from the amygdala of embryonic rats [117]. Lalmansingh et al determined that these cells express ERα and ERβ mRNA and protein [71]. We determined that these cells express the male genotype based on the detection of SRY (sex-determining region Y) mRNA (Appendix. Table 1). For all experiments, cell passage numbers were less than 15.

Cells were plated and grown in phenol red-free DMEM / Ham's F12 media (Hyclone Laboratories, UT). Phenol red can mimic biological action of estradiol and influence study results. Media was supplemented with 10% newborn calf serum (NCS) (Gemini Bioproducts), 1% penicillin/streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, and 1% L-glutamine (all from Cellgro, Mediatech Inc.). For all the studies, Nunc™ cell culture plates were used (Nalge Nunc International).

N27 cell line (gift from Dr. Rebecca Cunningham)

N27 is an immortalized cell line developed from female rat mesencephalic dopaminergic neurons. These cells closely mimic dopaminergic neurons of substantia nigra in that they produce dopamine, have tyrosine hydroxylase activity and are positive for dopamine transporter [118,119]. These cells express ERα mRNA and protein, but only ERβ mRNA [120]. For all experiments, cell passage numbers were less than 20.

Cells were plated and grown in phenol red-free and endotoxin free RPMI 1640 media. Media was supplemented with 10% fetal bovine serum (FBS) (Gemini Bioproducts), 1% penicillin/streptomycin, 1% nonessential amino acids, 1% sodium pyruvate, and 1% L-glutamine (all from Cellgro, Mediatech Inc.). For all the studies, Nunc™ cell culture plates were used (Nalge Nunc International).

Cell Treatments

Prior to all treatments, cells were maintained for 24 hours (h) in media containing charcoal-stripped NCS or FBS serum to remove endogenous hormones. Cells were treated either with 0.1% of ethanol or 0.1% of Dimethyl sulfoxide (DMSO) vehicle (VEH) or the following treatments: 17β-Estradiol (E2) (10^{-7} M) was obtained from Sigma-Aldrich Co. (St. Louis, MO). 2,3-*bis*(4-Hydroxyphenyl)-propionitrile (DPN) and 4,4',4''-(4-Propyl-[1H]-pyrazole-1,3,5-triyl)*tris*phenol (PPT), 4-[2-Phenyl-5,7-*bis*(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) all at 10^{-7}M were obtained from Tocris Bioscience (UK), Trichostatin A (TSA) and Phorbol 12-myristate 13-acetate (PMA) both at 100 nm and from Sigma-Aldrich Co. (St. Louis, MO).

B. Immunocytochemistry (ICC)

Cells were grown in Lab-Tek^{TM} II Chamber Slides (Nalge Nunc International). After fixation in 4% paraformaldehyde for 20 mins, cells were permeabilized with 0.1 % Triton X-100. Cells were washed three times 10 mins each with phosphate buffered saline (PBS). Cells were then incubated with a solution of 5% normal goat serum and 2% bovine serum albumin in PBS for 30 mins to block non-specific binding. After blocking, cells were incubated with primary antibodies overnight at 4 °C. Dilution for primary antibodies was 1:250. Cells were washed three times 10 mins each with PBS and incubated with 1:1000 dilution of a mixture of Alexa Fluor 594-goat anti-rabbit IgG and 488-goat anti-mouse (Molecular Probes Invitrogen, CA, USA) for 1h. After washing three times 10 mins each with PBS, cells were mounted in FluorSave reagent (Calbiochem, CA, USA). Images were captured using Olympus IX70 epifluorescence microscope with Simple PCI image acquisition software (Compix Inc., Hamamatsu Photonics Management, PA, USA). Digitized images were arranged using Adobe Photoshop (Adobe, CA, USA).

C. Protein sample preparation and Western Blot (WB) analysis

To extract whole cell lysates, cells were washed twice in ice-cold PBS and collected by scraping from plate. After centrifugation at 900 *g* for 5 mins, cells were lysed for 10 mins on ice using radioimmunoprecipitation assay buffer (RIPA) buffer (140mM NaCl, 10mM Tris-Cl (pH 8.0), 1mM EDTA, 1% Triton X-100, 0.1% SDS and 0.1% sodium deoxycholate) supplemented with 10 µL/mL protease inhibitor cocktail (contained 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatinA, E-64,bestatin, leupeptin, and aprotinin) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical, MO). Lysate was then centrifuged at 12,000 *g* for 15 min at

4 °C. Supernatant was transferred to fresh tubes. Protein was quantified using bicinchoninic acid assay (BCA assay, Thermo Scientific).

Protein samples were boiled in Laemmli buffer (Bio Rad Laboratories) with β-mercaptoethanol (Sigma-Aldrich) for 10 minutes (mins). Samples were separated by SDS-PAGE using 12% Mini-PROTEAN TGX precast gels (Bio Rad Laboratories). After transfer onto Immuno-Blot® PVDF membrane (Bio Rad Laboratories), the membrane was incubated with 5% nonfat dry milk in PBS for 1 h at room temperature to block non-specific binding. Subsequently, the membrane was incubated overnight at 4 ^{o}C with primary antibodies. A polyclonal anti-COX-2 (Abcam) and anti-β-actin (Cell signaling) at a dilution of 1:1000. β-actin was used as a loading control. The blots were washed three times 10 mins each with 5% nonfat dry milk in PBS and incubated with Horseradish peroxidase-conjugated antirabbit IgG (1: 5000, Millipore Corp, MA) for 1 h at room temperature. Following washing three times at 10 mins each with PBS, proteins were visualized using Enhanced Chemiluminescent Substrate (Pierce Biotechnology, Inc. IL) and imaged using UVP Biospectrum 500. Figures were arranged using Image J software (NIH).

D. RNA Isolation and Reverse transcription – qPCR

Following treatment, cells were collected using Tri reagent (Molecular Research Center, OH). To separate the aqueous phase containing RNA, chloroform was added. Total RNA was precipitated with isopropanol and washed with 75% ethanol. The RNA pellet was resuspended in nuclease free water followed by quantification. For reverse transcription (RT), 1μg of total RNA was used for cDNA synthesis performed at 42 °C for 30 min using the iScript™ cDNA synthesis kit (Bio Rad, USA). Changes in the levels of COX-2 mRNA and pre-mRNA were measured by real-time PCR (qPCR) on a CFX96™ Real-Time PCR detection system using IQ™ Sybr Green

Super Mix (Bio Rad Laboratories, Hercules, California). The primer sequences used to amplify COX-2 mRNA and COX-2 pre-mRNA are listed in Table 1. Thermal cycling parameters are as follows: initial denaturation at 95°C for 3 mins, followed by amplification (39 cycles) at 95°C for 5 seconds, 65°C for 30 seconds and 72°C for 60 seconds. Levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were used to normalize data. GAPDH mRNA primer sequences are listed in Table 1. To analyze the real time data, the $\Delta\Delta C_T$ method was used [121,122]:

Fold change = $2^{-\Delta\Delta C_T} = 2^{\wedge} - [(C_T$ gene of interest - C_T internal control) sample – (C_T gene of interest - C_T internal control) vehicle control]

The threshold cycle (CT) is the quantitative endpoint for qPCR. The CT is an arbitrarily placed threshold for the fluorescent signal to cross (i.e. above background level). Data was presented as fold difference of vehicle. Melt curve analysis were performed with each assay to ensure the amplification of a single product.

E. Chromatin Immunoprecipitation (ChIP)

AR-5 cells were grown to 80 – 90% confluency in 15 cm plates. Cells were directly fixed with 1% formaldehyde for 10 mins at room temperature with gentle mixing on the Belly Dancer™ orbital shaker. Fixation was stopped by the addition of 0.125 M glycine for 5 mins at room temperature and with gentle mixing on the Belly Dancer™ orbital shaker. After washing three times with 5ml of ice-cold PBS, cells were harvested by scraping and collecting in 5ml ice-cold PBS. The cells are immediately centrifuged at 700*g* for 5mins at 4° C. Cell pellet was resuspended in 1 – 1.5 mL cold cell lysis buffer (10mM Tris – HCl pH 8, 100mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.1% Na- deoxycholate, 0.5% SDS) supplemented with 10 µL/mL

protease inhibitor cocktail and 1 mM PMSF. Cells were lysed for 20mins on ice with frequent up and down pipetting. Cell lysate was aliquoted into 1.5 mL microcentrifuge tubes for sonication. An aliquot (about 50 µL) was saved as a pre-sonication control. Sonication was performed in an ice-water bath for 8 mins (30 seconds pulse ON and 45 seconds pulse OFF at 80% amplitude) using the Misonix Sonicator Q700 with cup horn (Q Sonica, LLC, Newtown, Connecticut). This sonication protocol ensured that the chromatin was sheared to 200- to 1000bp fragments. Fragment length was monitored by agarose gel electrophoresis. Chromatin lysate was cleared by centrifugation at maximum speed for 15 mins at 4 °C. Supernatant was transferred to fresh tubes.

To precipitate protein of interest, 100-150 µL of chromatin per antibody was used. A portion of the lysate (10%) was set aside as input. Chromatin was diluted with immunoprecipitation (IP) buffer (150mM NaCl, 50mM Tris-HCl (pH 7.5), 5mM EDTA, 0.5% IGEPAL, 1% Triton-X 100) supplemented with protease inhibitors and PMSF to a final volume of 1mL. Samples where incubated overnight on a rotator at 4 °C with 2 - 5 µg of antibody. Respective IgG were used as negative control for the IP. To isolate the protein-antibody complex, 20 µL of Magna ChIP™ Protein A+G Magnetic Beads (Millipore Corp, Massachusetts) were added to each sample and mixed by rotation at 4 °C for 2 h. Using a magnetic separation stand, beads were washed 4 times with 500 µL of RIPA buffer (50mM HEPES-KOH pH 7.5, 0.25M LiCl, 1mM EDTA 0.5% IGEPAL, 0.5% Na-deoxycholate) and 2 times with 500 µL Tris-EDTA (TE) buffer with 50mM NaCl on a rotator at 4 °C.

To reverse crosslink and isolate DNA, 100 µL of 10% Chelex® 100 Resin (Bio Rad Laboratories, CA) was added directly to samples. The slurry was briefly vortexed (10 sec) and boiled for 10 mins at 100 °C. Samples were then placed on ice to cool followed by incubation with 2 µL of proteinase K for 30 mins at 55 °C. To inactivate proteinase K, samples were boiled

for 15 mins. Samples were centrifuged at 12, 000*g* for 3 mins at room temperature. Supernatant containing DNA (50 µL) was collected. For qPCR 2 µL of DNA was used. Primers targeting the *cox-2* NF-κB p65 site and *c-fos* ERE promoter region were used (Table 1). As a negative control for binding, primers targeting the far upstream regions of the *cox-2* promoter (−2700 through −2500 bp) were used (Table 1). To calculate enrichment, the percent input method was used (Invitrogen, Carlsbad, CA, USA).

Percent of input = 100 * 2^ (Adjusted input) - C_T (IP)

(Adjusted input = adjusted to 100% = C_T input – 3.32)

(3.32 cycles = $\log_2$ of 10 (dilution factor of input))

Background was subtracted and data presented as fold occupancy in relation to vehicle.

F. DNA Methylation analysis

Genomic DNA Purification, Sodium bisulfite treatment, cloning and methylation analysis

Genomic DNA was extracted using *Quick-gDNA*™ MiniPrep kit (Zymo Research, Irvine, CA). Genomic DNA (1 µg) was then subjected to bisulfite conversion using EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA) according to the manufacturer's guidelines. Modified DNA was then purified and concentrated using ChIP DNA Clean & Concentrator™ kit ((Zymo Research, Irvine, CA). Converted DNA (5 µL) was subjected to first round of PCR amplification using PCR master mix (Promega, WI) and outside primers (Table 1). The protocol involved initial denaturation at 95°C for 5 mins, followed by 34 cycles of denaturation (95°C, 60 sec), annealing (54°C, 60 sec), and extension (72°C, 60 sec) with a final extension cycle (72°C, 10 min). The PCR product was purified using the ChIP DNA Clean & Concentrator™ kit and 5 µL was used as a template for the second round of PCR using nested

primers (Table 1). Primers were designed using the MethPrimer software to amplify the *cox-2* proximal promoter region containing a CpG island (Table 1). The PCR product was purified and analyzed by agarose gel. The 230 bp PCR product was cloned into pCR4-TOPO vector using TOPO-TA Cloning Kit (Life Technologies, Gaithersburg, Maryland) according to manufacturer's guideline. Recombinant clones from at least 3 independent experiments were selected for inoculation. Plasmid DNA was isolated using the PureLink® Quick Plasmid Miniprep Kit (Life Technologies, Gaithersburg, Maryland). DNA was sequenced and methylation data was analyzed by comparison with original DNA sequence to identify modified cytosine residues. Data was compiled using the BISMA software available online (http://biochem.jacobs-university.de/ BDPC/BISMA).

G. Statistical Analysis

Data was analyzed using one-way analysis of variance (ANOVA) with treatment as a factor. For post-hoc analyses, Bonferroni was used. $p \leq 0.05$ was considered as statistically significant for all comparisons. All data was presented as mean ± standard error of the mean (SEM) for at least three experiments. The software used for the analyses was IBM SPSS Statistics 21 software (IBM, USA).

Table 1. List of forward and reverse primers used for qPCR and their product size

Amplified Gene/region	**Forward primer**	**Reverse Primer**	**Product size (bp)**
mRNA COX-2	AAAGCCTCGTCCAGATGCTA	ATGGTGGCTGTCTTGGTAGG	249
Pre-mRNA COX-2	AAGGCATTTGTTGAGCTTGC	GCATGCCTGGTACCCTAAAA	175
GAPDH	TGGAGTCTACTGGCGTCTT	GCTGACAATCTTGAGGGAG	200
***Cox-2* proximal promoter flanking the p65 binding site**	CGGTAACTGTGTGCGTGCT	CGGAGGAGCAAGAGAATGTC	130
***Cox-2* promoter far upstream region (negative control)**	TGCGTTTCCTCATTTTCCTT	AGCGCGATGATAAAGATGCT	185
***c-fos* ERE**	GGCGAGCTGTTCCCGTCAATCC	GCGGGCTCCCTGTCATCAACTCTA	240
Outside primers for methylation PCR	TTTGTTTTTATGGGTATTATGTAATTGG	AAAAAAATCCCTCCAAAAATACTTC	400
Nested primers for methylation PCR	TTGTTTTTATGGGTATTATGTAATTGG	AACAAAACACAAAACTAAATTCCTTC	230

CHAPTER 3

RESULTS

A. E2 REGULATION OF *cox-2* EXPRESSION IN AR-5 NEURONAL CELLS

AR-5 cells express COX-2 immunoreactivity (IR)

To determine whether the AR-5 cell line express COX-2, ICC was performed using an antibody directed against COX-2 (Fig. 1A). To determine whether COX-2 antibody recognized protein at correct molecular weight, Western Blots were performed using duplicate samples. The antibody recognized an immunoreactive band at approximately 69 kDa (Fig. 1D). Thus, in our hands, the AR-5 cell line serves as a suitable model for examining the effects of E2 on neuronal *cox-2* expression.

E2 and DPN suppress COX-2 mRNA and pre-mRNA

To determine the effect of E2 on COX-2 mRNA, we analyzed its levels by RT-qPCR at various time points. Additionally, to analyze receptor-mediated mechanism, we used subtype selective agonists DPN and PPT. DPN has a greater affinity for ERβ while PPT has an affinity for ERα. E2, DPN and PPT had no effect on COX-2 mRNA levels before 24 h (Fig. 2A). However, E2 and DPN repressed COX-2 mRNA levels to 50% at 24 h (Fig. 2B). In distinction, PPT had no effect on COX-2 mRNA at any time. Furthermore, the effect of ligands on mRNA levels was indistinguishable from their effects on pre-mRNA (Fig. 3). Taken together, these

pharmacologic data suggest that ERβ but not ERα suppress expression of *cox-2*, and that the point of regulation occurs at the level of mRNA or before.

E2 and DPN suppression of COX-2 pre-mRNA is reversed by PHTPP

To corroborate the conclusion that ERβ but not ERα leads to the suppression of *cox-2* expression, cells were treated with E2 or DPN in the presence of the selective ERβ antagonist PHTPP. Both E2 and DPN suppression were reversed by PHTPP (Fig. 4) further validating that ERβ suppresses *cox-2* expression. Given that E2 and DPN suppress RNA levels to the same extent, and that PHTPP blocks E2 and DPN equally well, the data indicated that E2 suppression of *cox-2* expression is mediated by ERβ.

ERβ fails to occupy the cox-2 promoter

We next asked whether E2 and DPN elicited changes in ERβ occupancy in the region of the NF-κB site (Fig. 5). Surprisingly, ChIP analysis failed to reveal ligand-induced increased ERβ occupancy (Fig. 6A). In distinction, analysis of the *c-fos* promoter in the region with known ERE (-309 to -80 bases) showed that both ligands induced ERβ occupancy by 4.5-fold (E2) and 2-fold (DPN) (Fig. 6B). Thus, ligand induced recruitment of ERβ is promoter specific. Furthermore, this data suggests that E2 down regulates *cox-2* expression in a manner that is independent of ERβ occupancy.

E2 and DPN decrease p65 occupancy and Ac-H4 levels

Given that neither E2 nor DPN induced ERβ recruitment, we then sought to determine if ERβ could be affecting another point of regulation secondarily. To that end, we targeted changes in occupancy of p65 in response to E2 and DPN. Indeed, ChIP analysis revealed that E2 and DPN decreased p65 occupancy by about 40% and 70% respectively (Fig. 7A). p65 occupancy to this region was specific to the extent that it was not detected in far upstream regions of the *cox-2*

promoter at −2700 through −2500 in the absence or presence of ligand (Fig. 7B). Thus, rather than down-regulate *cox-2* expression via increasing ERβ interaction with the promoter region, ERβ reduces *cox-2* expression indirectly through a mechanism that involves reduced p65 occupancy. In keeping with the decrease in RNA levels and p65 occupancy at the promoter, E2 and DPN led to decreased levels of Ac-H4 (pan acetylated H4) by about 20 % and 40% respectively (Fig. 7C). There was specificity in the histone deacetylation in that both ligands decreased H4 but not H3 (Fig. 7C and 7E). The effect of both ligands on H4 levels is specific to this region in that it was not detected at the upstream region of the *cox-2* promoter at -2700 through -2500 (Fig. 7D).

E2 and DPN increases HDAC1 and Sin3A occupancy

We next sought to determine whether E2 would regulate occupancy of other factors associated with gene repression. To investigate the role that HDACs might play in ligand induced *cox-2* suppression, cells were treated with the HDAC inhibitor TSA. TSA elicited almost 2-fold increase in COX-2 pre-mRNA and as before, E2 and DPN suppressed *cox-2* expression; however, TSA blocked their repressive effects (Fig. 8). Accordingly the effect of E2 and DPN on HDAC promoter occupancy was determined by ChIP. Here too there was specificity in that ligands increased HDAC1 (3.5-fold for E2 and 2-fold for DPN) albeit to differing degrees but not HDAC3 occupancy (Fig. 9A and 9C). Given that HDAC1 has been shown to interact with Sin3A in a repressive complex [106,108,123-125], we next sought to determine whether or not E2 and/or DPN increased Sin3A occupancy. Both ligands increased Sin3A occupancy to the same degree; approximately 5-fold (Fig. 9E). As was the case for p65, neither HDAC1 nor Sin3A occupancy was detected at the far upstream region of the *cox-2* promoter at -2700 through -2500 in the absence or presence of ligands (Fig. 9B and 9F).

Taken together the data reveal that ERβ reduces p65 occupancy, and simultaneously leads to formation of a functional HDAC1/Sin3A complex. Occupancy by members of the complex, however are differentially regulated by E2 and DPN, suggesting that ERβ is not the only receptor mediating the changes.

E2 increases the overall methylation of *cox-2* proximal promoter

To determine whether or not CpG methylation could complement the ligand-induced reduction in histone acetylation, we measured changes in *cox-2* promoter methylation. Identification of the CpG island was performed by bioinformatic analysis of GC content (Fig. 10A). Eight CpG sites are clustered in the *cox-2* proximal promoter and none are in the NF-κB site (Fig. 10B). In the absence of ligand, none of these sites are methylated (Fig. 11A). E2 increases the overall methylation of the region to 7.6% (Fig. 11A and B); however, no single site is preferentially methylated (Fig. 11A). Site 1 and 4 are always unmethylated (Fig. 11C). Curiously, DPN failed to alter the status of promoter methylation, suggesting that even though ERβ regulates RNA levels, methylation is due to a different mechanism.

B. E2 REGULATION OF *cox-2* EXPRESSION IN N27 NEURONAL CELLS

E2 fails to suppress COX-2 pre-mRNA

To determine the effects of E2 on COX-2 pre-mRNA, N27 cells were treated with E2, DPN and PPT at various time points. The ligands failed to alter COX-2 pre-mRNA levels at any time point (Fig. 12A and 12B). The data suggests that the point of E2 regulation of *cox-2* expression in N27 occurs at a different level rather than RNA level.

FIGURES AND FIGURE LEGENDS

E2 REGULATION OF *cox-2* EXPRESSION IN AR-5

Figure 1. AR-5 express COX-2 immunoreactivity (IR). (A) COX-2 IR, (B) COX-2 staining merged with Hoechst (C) phase contrast showing presence of cells (D) COX-2 protein band detected at the appropriate molecular weight of approximately 69 kDa (duplicate samples). Scale bar 50μm.

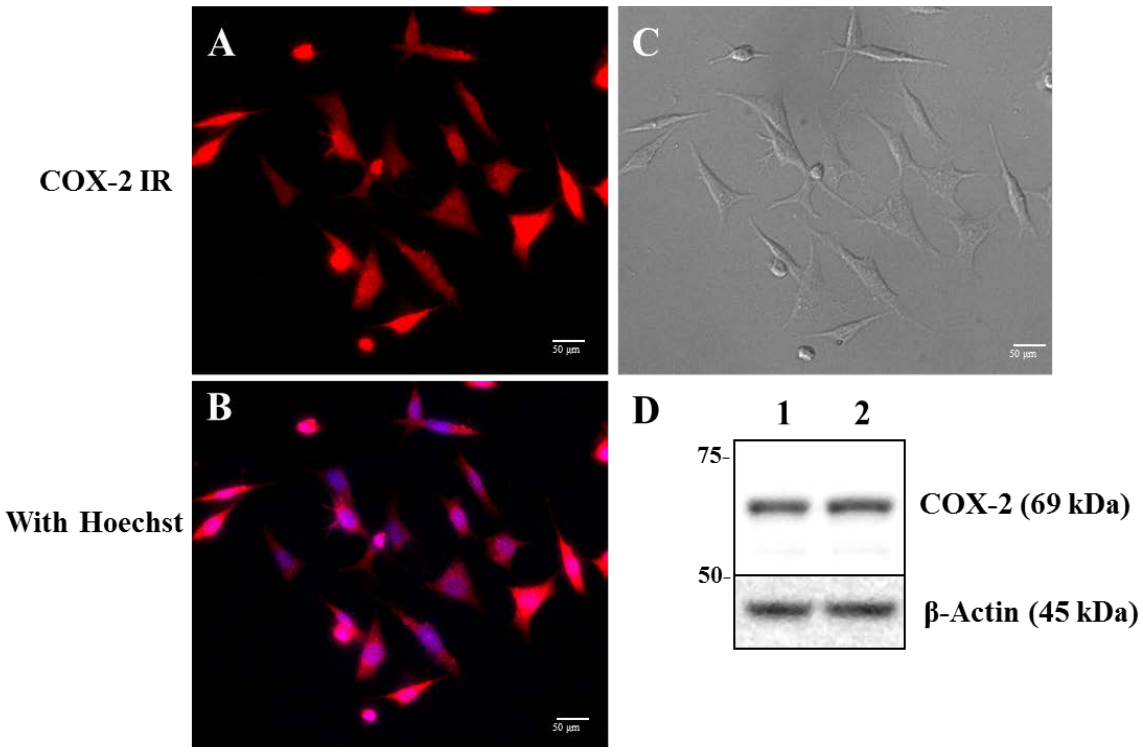
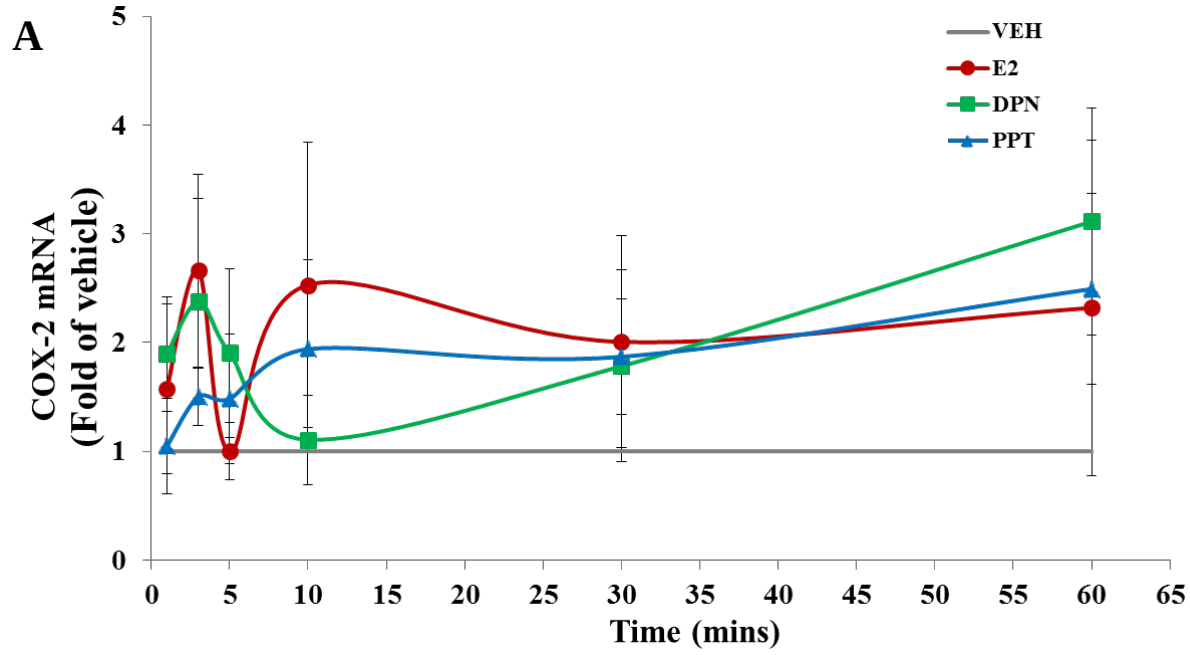

A
C
COX-2 IR
B
D
1
2
75-
50-
COX-2 (69 kDa)
β-Actin (45 kDa)
With Hoechst

Figure 2. E2 and DPN suppress COX-2 mRNA levels at 24 h. Cells were treated with E2, DPN, and PPT (all 10^{-7} M): A, up to 1 h. B, up to 24 h. Expression of COX-2 mRNA was measured by RT-qPCR ($n \geq 3$). Data represents mean ± SEM. B, *p = 0.03 E2 and DPN compared to VEH (Vehicle).


A
VEH
E2
DPN
PPT
COX-2 mRNA
(Fold of vehicle)
0
1
2
3
4
5
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time (mins)

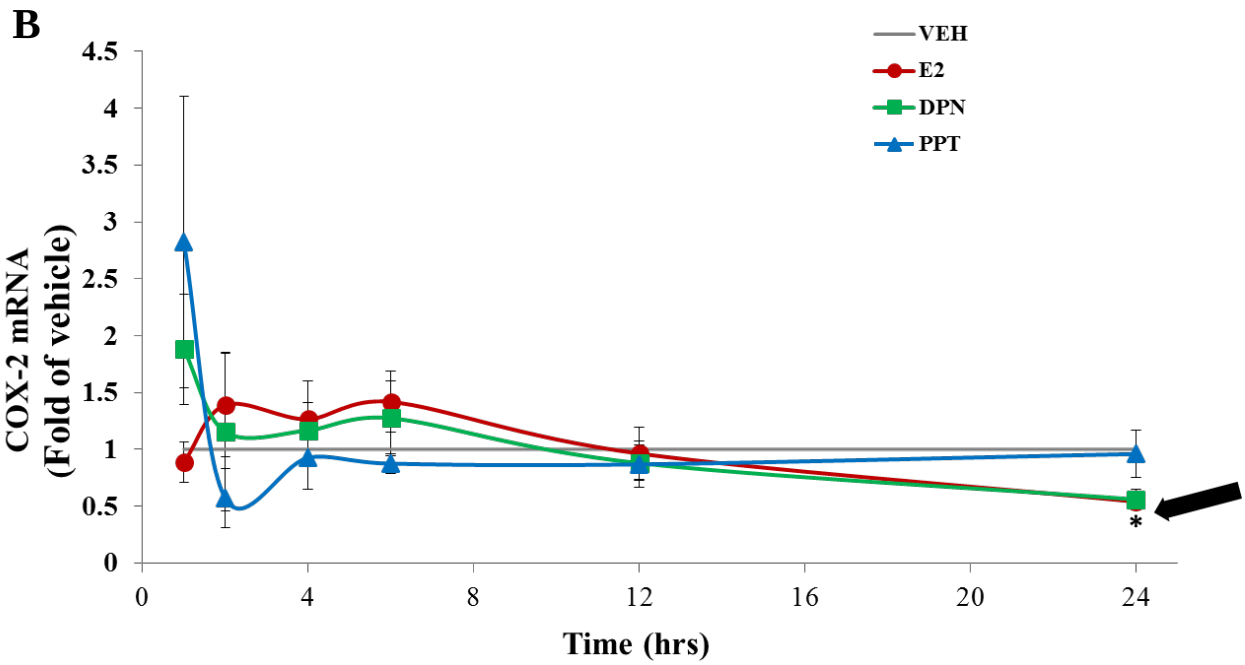

B
VEH
E2
DPN
PPT
COX-2 mRNA
(Fold of vehicle)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
*
0
4
8
12
16
20
24
Time (hrs)

Figure 3. E2 and DPN suppress COX-2 pre-mRNA. Cells were treated with E2, DPN and PPT for 24 h here and in subsequent experiments. Pre-mRNA were measured by RT-qPCR. ($n \geq 3$). Data represents mean ± SEM. *p = 0.01 E2 compared to VEH, *p = 0.002 DPN compared to VEH

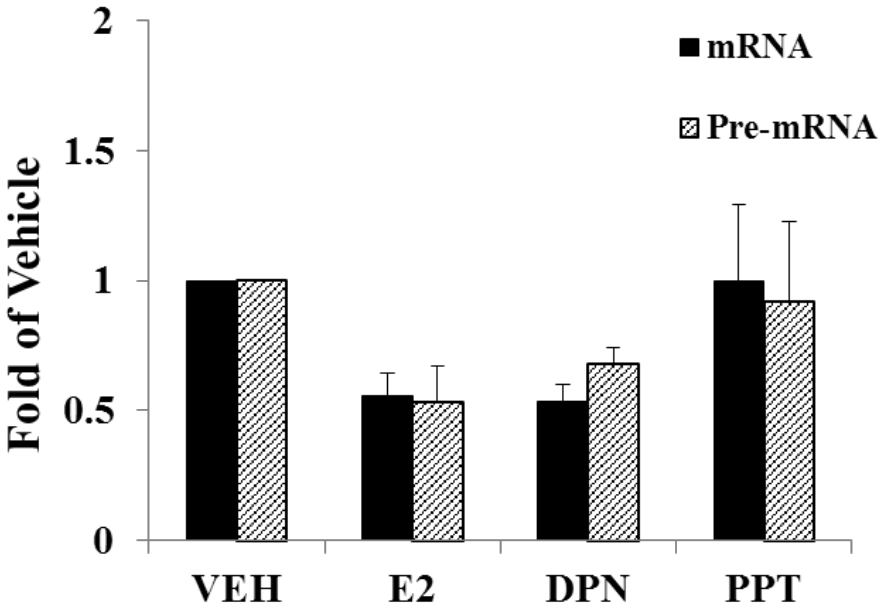

2
1.5
1
0.5
0
Fold of Vehicle
mRNA
Pre-mRNA
VEH
E2
DPN
PPT

Figure 4. PHTPP reverses E2 and DPN suppression of COX-2 pre-mRNA. Cells were treated for 24 h with PHTPP, E2 + PHTPP or DPN + PHTPP. (n=5). Data represents mean ± SEM. *p = 0.001 E2 and DPN compared to VEH, #p = 0.023 E2+PHTPP compared to E2, ^p = 0.026 DPN+PHTPP compared to DPN. VEH (Vehicle)

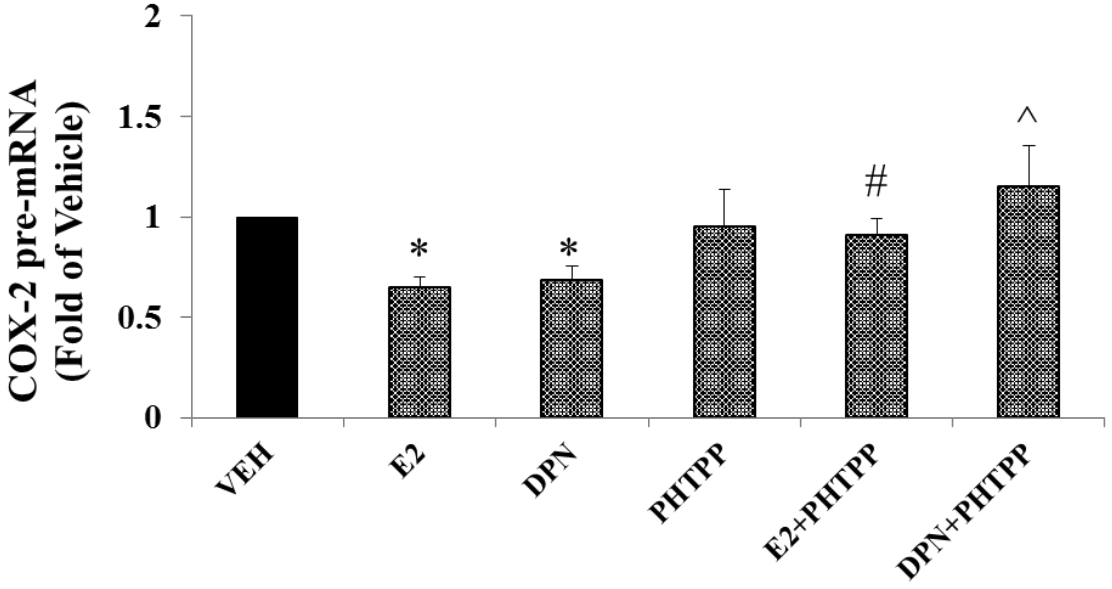

COX-2 pre-mRNA
(Fold of Vehicle)
2
1.5
1
0.5
0
*
*
#
^
VEH
E2
DPN
PHTPP
E2+PHTPP
DPN+PHTPP

Figure 5. Schematic of *cox-2* proximal promoter showing the region of the NF-kB element. The numbers indicate the bases from the transcription start site (arrow). ChIP primers P1 and P2 used in subsequent experiments are shown.

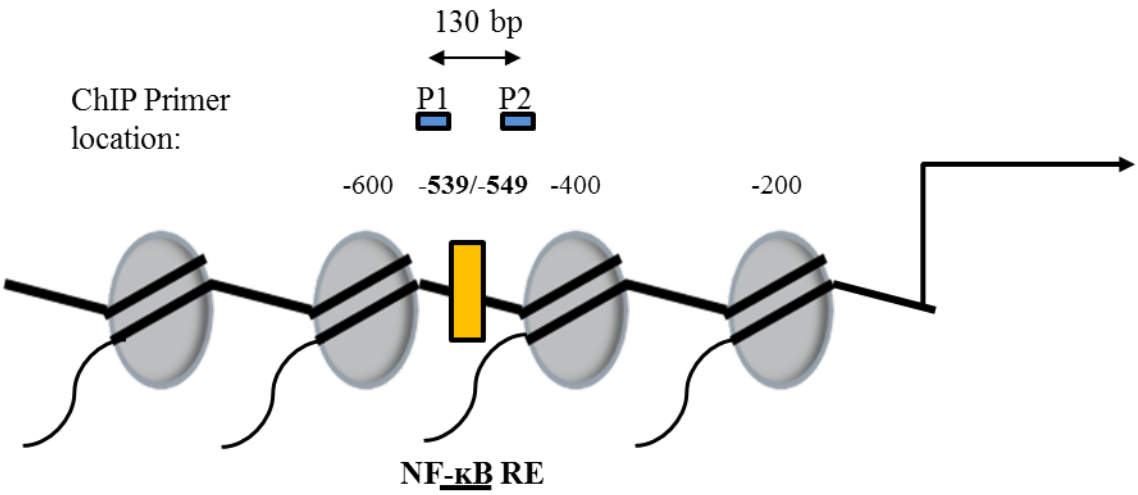
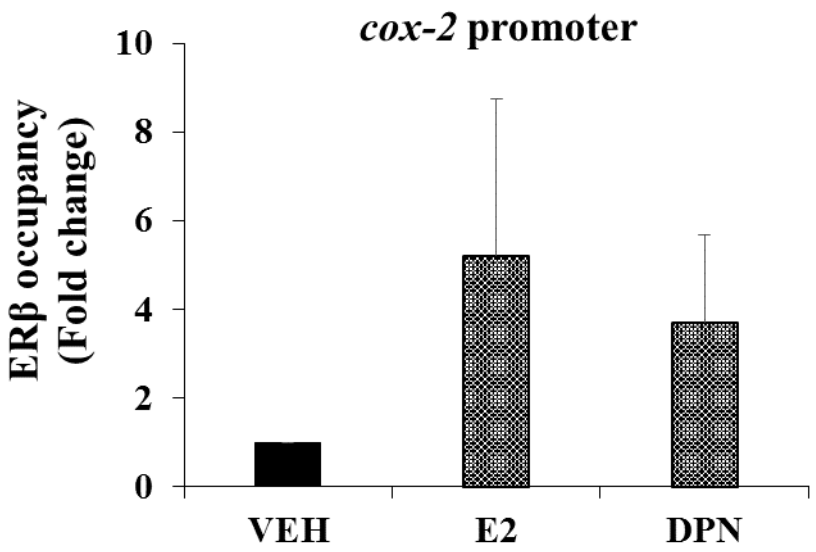

130 bp
ChIP Primer location:
P1
P2
-600
-539/-549
-400
-200
NF-κB RE

Figure 6. E2 and DPN fail to induce ERβ occupancy at the *cox-2* proximal promoter in the region of the NF-kB element. A, ChIP analysis of *cox-2* NF-kB region. B, ChIP analysis of *c-fos* ERE region. A polyclonal anti-ERβ was used (Abcam). (All experiments are n=4). Data represents mean ± SEM and expressed as fold of VEH. B, *p = 0.043 E2 compared to VEH, *p = 0.008 DPN compared to VEH. VEH (Vehicle)

A


cox-2 promoter
ERβ occupancy
(Fold change)
10
8
6
4
2
0
VEH
E2
DPN

B

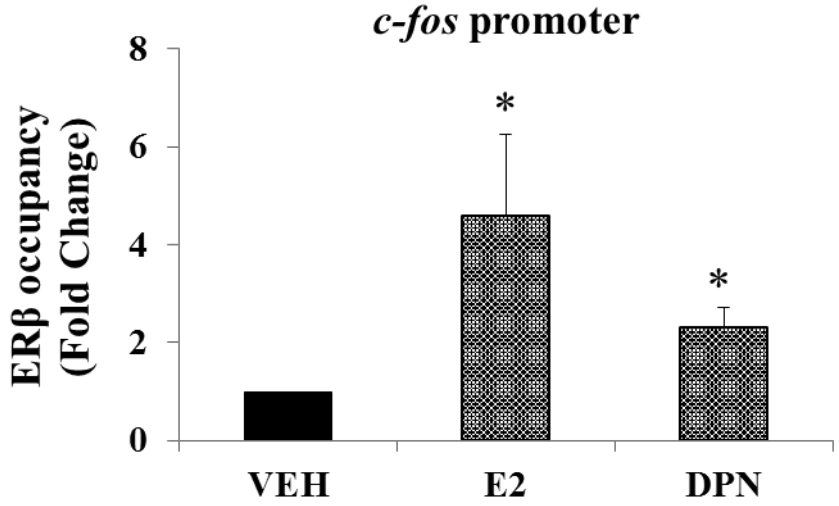

c-fos promoter
ERβ occupancy
(Fold Change)
8
6
4
2
0
*
*
VEH
E2
DPN

Figure 7. E2 and DPN decrease p65 occupancy and Ac-H4 levels. All data is from ChIP analyses. All antibodies were polyclonal. A, B p65 (Santa Cruz). C, D H4 pan-acetylation (Active Motif). E, F H3 pan-acetylation (Millipore; for all experiments n=3). Analysis of far upstream region B, D and F (n=2). Data represents mean ± SEM and expressed as the fold of VEH. A, *p =0.042 E2 compared to VEH; *p =0.003 DPN compared to VEH. B, *p = 0.013 E2 compared to VEH, *p =0.001 DPN compared to VEH. #p = 0.05 E2 compared to DPN.

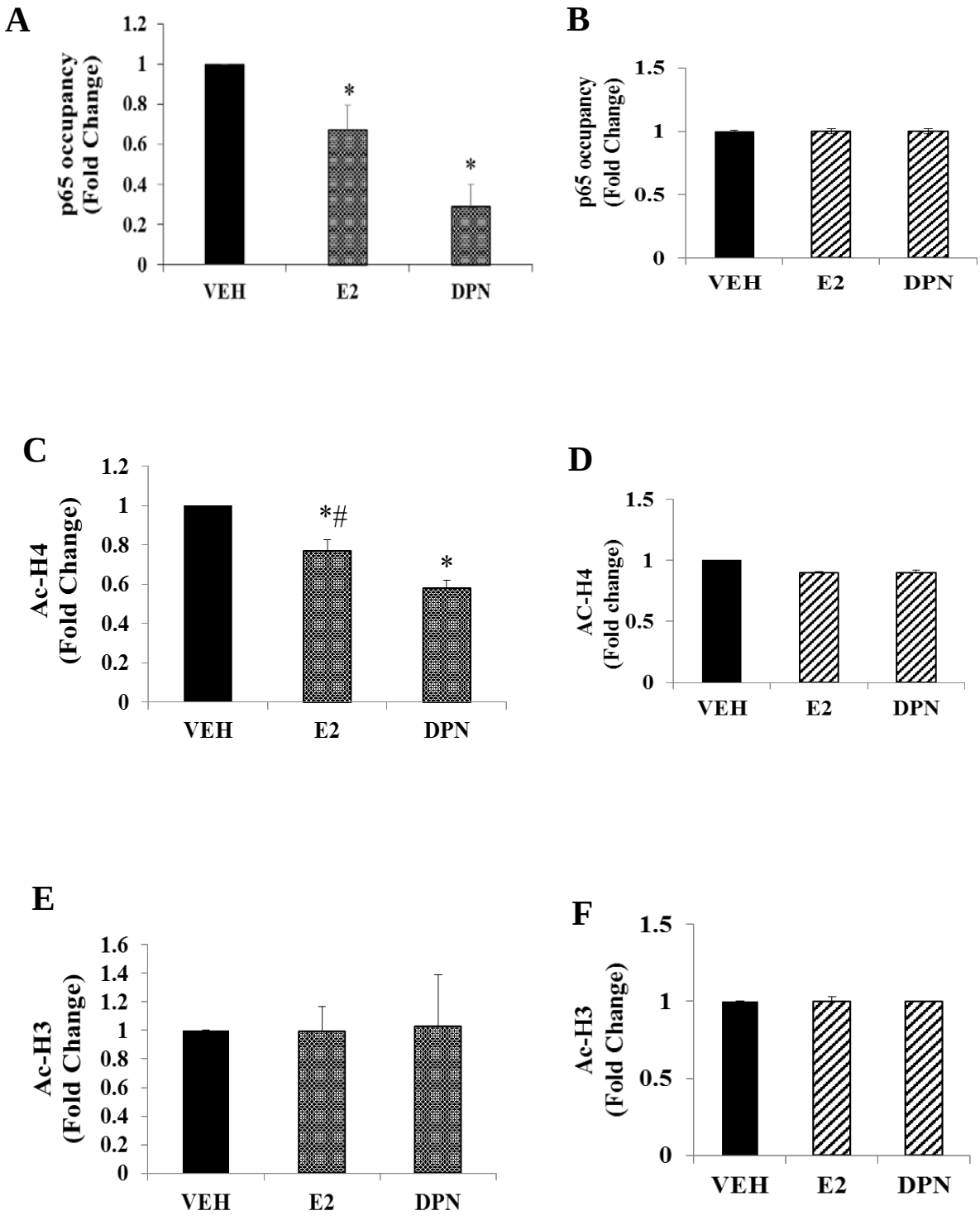

A
p65 occupancy (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
*
*
VEH
E2
DPN
B
p65 occupancy (Fold Change)
1.5
1
0.5
0
VEH
E2
DPN
C
Ac-H4 (Fold Change)
1.2
1
0.8
0.6
0.4
0.2
0
*#
*
VEH
E2
DPN
D
AC-H4 (Fold change)
1.5
1
0.5
0
VEH
E2
DPN
E
Ac-H3 (Fold Change)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
VEH
E2
DPN
F
Ac-H3 (Fold Change)
1.5
1
0.5
0
VEH
E2
DPN

Figure 8. TSA increases COX-2 pre-mRNA and blocks E2 and DPN suppressive effects. Cells were treated for 24 h with E2, DPN, TSA (100 nm) or E2+TSA and DPN+TSA (n=5). Data represents mean ± SEM and expressed as fold of VEH. *p =0.007 E2 compared to VEH, *p = 0.050 DPN compared to VEH, *p = 0.025 TSA compared to VEH, *p = 0.013 E2+TSA compared to VEH, *p = 0.005 DPN+TSA compared to VEH, #p = 0.004 E2+TSA compared to E2, ^p = 0.003 DPN+TSA compared to DPN.

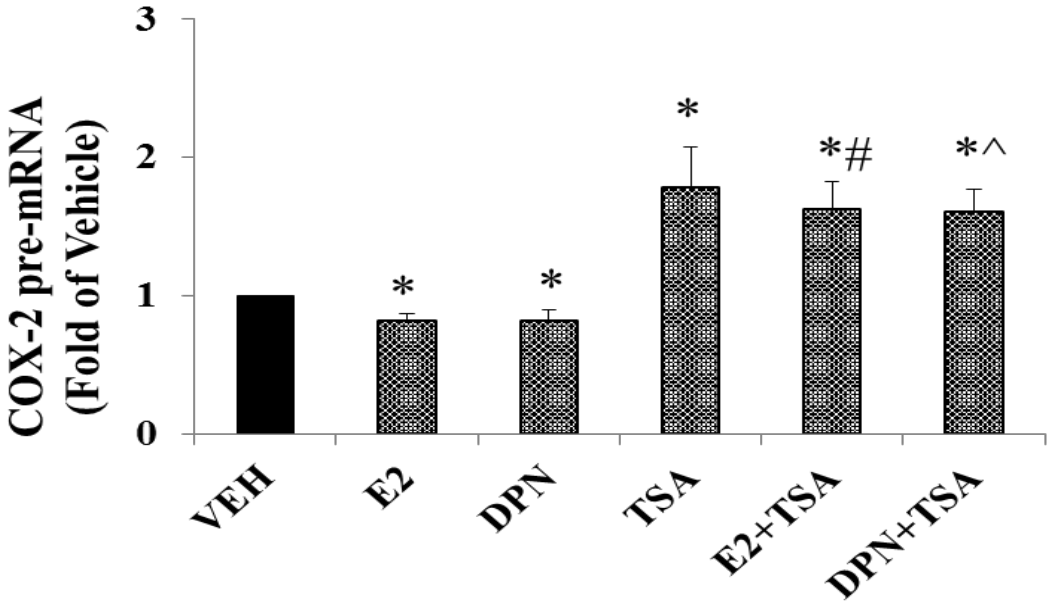

COX-2 pre-mRNA
(Fold of Vehicle)
3
2
1
0
*
*
*
*#
*^
VEH
E2
DPN
TSA
E2+TSA
DPN+TSA

Figure 9. E2 and DPN increase HDAC1 and Sin3A occupancy. ChIP analysis was performed for: A, HDAC1 (mAb, Abcam). C, HDAC3 (mAb, Cell Signaling). E, Sin3A (polyclonal, Sigma-aldrich). (n≥3).). Analysis of far upstream region B, D and F (n=2). Data represents mean ± SEM and expressed as fold of VEH. A, *p = 0.008 E2 compared to VEH, *p = 0.002 DPN compared to VEH, #p= 0.04 E2 compared to DPN. E, *p = 0.002 E2 compared to VEH, *p = 0.048 DPN compared to VEH.

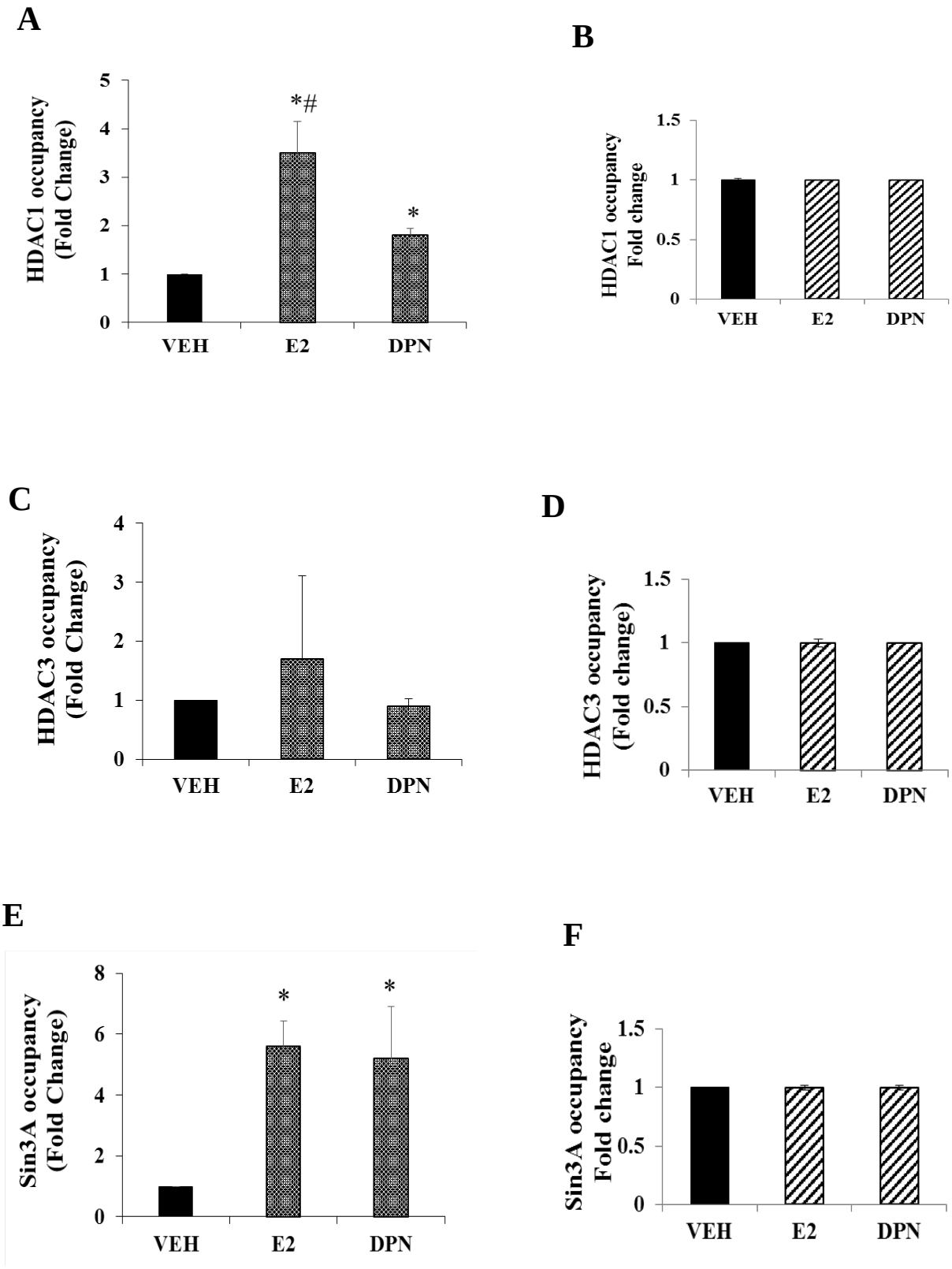
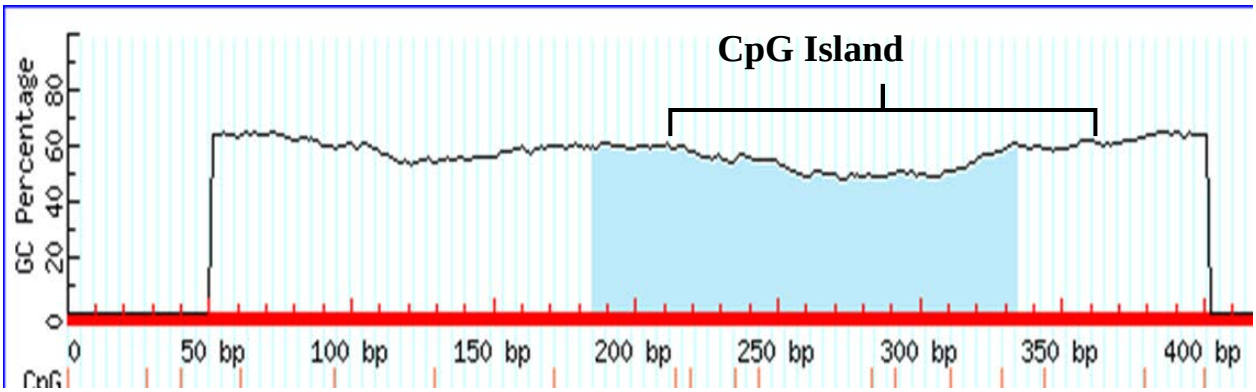

A
HDAC1 occupancy (Fold Change)
5
4
3
2
1
0
*#
*
VEH
E2
DPN
B
HDAC1 occupancy Fold change
1.5
1
0.5
0
VEH
E2
DPN
C
HDAC3 occupancy (Fold Change)
4
3
2
1
0
VEH
E2
DPN
D
HDAC3 occupancy (Fold change)
1.5
1
0.5
0
VEH
E2
DPN
E
Sin3A occupancy (Fold Change)
8
6
4
2
0
*
*
VEH
E2
DPN
F
Sin3A occupancy Fold change
1.5
1
0.5
0
VEH
E2
DPN

Figure 10. CpG sites in the *cox-2* proximal promoter. A, Bioinformatic analysis showing the predicted CpG island. B, Schematic of the *cox-2* proximal promoter indicating the 8 CpG clusters (small numbers). Arrow indicates the start of transcription

A


CpG Island
GC Percentage
0
20
40
60
80
0
50 bp
100 bp
150 bp
200 bp
250 bp
300 bp
350 bp
400 bp
CpG

B

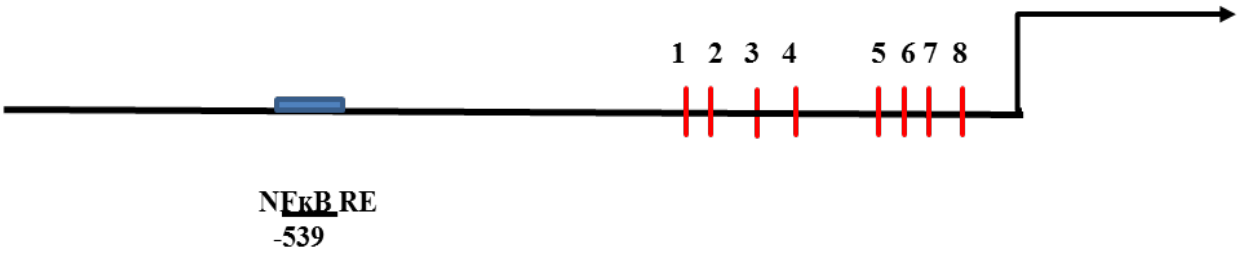
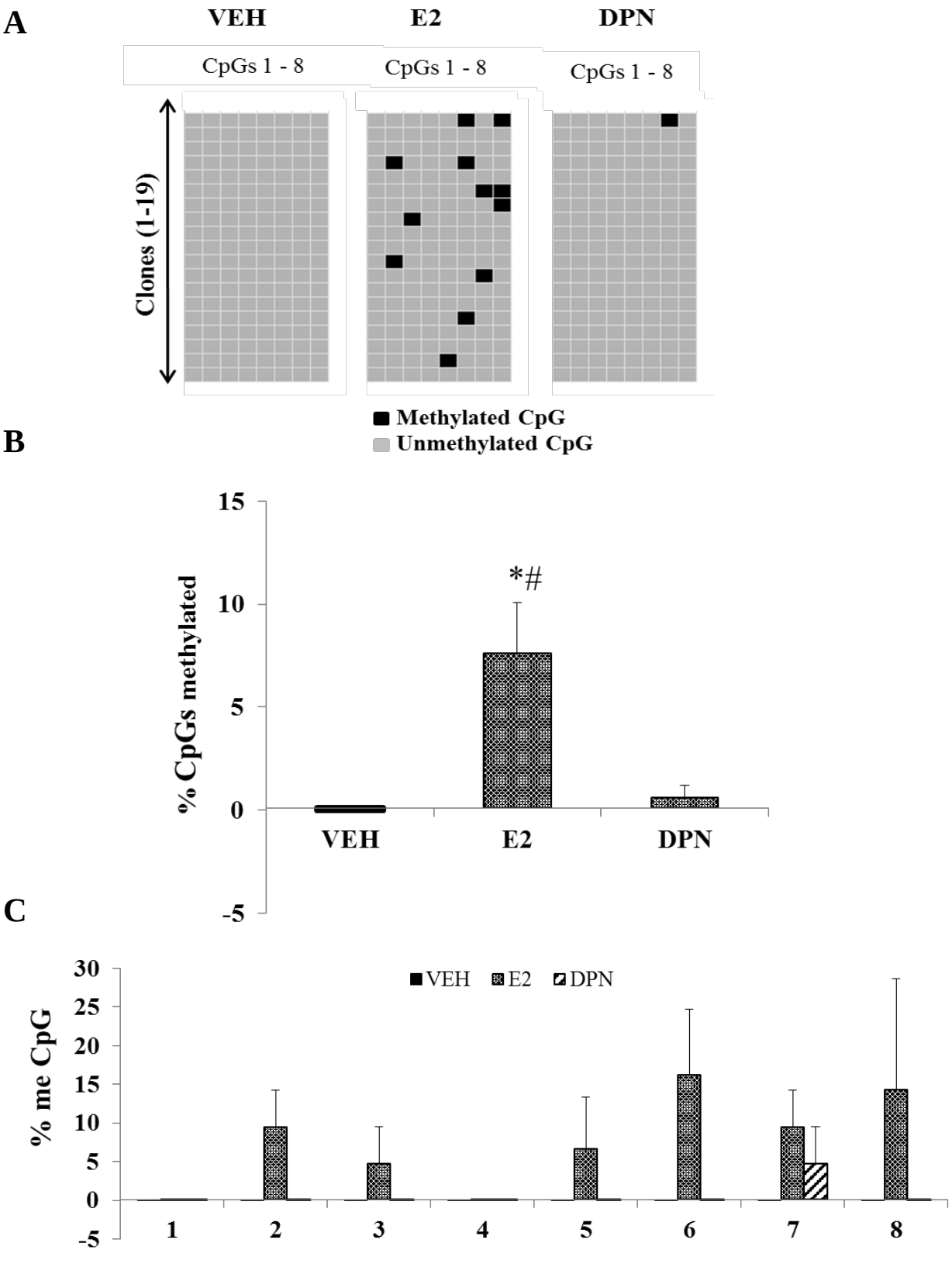

1 2 3 4
5 6 7 8
NFκB RE
-539

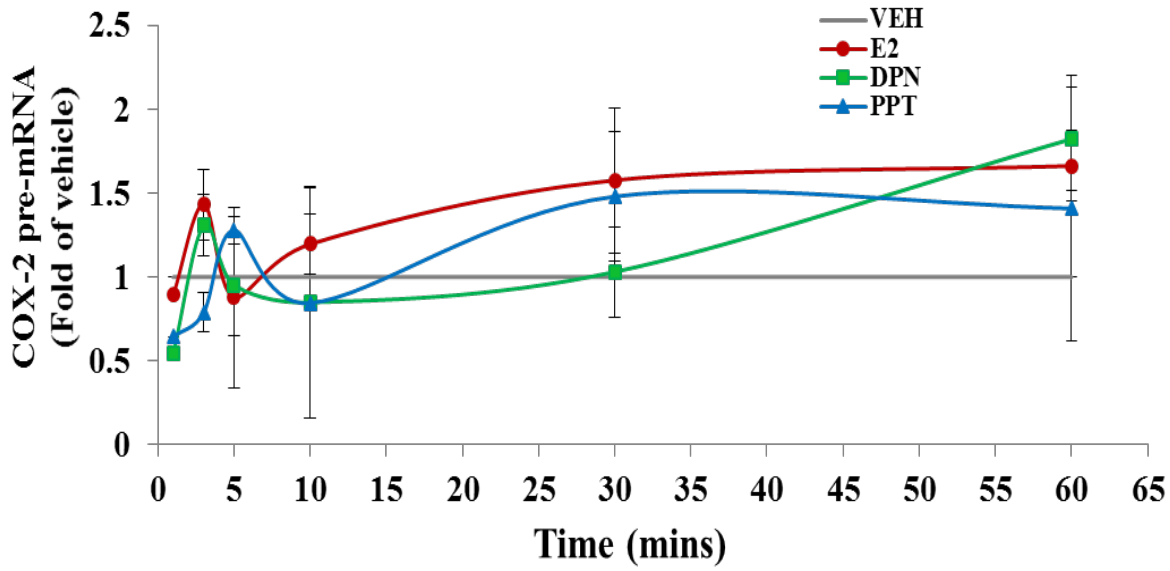
Figure 11. E2 increases the overall methylation of *cox-2* proximal promoter. A, Raw sequencing data for promoter methylation. Independent clones represented by each row. B, Analysis of overall methylation of CpGs. C, Percent methylation at each CpG site. (n=3) Data represents mean ± SEM. *p = 0.035 E2 compared to VEH, #p = 0.049 E2 compared to DPN.


A
VEH
E2
DPN
CpGs 1 - 8
CpGs 1 - 8
CpGs 1 - 8
Clones (1-19)
Methylated CpG
Unmethylated CpG
B
% CpGs methylated
15
10
5
0
-5
*#
VEH
E2
DPN
C
% me CpG
30
25
20
15
10
5
0
-5
VEH
E2
DPN
1
2
3
4
5
6
7
8

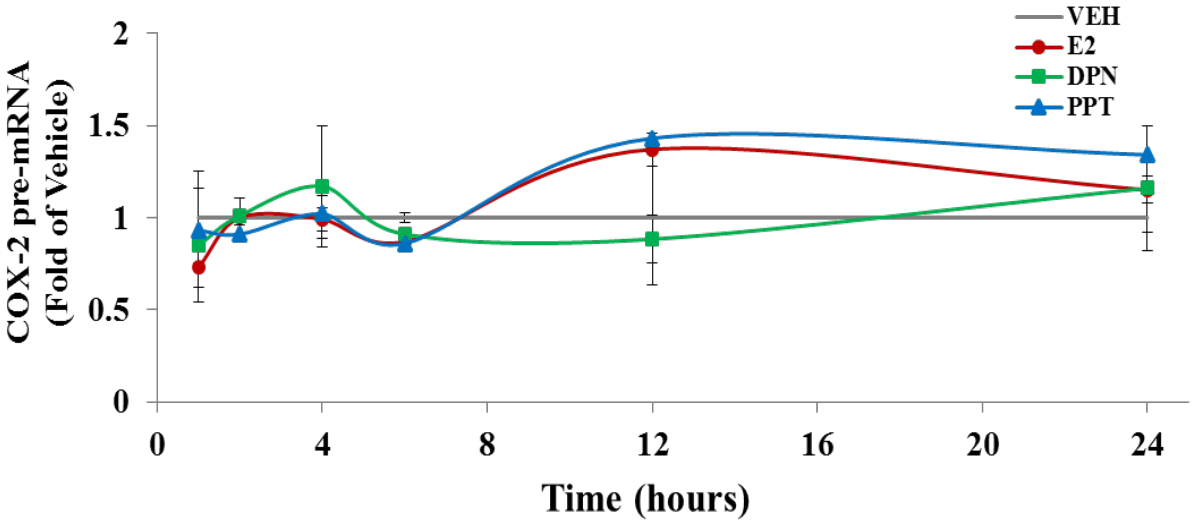
E2 REGULATION OF *cox-2* EXPRESSION IN N27

Figure 12. E2 fails to suppress COX-2 pre-mRNA. Cells were treated with E2, DPN, and PPT (all 10^{-7} M). A, up to 1h. B, up to 24h. Expression of COX-2 pre-mRNA was measured by RT-qPCR (n =3). Data represents mean ± SEM.

A


COX-2 pre-mRNA
(Fold of vehicle)
2.5
2
1.5
1
0.5
0
0
5
10
15
20
25
30
35
40
45
50
55
60
65
Time (mins)
VEH
E2
DPN
PPT

B


COX-2 pre-mRNA
(Fold of Vehicle)
2
1.5
1
0.5
0
0
4
8
12
16
20
24
Time (hours)
VEH
E2
DPN
PPT

CHAPTER 4

DISCUSSION

The anti-inflammatory properties of E2 are well established. Studies have indicated that E2 can suppress various pro-inflammatory genes such as *TNF-α* in U2OS osteosarcoma cells [126], *IL-6* in MCF-7, Ishikawa and HeLa cells [73,74,88], *iNOS* and *IκBα* in Raw 264.7 cells [91] and *cox-2* in rat cerebral blood vessels [127]. Furthermore, E2 can repress NF-κB regulated genes in macrophages, microglia [91] and in rat aortic smooth muscle cells [80]. However, to our knowledge there are no studies on whether E2 suppresses neuronal *COX-2* expression human or in rodent (*cox-2*).

The goal of this study was to elucidate mechanisms by which E2 suppresses neuronal *cox-2* expression. We first characterized an amygdalar cell line for COX-2 expression and determined that it was an appropriate model. We further assessed the effects of E2 on COX-2 mRNA and pre-mRNA. The E2 effect was not mediated by PPT, an ERα agonist, in so far as it had no effect on COX-2 mRNA and pre-mRNA levels. In contrast, the ERβ agonist, DPN, decreases COX-2 mRNA and pre-mRNA to the same extent as E2, by 24 h. The importance of ERβ to the E2 repressive effect was underscored by the ability of the selective ERβ antagonist PHTPP to completely reverse the suppressive effects of E2 and DPN. Given that DPN, accounted for all of the E2 effects, we focused the subsequent studies on ERβ.

To parse the molecular mechanisms that underlie E2 suppression of *cox-2* expression, we performed ChIP analysis of the proximal region of the *cox-2* promoter. Because the *cox-2* promoter lacks a palindromic ERE, we focused on a region containing an NF-κB p65 response element, an important site of *cox-2* activation [51,53]. Surprisingly, ChIP analysis revealed that neither E2 nor DPN increased ERβ occupancy in the proximal region of the *cox-2* promoter. In distinction, E2 and DPN increased ERβ occupancy of the *c-fos* promoter, in the region of its palindromic ERE. Taken together, the data underscores two key aspects of ER action. First ER occupancy is promoter dependent. Second, ER can down-regulate genes in a manner independent of promoter occupancy and/or EREs. In fact, about one third of E2 responsive genes are believed to lack ERE-like sequences [62]. For example, E2 repression of *IL-6* does not involve high affinity binding to the promoter but rather inhibition of the DNA-binding ability of transcription factors such as C/EBPβ and NF-κB [74]. It is not surprising then that rather than an ERE-dependent signaling or direct DNA binding, that the ER effect is more indirect than even the prototypic ERα and ERβ regulation through an AP-1 site [128].

In vitro studies have reported E2 through ERβ inhibits p65 binding to the promoters of cytokine-induced neutrophil chemoattractant *(CINC)-2β* and monocyte chemotactic protein *(MCP)-1*genes [80]. In their study, Xing et al. showed that ERβ presence at the *MCP-1* and *CINC-2β* promoters coincided with reduced levels of p65. Interestingly, these genes lack the prototypic EREs in their promoters. Likewise, E2 through ERα has been reported to interfere with DNA binding activity of p65 in *iNOS*, *IκBα*, macrophage inflammatory protein 2 (*MIP-2*) [91] and *IL-6* [129] promoters. In vivo studies further corroborate E2 inhibition of p65 DNA binding activity to suppress induction of *COX-2* and *iNOS* expression [89,90]. However, there are discrepancies in the literature regarding ER inhibition of p65. Some studies report a direct

interaction between ER and p65 but no effect on p65 DNA binding in *MCP-1*, *IL-8* [129] and major histocompatibility complex (MHC) class 1 promoters [130]. Rather ER interferes with histone acetyltransferase (HAT) activity needed for p65 transcriptional activity. In contrast, ER interaction with p65 and subsequent displacement of p65 is indicated in *IL-6* and *IL-2* promoters [74,88,129,131]. Thus, it seems that the mechanisms by which ER suppress p65 activity may vary by gene and cell type. In keeping with the reported data, E2 and DPN decrease p65 binding in the region of the *cox-2* promoter. Whether ERβ physically interacts with p65 in the present context remains to be elucidated.

Acetylation of histone tails permits an open chromatin to allow regulatory proteins to bind [132]. There is evidence that p65 plays a critical role in histone acetylation by recruiting histone acetyl transferases that acetylate histone tails [133]. Moreover, recruitment of p65 to the promoter of a gene correlates with increased acetylated H4 and consequently, increased gene expression [134-136]. Therefore, it is not surprising that E2 and DPN decrease levels of Ac-H4. In contrast to our findings, some studies have shown that E2 either increases acetylated H4 levels [71] or had no effect [80]. This could be explained by different gene promoters and/or cellular context.

TSA is a broad spectrum HDAC inhibitor [137]. One interpretation of the TSA data is that HDACs are necessary for E2 and DPN effects on *cox-2* expression at the level of promoter recruitment. Indeed, HDAC1 but not HDAC3 was recruited. This is not surprising given that numerous studies have reported HDAC1 as a corepressor protein for p65 [138-141]. In those studies, HDAC1 but not HDAC2 was shown to directly interact and inhibit p65 activity in the context of *IκBα* repression. Even though a direct interaction between HDAC1 and p65 was observed, at present we do not know if HDAC1 directly interacts with p65 in the context of the

cox-2 promoter. Furthermore, HDAC1 is co-recruited with Sin3A, a master scaffold protein, which is thought to be in a complex that does not include NCoR and SMRT [106,108,123]. HDAC3 is associated with NCoR or SMRT in mediating transcriptional repression by thyroid hormone receptor (TR) and tamoxifen-bound ER [104,142]. Thus, it is plausible that at the *cox-2* proximal promoter, E2 induces a repressive complex composed of ERβ, p65, HDAC1 and Sin3A.

In regards to CpG methylation, previous studies indicate that the hypermethylation of the *COX-2* 5' CpG island correlates with its degree of expression [113,114,143]. For example, in the context of human gastric carcinoma cells, Song et al reported that the human *COX-2* promoter is hyper-methylated in the region from -590 to +186, and that this hyper-methylation correlates with transcriptional silencing of *COX-2* expression [114]. This finding is in accord with the data presented here. A distinction is that the data presented involves a shorter region than did the Song study. Here the fragment extends from -226 to -111 and contains a CpG island of eight CpG sites. Thus, at least in the context of the experiments presented, E2-mediated *cox-2* repression can be supported by a more focal region of the *cox-2* promoter.

Another intriguing finding is the lack of ERβ dominance at every level of regulation. At the RNA levels, the E2 effect is fully accounted for by DPN, and thus by inference, by ERβ. This is not the case either for all factors recruited to the region of the proximal promoter nor for the pattern of E2 induced increase in methylation status (Table 2). Even though ERβ fully accounts for the E2 changes in RNA levels, it does not account for the decrease in Ac-H4, recruitment of HDAC1 and overall methylation of CpGs suggesting a possible involvement of another receptor. The most obvious candidate is ERα; however other ER receptors could be involved; for example, the membrane bound receptors or the GPR30. The involvement of another receptor is bolstered

by several studies that indicate that both ERα and ERβ can be involved in suppression of inflammatory genes [84,98,131]. In their *in vivo* study of ectopic lesions in mice, Zhao et al. showed that chloroindazole (CLI), an ERβ-dependent ligand, and oxabicycloheptene sulfonate (OBHS), an ERα ligand, equally suppressed COX-2 protein [144]. This evidence and our results indicate that another receptor is possibly involved at regulating neuronal *cox-2* expression.

In summary, as pharmacologically defined, our data indicate that E2 represses neuronal *cox-2* expression and does so through an ERβ-mediated mechanism. ERβ accounts for the entire effect at the level of RNA, and at the level of Sin3A recruitment. The mechanism does not involve direct ERβ binding to the *cox-2* promoter nor does it involve ERβ directed promoter methylation. ERβ contributes to decreased p65 and increasing HDAC1 occupancy, but requires other factors for this activator/repressor exchange. Thus, ERβ regulates *cox-2* expression via a mechanism independent of specific DNA binding, one that involves functional titration of p65 and recruitment of HDAC1:Sin3A. Lastly, it does so in cells that display a neuronal phenotype, cells usually thought of being targets rather than participants of inflammatory processes.

The models of the mechanisms of E2 suppression of neuronal *cox-2* expression in AR-5 are demonstrated in Figure 1. Fig. 1A. shows that the presence of p65 at its site and acetylated H4 may contribute to the constitutive expression of *cox-2* in AR-5. Even though the order of events is not yet known, we speculate that E2 through ERβ alone or with an unknown ER either: first decreases p65 occupancy, Fig. 1B, which then results in recruitment of HDAC1 and Sin3A and subsequent deacetylation of H4. Or recruits HDAC1 and Sin3A first to deacetylate H4 which results in decreased p65 occupancy, Fig.1C. It is still unknown whether ERβ interacts directly with these proteins.

The goal for our study using the N27 neuronal cell line was to investigate the effects of E2 on *cox-2* expression in dopaminergic neurons of SN. E2 failed to suppress *cox-2* expression in N27. In distinction, E2 suppressed *cox-2* expression at the RNA levels in AR-5. It is possible that the E2 point of regulation in N27 is not at the RNA level. Furthermore, unlike in amygdala neurons, COX-2 is normally expressed in low levels in nigral dopaminergic neurons but is upregulated in AD, PD and PD animal models [49,145]. Thus, it is possible that E2 effects would be evident in the case of induced *cox-2* expression rather than on constitutive expression as the case in AR-5. Here we see a disparity in effects of E2 on neuronal *cox-2* expression indicating that the mechanisms may be cell-specific.

Table 2. Differential effects of E2 and DPN on activators and repressors and on methylation status in the *cox-2* proximal promoter region

Summary of data shown in Figs. 7, 9 and 11. Data represents mean ± SEM and expressed as fold of vehicle.

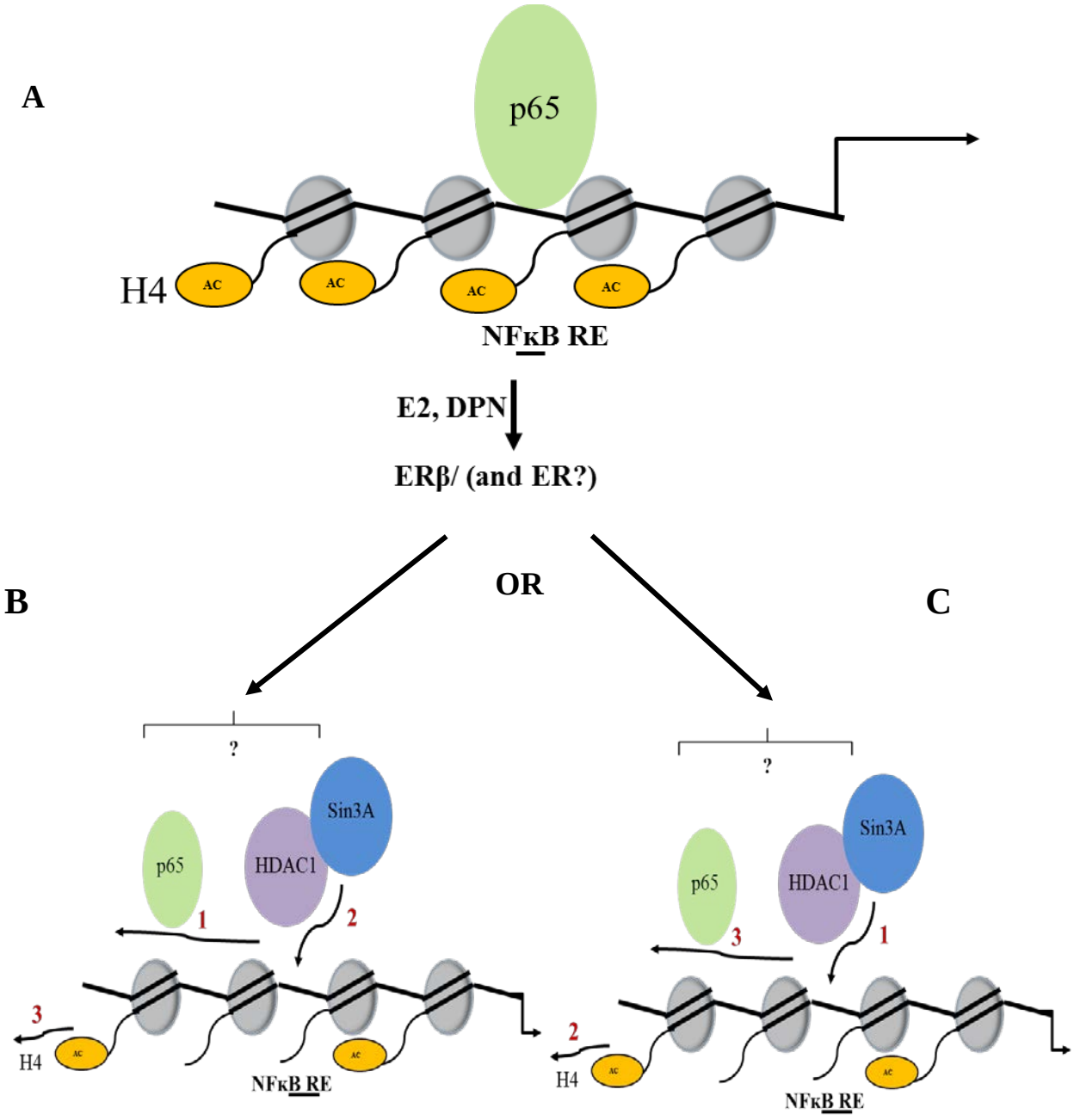
Table 2. Differential effects of E2 and DPN on activators and repressors and on methylation status in the *cox-2* proximal promoter region

Activators				Repressors			
		Fold change (p-value)	E2 vs DPN Effect (p-value)			Fold Change (p-value)	E2 vs DPN Effect (p-value)
p65	E2	0.67 (**0.042**)	Same (0.086)	**HDAC1**	E2	3.50 (**0.008**)	Different (**0.040**)
	DPN	0.29 (**0.003**)			DPN	1.80 (**0.002**)	
Ac-H4	E2	0.77 (**0.013**)	Different (**0.050**)	**Sin3A**	E2	5.60 (**0.002**)	Same (0.841)
	DPN	0.58 (**0.001**)			DPN	5.20 (**0.048**)	
Ac-H3	E2	0.99 (0.971)	Same (0.937)	**HDAC3**	E2	1.80 (0.583)	Same (0.548)
	DPN	1.03 (0.944)			DPN	0.90 (0.612)	
				Overall CpG methylation	E2	7.63 (**0.035**)	Different (**0.049**)
					DPN	0.60 (0.374)	

Figure 1. Models of E2 suppression of neuronal *cox-2* expression. A, p65 occupancy in the proximal *cox-2* promoter region and acetylation of H4 drives the constitutive expression of *cox-2* in AR-5. Ligand-activated ERβ alone or with an unknown ER either:

B. decrease p65 occupancy allowing HDAC1 and Sin3A recruitment to the promoter, and consequently decreasing Ac-H4 levels, or

C, increase HDAC1 and Sin3A occupancy promoting decrease in Ac-H4 levels and subsequently decrease in p65 occupancy. At present, the order of events is not yet known.


A
p65
H4
AC
AC
AC
AC
NFκB RE
E2, DPN
ERβ/ (and ER?)
OR
B
C
?
Sin3A
p65
HDAC1
1
2
3
H4
AC
NFκB RE
?
Sin3A
p65
HDAC1
3
1
2
H4
AC
NFκB RE

CHAPTER 5

CONCLUSION

General Conclusions

Neuroinflammation is a common feature in the pathology and progression of neurodegenerative diseases including AD and PD [8,12]. With mounting evidence supporting the contributory role of COX-2 overexpression in neurodegenerative diseases, it is imperative to understand how this gene is suppressed. A majority of studies have focused on expression of COX-2 in hippocampus and cerebral cortex [24,30,31]. The amygdala is less appreciated even though studies indicate that the amygdala undergoes substantial neuronal loss in AD [32,33] and PD [146], which may partly justify the emotional disturbances including increased or decreased fear and anxiety, behavioral, and cognitive deficits exhibited in these disorders. Further evidence suggest that COX-2 expression is elevated in dopaminergic neurons of SN in AD and PD which may contribute to the neuronal loss evident in these disorders [49,145].

E2, the principle estrogenic hormone, plays a pivotal role in regulating inflammatory processes in the brain by suppressing inflammatory mediators and preventing their induction [85,98]. E2 deprivation as evident in menopausal women has detrimental effects including increased susceptibility to AD and PD [82]. The importance of the present study is that, to our

knowledge, no studies have addressed whether E2 suppresses neuronal *cox-2* expression. The main aim of this study was to delineate the mechanisms by which E2 suppressed *cox-2* expression in amygdala. Secondarily, this study investigated the effects of E2 on *cox-2* expression in substantia nigra neurons.

In this study, we examined the effects of E2 on *cox-2* expression in two neuronal cell lines, AR-5, an amygdala neuronal cell line and N27, a dopaminergic substantia nigra cell line. Interestingly, E2 suppressed *cox-2* expression in AR-2 but not in N27 cells. Our study indicates that ERβ mediates the E2 suppression of *cox-2* expression at RNA levels. Intriguingly, even though ERβ fully accounted for all the effects at RNA levels, it did not account for all the changes in the *cox-2* proximal promoter region. ERβ fails to account for decreased levels of Ac-H4 and increased HDAC1 occupancy. Furthermore ERβ had no effect on the degree of methylation.

In conclusion, in this study, we characterized the neuronal cell line in terms of *cox-2* expression and showed that it would be a suitable model for molecular studies on neuronal *cox-2* regulation. Furthermore, from this study, the evidence indicates that E2 suppression of neuronal *cox-2* is ER-β mediated and involves mechanisms independent of direct DNA binding. At the level of the chromatin environment, other factors must be involved in E2 downregulation of *cox-2* expression. Results from this study will pave the way for *in vivo* studies and may further lead to identification of novel therapeutic targets, and ultimately agents that could selectively target ER-mediated *cox-2* suppression in the brain.

Future Directions

Even though the present study elucidated the mechanisms by which E2 suppressed neuronal *cox-2* expression, a number of questions remain unanswered. One of the main questions is, given that ERβ fails to account for all E2 effects at all levels, could another ER be involved in mediating the decrease in Ac-H4 levels, increase in HDAC1 and methylation changes in the *cox-2* proximal promoter? Future studies can be directed to identify if ERα and/or GPR30 work in concert with ERβ to mediate E2 effects at some of the steps.

Other unanswered questions are: Is there a physical interaction between ERβ, p65, HDAC1 and Sin3A? What is the order of events occurring at the *cox-2* proximal promoter? Thus, future experiments need to identify how ERβ regulates these proteins and the order of their regulation. It would be interesting to know if the methylation changes correlate with upstream changes in occupancy of p65, HDAC1 and Sin3A. Also, more studies are needed to rule in or out whether other corepressors such as DNMTs, NCOR and SMRT are involved in *cox-2* repression.

In the present study, E2 failed to suppress *cox-2* expression at the RNA level in N27. This is not surprising since *cox-2* expression is not constitutively expressed in dopaminergic neurons of substantia nigra. It is possible that *cox-2* expression needs to be induced in N27 cells in order to see suppression by E2. Thus, a future study would elucidate E2 regulation of constitutive and inducible neuronal *cox-2* expression.

In summary, the present study demonstrated that E2 suppresses neuronal *cox-2* expression. The data suggests that the mechanism for E2 down-regulation of neuronal *cox-2* expression involves an alternate pathway, and that part of the mechanism is accounted for by ERβ, as pharmacologically defined.

Appendix

Figure 1. E2 and DPN increase CBP occupancy. ChIP analysis using a polyclonal anti-CBP (SC-369; Santa Cruz). (n=4). Data represents mean ± SEM and expressed as fold of VEH. *p = 0.0001 E2 compared to VEH, *p = 0.05 DPN compared to VEH.

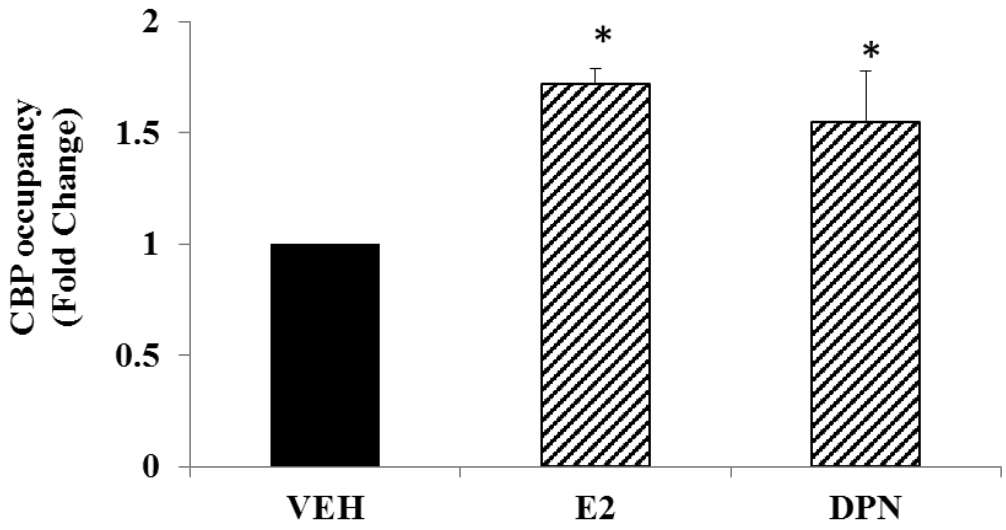

CBP occupancy
(Fold Change)
2
1.5
1
0.5
0
*
*
VEH
E2
DPN

Figure 2. Continuous E2 and DPN treatment suppresses COX-2 pre-mRNA. AR-5 cells were treated with E2 or DPN (10^{-7} M) when plated at 0 h. At 24 h, in A, they were treated with charcoal stripped serum (CSS) or B, with E2 or DPN. RNA was extracted at 48 h. COX-2 pre-mRNA was analyzed by RT-qPCR. (n=5). Data represents mean ± SEM. *p = 0.036 E2 compared to normal serum (NS). *p= 0.012 DPN compared to NS. #p = 0.006 DPN compared to DPN+CSS.

A

E2/DPN charcoal strip serum (CSS) Endpoint

0 hrs---------- 24hrs---------- 48hrs

B

E2/DPN E2/DPN Endpoint

0 hrs----------24hrs----------48hrs

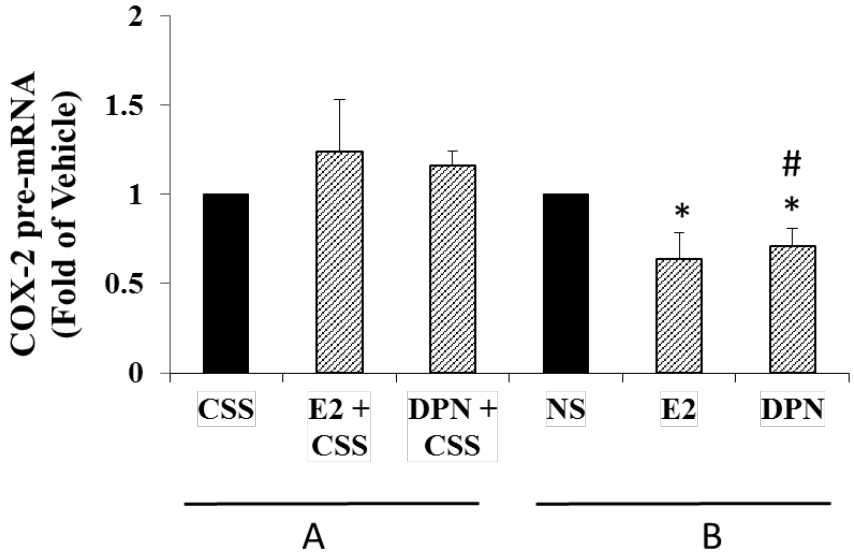


Figure 3. Phorbol 12-myristate 13-acetate (PMA) fails to increase COX-2 pre-mRNA. AR-5 cells were treated with 100 nM of PMA for up to 6 h. Pre-mRNA was analyzed using real time RT-PCR. (n=3). Data represents mean ± SEM.

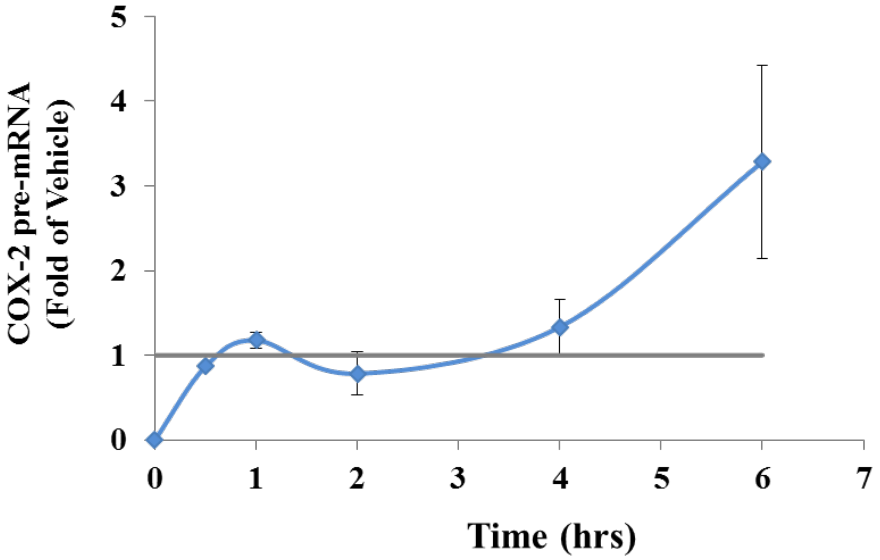

COX-2 pre-mRNA
(Fold of Vehicle)
5
4
3
2
1
0
0
1
2
3
4
5
6
7
Time (hrs)

Figure 4. E2 and DPN induce HOTAIR (HOX antisense intergenic RNA) expression in AR-5. Cells were treated with E2 and DPN (10^{-7} M) for 24h. RNA was analyzed using qRT-PCR primers specific to HOTAIR. (n=3). Data represents mean ± SEM. *p= 0.03 E2 compared to VEH. *p = 0.01 DPN compared to VEH. #p = 0.02 E2 compared to DPN.

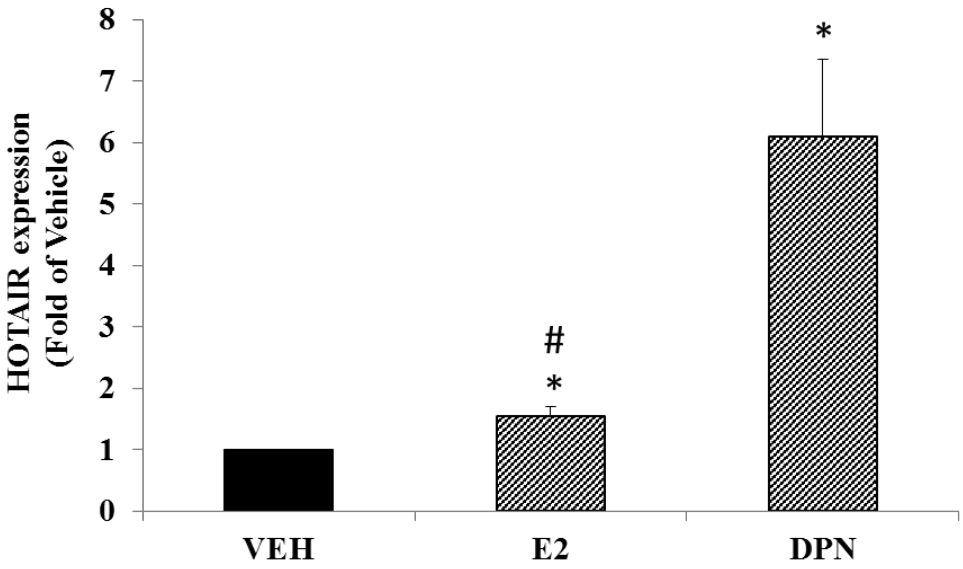

HOTAIR expression
(Fold of Vehicle)
8
7
6
5
4
3
2
1
0
#
*
*
VEH
E2
DPN

Figure 5. E2 and DPN fail to increase inhibitor of kappa B α (IκBα) expression in AR-5 cells. Cells were treated with E2 and DPN (10^{-7} M) for 24h. Pre-mRNA and mRNA were analyzed using real time RT-PCR. (n=2-4). Data represents mean ± SEM.

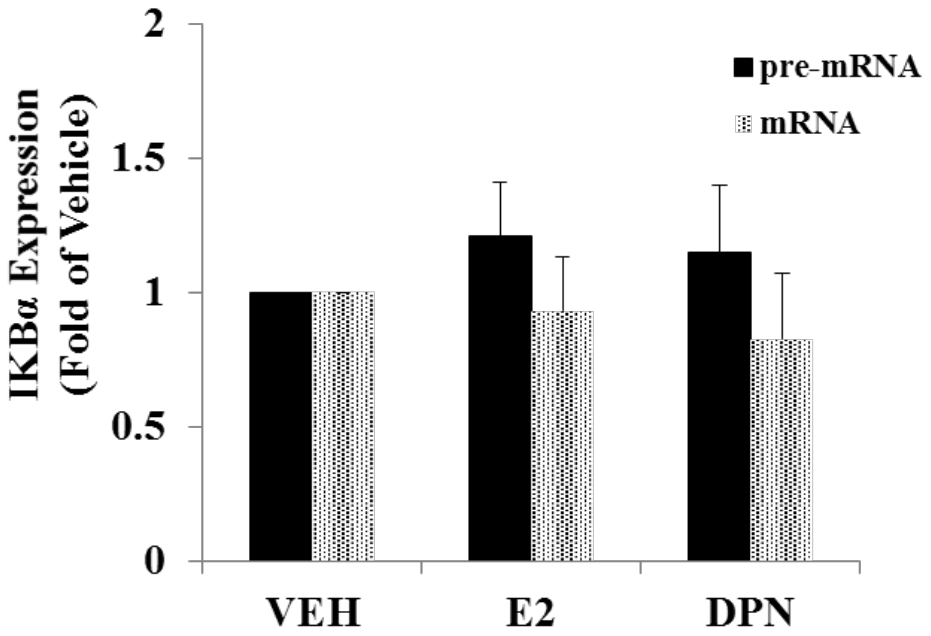

IKBα Expression
(Fold of Vehicle)
2
1.5
1
0.5
0
pre-mRNA
mRNA
VEH
E2
DPN

Table 1: Expression of *Sry* (sex-determining region Y) mRNA, a male-specific gene in rat embryo, in AR-5 cells.

Gene of Interest	Primer Sequence	C_T (Means ± SEM)
SRY	Forward: 5′-AAGCGCCCCATGAATGCATTTATGGT-3′ Reverse: 5′-ACACTTTAGCCCTCCGATGAGGCTGA-3′	26.76 ± 0.20

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