Spontaneous Glutamatergic Synaptic Activity Regulates Constitutive COX-2 Expression in Neurons

OPPOSING ROLES FOR THE TRANSCRIPTION FACTORS CREB (cAMP RESPONSE ELEMENT BINDING) PROTEIN AND Sp1 (STIMULATORY PROTEIN-1) *1

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Burgeoning evidence supports a role for cyclooxygenase metabolites in regulating membrane excitability in various forms of synaptic plasticity. Two cyclooxygenases, COX-1 and COX-2, catalyze the initial step in the metabolism of arachidonic acid to prostaglandins. COX-2 is generally considered inducible, but in glutamatergic neurons in some brain regions, including the cerebral cortex, it is constitutively expressed. However, the transcriptional mechanisms by which this occurs have not been elucidated. Here, we used quantitative PCR and also analyzed reporter gene expression in a mouse line carrying a construct consisting of a portion of the proximal promoter region of the mouse COX-2 gene upstream of luciferase cDNA to characterize COX-2 basal transcriptional regulation in cortical neurons. Extracts from the whole brain and from the cerebral cortex, hippocampus, and olfactory bulbs exhibited high luciferase activity. Moreover, constitutive COX-2 expression and luciferase activity were detected in cortical neurons, but not in cortical astrocytes, cultured from wild-type and transgenic mice, respectively. Constitutive COX-2 expression depended on spontaneous but not evoked excitatory synaptic activity and was shown to be N-methyl-D-aspartate receptor-dependent. Constitutive promoter activity was reduced in neurons transfected with a dominant-negative cAMP response element binding protein (CREB) and was eliminated by mutating the CRE-binding site on the COX-2 promoter. However, mutation of the stimulatory protein-1 (Sp1)-binding site resulted in an N-methyl-D-aspartate receptor-dependent enhancement of COX-2 promoter activity. Basal binding of the transcription factors CREB and Sp1 to the native neuronal COX-2 promoter was confirmed. In toto, our data suggest that spontaneous glutamatergic synaptic activity regulates constitutive neuronal COX-2 expression via Sp1 and CREB protein-dependent transcriptional mechanisms. The first committed reaction in the metabolism of arachidonic acid to prostaglandins and thromboxanes is catalyzed by two related heme-containing bis-oxygenases, cyclooxygenase (COX) 1 and 2. These enzymes share 90% similarity in amino acid sequence and exhibit nearly identical enzyme kinetics (1, 2). Both catalyze two separate reactions, the first metabolizing arachidonic acid to PGH22 (cyclooxygenase reaction), an intermediate that is subsequently reduced in the second reaction to the product, PGH3 (peroxidase reaction). PGH3 is the substrate for various synthases that generate individual biologically active prostaglandins and thromboxanes, often in a cell type-specific manner (3). Although both metabolize arachidonic acid to PGH3, the transcriptional regulation of each isoform differs. The PTGS1 gene encoding COX-1 lacks a TATA box motif in its 5′ promoter region and is generally constitutively active in cells (4, 5). In contrast, the promoter regulatory region of the PTGS2 gene encoding COX-2 is not typically active but can be strongly and rapidly induced under specific conditions by growth factors and proinflammatory mediators (1, 6). An important exception to this occurs in the central nervous system of many species, including rodents and humans. In the brain, for example, COX-2 is constitutively expressed in specific neuronal populations of the cortex and hippocampus (7–9). The molecular and cellular mechanisms that contribute to the constitutive expression of COX-2 in these neurons are not understood. Hence, the goal of this study was to determine the transcriptional mechanisms regulating basal COX-2 expression in cortical neurons and to examine the relationship between transcriptional regulation and neuronal activity.

Results

COX-2 Is Constitutively Expressed in the Brain of PLuc371 Transgenic Mice—Mice harboring the PLuc371 transgenic construct consisting of −371/+70 bp of the PTGS2 gene promoter fused 5′ of a luciferase transgene were used herein to analyze constitutive transcriptional regulation of COX-2 expression. Quantitation of luciferase activity (PTGS2 activity) in different

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3 The abbreviations used are: PG, prostaglandin; DIV, days in vitro; NR, NMDA receptor; PNC, purified neuronal culture; APV, 5-amino-phosphonovaleric acid; CREB, cAMP response element (CRE)-binding protein; Sp1, stimulatory protein-1; RLU, relative light units; TeNT, tetanus toxin.
tissues revealed that central nervous system (CNS) possessed the highest level of constitutive activity with little to no luciferase detected in the liver, heart, kidneys, and lungs (Figs. 1A and 2A). Analysis of isolated CNS areas demonstrated high activity in numerous regions including cerebral cortex, hippocampus, olfactory bulb, spinal cord, and cerebellum (Fig. 1A). For the most part, luciferase activity reflected endogenous COX-2 expression assessed by quantitative PCR, with the notable exception of cerebellum and spinal cord (Fig. 1B). Importantly, endogenous COX-2 expression was faithfully represented by the reporter construct in cortex (Fig. 1).

Primary cortical astrocytes cultured from transgenic animals expressed little to no luciferase activity, whereas cortical neuronal cultures, plated atop of non-transgenic astrocytes, showed abundant luciferase activity, suggesting that the majority of the activity in the total brain homogenates was neuronal (Fig. 2A). Astrocyte factors do not appear to be involved in the regulation of constitutive neuronal expression of COX-2 as the temporal expression of luciferase activity in pure neuronal cultures derived from transgenic animals was identical to that found in mixed cultures composed of transgenic neurons and wild-type astrocytes (Fig. 2B). Temporally, activity was low on days in vitro (DIV) 1–4, increased substantially between DIV 4–7, and peaked at DIV 10 (Fig. 2). This was not just a transgenic phenomenon as purified neurons derived from wild-type animals cultured with or without astrocyte conditioned media demonstrated similar COX-2 mRNA levels as do mixed cultures from wild-type animals (data not shown).

**PLuc371 Activity Parallels the Physical and Functional Expression of NMDA Receptors**—Neuronal luciferase activity near perfectly paralleled the temporal expression of the obligate NMDA receptor (NR) subunit, NR1, as well as its functional activity. Neuronal COX-2 luciferase activity (Fig. 2B) and surface NR1 expression (Fig. 3A) as well as c-Fos mRNA levels (Fig. 3B), the latter used here as marker of neuronal activity (10), increased steadily from DIV 1 through DIV 10. To determine if NMDA receptor activity was required for constitutive neuronal COX-2 expression, COX-2 mRNA levels were measured in purified neuronal cultures (PNCs) that were either wild-type (NR1+/+) or null (NR1−/−) for NR1 (Fig. 4). Strikingly, COX-2
mRNA levels measured in NR1−/− cultures at DIV 7 were dramatically reduced when compared with that found in NR1+/+/ cultures (Fig. 4), suggesting that indeed NMDA receptor activity was necessary for constitutive expression of neuronal COX-2 mRNA. In support, the addition of 30 μM NMDA receptor antagonist 5-amino-phosphonovaleric acid (APV), a concentration that reduced NMDA receptor activity in pure neuronal cultures as evidenced by block of c-Fos mRNA production (Fig. 5A), also significantly decreased the levels of COX-2 mRNA (Fig. 5B). Interestingly, neither c-Fos nor COX-2 mRNA expression in cultured neurons was affected by pretreatment with tetanus toxin (300 ng/ml), a Clostridium toxin that prevents evoked but not spontaneous, synaptic vesicle exocytosis by cleaving the SNARE protein, synaptobrevin-2 (11) (Fig. 6).

Transcriptional Regulation of Constitutive Neuronal COX-2 Expression in Neuronal Cultures—Several putative cis-acting elements are found in the 5′-flanking region of the PTGS2 gene used to make the transgenic mouse. These include binding sites for nuclear factor-IL-6, glucocorticoid receptor, activator protein-1 and activator protein-2, CAAT box/enhancer-binding protein, cAMP response element-binding protein site (CREB), and stimulatory protein-1 (Sp1). Of these, CREB is known to specifically regulate numerous NMDA receptor-dependent genes (12, 13). To test whether CREB was involved in the constitutive expression of COX-2 in neurons, we co-transfected neurons with the Pluc371 plasmid and a dominant negative CREB (A-CREB) plasmid. The results demonstrate that A-CREB significantly attenuated Pluc371 promoter luciferase activity (Fig. 7). To confirm that the CREB site was functionally involved in the transcriptional regulation of constitutive COX-2 expression in neurons, we mutated the CREB-binding CRE site of the Pluc371 plasmid and measured the effects on luciferase activity. Additionally, as Sp1 has been demonstrated to be essential for the constitutive expression of numerous genes, we chose to mutate its binding site as well. Mutation of the CRE site completely abolished expression of luciferase activity from Pluc371
(Fig. 8), confirming a role for CREB. Surprisingly, mutation of the Sp1-binding site resulted in a significant elevation of luciferase activity, suggesting Sp1 acts as a transcriptional repressor of COX-2 expression in neurons (Fig. 8A). Interestingly, NMDA receptor antagonism reduced the increase mediated by mutation of the Sp1 site back down to control levels (Fig. 8A).

To examine a direct association of CREB and Sp1 with the endogenous PTGS2 gene promoter in neurons, ChIP analysis was performed on extracts from primary neurons at DIV 7. As shown in Fig. 8B, PTGS2 gene promoter fragments were successfully amplified from neuron genomic DNA samples using antisera directed against Sp1 or CREB. In each preparation, of COX-2 expression in neurons (Fig. 8A). Interestingly, NMDA receptor antagonism reduced the increase mediated by mutation of the Sp1 site back down to control levels (Fig. 8A). To examine a direct association of CREB and Sp1 with the endogenous PTGS2 gene promoter in neurons, ChIP analysis was performed on extracts from primary neurons at DIV 7. As shown in Fig. 8B, PTGS2 gene promoter fragments were successfully amplified from neuron genomic DNA samples using antisera directed against Sp1 or CREB. In each preparation,
strong binding was observed for CREB, whereas the SP1 appeared to be considerably weaker (Fig. 8B). When IgG antiserum was used for immunoprecipitation, a faint band was observed in 2/4 gels for SP1 and 1/4 for CREB (supplemental Figs. 1 and 2). Although, the exact reason for the small amount of template in the IgG control is not known, such background is not unusual (14, 15). Nevertheless, in all cases we see a significant -fold enrichment of the positive binding region for anti-SP1 as well as for anti-CREB antibodies over the IgG control when expressed as a ratio of bound sequence over input (Fig. 8B). Together these data provide compelling evidence for the binding and function of these transcription factors in the control of neuronal constitutive COX-2 gene expression.

Discussion

In contrast to various other tissues, COX-2 expression occurs constitutively in the central nervous system (7–9, 16, 17). Despite knowing that the PTGS2 gene promoter contains a TATA box motif and a number of cis-acting elements, including CREB, C/EBP, NF-IL6, AP-1, SP1, and NF-kB consensus sequences (18, 19), the molecular mechanisms underlying constitutive neuronal COX-2 expression remain largely unknown. With respect to this, this study presents with several original findings. First, results indicate that the factor(s) regulating constitutive neuronal COX-2 expression were inherent to neurons, as co-culture with astrocytes or treatment with astrocyte-conditioned media4 fail to regulate either Pluc371 gene reporter activity or COX-2 mRNA levels in neurons. Second, constitutive expression depends largely on the presence of functional NMDA receptors as genetic loss of the protein or functional block of NMDA receptors prevents the constitutive neuronal expression of COX-2. Third, the lack of effect of tetanus toxin reveals the involvement of spontaneous glutamatergic synaptic activity. Fourth, ChIP analysis demonstrates that both SP1 and CREB bind to native COX-2 promoter, whereas promoter mutation studies indicate that constitutive neuronal COX-2 expression is regulated by both SP1 and CREB family-dependent mechanisms.

Consistent with the COX-2 mRNA expression data found in the Allen Brain Atlas (20), brain tissue extracts from transgenic mice exhibit high constitutive luciferase activity in hippocampus, olfactory bulbs, and cortex (Fig. 1A). We additionally demonstrate constitutive activity in the cerebellum and spinal cord (Fig. 1A). This expression closely mirrored tissue mRNA levels, with the notable exception of cerebellum and spinal cord, suggesting areas of the promoter that suppress expression may be missing from our construct and/or that native expression in these tissues is influenced by elements in the COX-2 3′-UTR. Indeed, underscoring this point, analysis of basal expression of luciferase activity driven from the endogenous PTGS2 gene in a knock-in mouse also shows high expression in cortex and hippocampus, with little expression in cerebellum; the spinal cord was not assessed (21).

With respect to cortex, where the construct does faithfully reproduce native expression, this activity likely reflects neuronal expression as cultured cortical neurons from transgenic mice exhibit high luciferase activity, whereas activity in purified cultures of transgenic cortical astrocytes is negligible (Fig. 2A). This is congruent with previous immunocytochemical data from our laboratory demonstrating constitutive COX-2 protein expression in neurons but not astrocytes (17) and rat in vivo data demonstrating the same (9). Interestingly, astrocytic factors appear to have little or no influence on neuronal COX-2 expression, as evidenced by the fact that there was no change in the profile of constitutive luciferase expression in neurons when astrocytes were either in direct contact (Fig. 2B) or when astrocyte-conditioned medium was added to purified neuronal cultures.4 These findings suggest that the factors controlling constitutive COX-2 expression are intrinsic to neurons.

The constitutive COX-2 expression found in our cultures occurs in a temporal pattern (Fig. 2B), one that coincides with reported increases in the development of spontaneous post-synaptic currents in cultured cortical neurons (22). Additionally, this increase precisely mirrors the developmental expression of NMDA receptor protein and activity in our cultures as we reported previously (23) and confirm herein (Fig. 3A), raising the intriguing possibility that constitutive COX-2 expression is NMDA receptor-dependent. Indeed, functional block (genetic or pharmacological) of NMDA receptors effectively suppressed neuronal COX-2 mRNA expression (Figs. 4 and 5). The failure of tetanus toxin, a Clostridium toxin that prevents evoked, but not spontaneous, synaptic vesicle exocytosis in our and other systems (23–26), to reduce both constitutive neuronal activity and basal COX-2 mRNA expression in our cultures (Fig. 6) led us to conclude that spontaneous synaptic activity regulates basal neuronal COX-2 expression. This is not just a culture phenomenon, as basal COX-2 mRNA expression increased in rat forebrain and hippocampus over a developmental time frame in vivo and brain COX-2 basal mRNA expression can be reduced by neuronal deafferentation or by NMDA receptor inhibition but only slightly by intrahippocampal administration of tetrodotoxin (9).

Many of the regulatory elements driving constitutive neuronal expression must be found within −371 base pairs of the COX-2 promoter, as we found robust gene expression of constitutive COX-2 using the Pluc371 transgenic construct both in vivo and in vitro. This is an area that lacks the NF-κB site, which is located −400 bp upstream of the transcriptional start site (27). Hence, constitutive neuronal PTGS2 transcription appears to be independent of NFκB. This is in line with a recent study demonstrating both negligible constitutive NFκB activity in brain and a lack of effect of NFκB inhibition on PTGS2 promoter activity in knock-in reporter mice (21). Interestingly, stimulus-induced increases in neuronal COX-2 expression do appear to involve NFκB binding. For instance, Kalschmidt et al. (28) show that exposure of neurons to the phorbol 12-myristate 13-acetate (PMA) increases neuronal COX-2 expression in an NF-κB-dependent manner. Additionally, NFκB is regarded as a strong regulator of inducible COX-2 expression in neurons from diseased brain (29, 30) and spinal cord (31, 32). Thus, synaptic activity-dependent constitutive COX-2 expression is mechanistically different from injury-induced COX-2 expression.

4 K. Dhandapani, unpublished observation.
Transcriptional Regulation of Constitutive Neuronal COX-2

NMNMA receptor activation stimulates neuronal gene expression via several signaling pathways, including the Ras-MAP kinase (MAPK) pathway, tyrosine kinases, and via activation of the transcription factors, including Fos, Fra, and CREB (33–40). With respect to the latter, sequence analysis revealed that there is one cAMP-response element (CRE) site on the murine COX-2 promoter located between −85 and −75 bp upstream of the transcription start site. CREB and its family members contain a bZIP domain that mediates DNA binding and dimerization to CRE site and enhances gene transcription (41–43). Transfection of our neuronal cultures with a dominant-negative inhibitor of CREB (A-CREB), which interacts with CREB at its bZIP domain binding (44, 45), resulted in a significant decrease in the transgenic PTGS2 promoter-driven luciferase expression (Fig. 7), suggesting an important role for CREB in regulation of constitutive COX-2 expression in neurons. The fact that mutation of the CRE site on the promoter completely abolished luciferase activity (Fig. 8A) further supports this idea.

As a putative negative control, we also mutated the putative Sp1-binding site found between −265 and −255 upstream of the transcriptional start site. We chose Sp1 as it is a ubiquitously expressed transcription factor that is required for the expression of a variety of constitutively expressed genes (46), including endothelial COX-1 expression (47). Sp1 also promoted hypoxia-induced COX-2 expression in the vascular endothelium and oxidative and DNA damage-induced COX-2 expression in neuronal cells (27, 48). To our surprise, this mutation resulted in a significant increase in COX-2 luciferase activity (Fig. 8A), which demonstrates that Sp1 does not promote but rather represses basal COX-2 expression in neuronal cells. Indeed, Sp1 does constitutively bind to the native cortical neuron PTGS2 gene promoter (Fig. 8B). Several other studies describe Sp1 as a negative regulator of transcription as well. For instance, disulfide bond A oxidoreductase-like protein (DsbA-L) in preadipocytes (49) and Myc-associated zinc finger protein (MAZ) expression in hepatocytes (50) have also been demonstrated to be negatively regulated by Sp1. Gene expression can also be repressed through recruitment of histone deacetylase or methyltransferase to Sp1 sites (51, 52). The fact that mutation of the CRE site on the promoter completely abolished luciferase activity (Fig. 8A) further supports this idea.

In our initial studies with neuronal cells, we found that the PTGS2 promoter-driven luciferase activity was significantly lower in cultured neurons compared with astrocytes. This result is consistent with the idea that COX-2 expression is cell-autonomous in that it does not rely on astrocyte signaling. Finally, we identified two molecular players that govern constitutive PTGS2 transcription in cortical neuron via repression (Sp1) as well as enhancement (CREB). Our ongoing studies are devised to elucidate the signaling mechanism by which CREB positively and Sp1 negatively regulates COX-2 expression in neuronal cells.

Given the burgeoning evidence in support of a role for COX-2 metabolites in regulating membrane excitability in various forms of synaptic plasticity (54–65), the present findings add to our understanding of the dynamic gene expression landscape occurring in brain development and its underlying synaptic connectivity.

Materials and Methods

Animals

This study was conducted in accordance with the National Institutes of Health guidelines for the use of experimental animals and was approved by the Institutional Animal Care and Use Committee of both the University of Connecticut Health Center and Syracuse University.

Transgenic COX-2 Reporter Mice

Mice transgenic for the Pluc371 DNA construct (66, 67), containing the DNA construct −371 to +70 bp of the murine COX-2 promoter fused to a luciferase reporter gene, were developed in a CD-1 background by the Transgenic Animal Facility at the University of Connecticut Health Center, as described previously (66). This region of the murine COX-2 gene contains numerous known cis-acting transcriptional elements whose elements have been demonstrated to contribute to COX-2 transcriptional induction in non-neuronal cells under various conditions (68–70).

Cortical Cell Culture

Purified Primary Astrocytes—Astrocytes were cultured from the cortices of postnatal day 1–3 mouse pups as described in detail (71). Cells were plated 400 μl/well (2 hemispheres/10 ml/plate) (Falcon Primaria 24-well plates). Once confluent, astrocyte monolayers were treated with 8 μM β-2-caffeine arabinofuranoside (AraC) once for 4–7 days to reduce the number of microglia.

Mixed Cortical Cultures—Mixed cortical cultures containing both astrocytes and neurons were prepared from postnatal and fetal mice, respectively, as described in detail previously (17, 72). In brief, confluent astrocyte monolayers (9–11 DIV) were first established. Cortical cells from fetal mice were then plated at a density of 3.0–3.8 hemispheres/plate on an established astrocytic monolayer (12–24 DIV). After 5–7 days in culture, mixed cultures were treated with 8 μM Ara-C once for 2 days. Experiments were performed on mixed cultures containing ~50% neurons and 50% astrocytes.

PNCs—PNCs were cultured from cortices derived from the embryos of day 15 mouse and plated at 3.0–3.5 hemispheres/plate or 1.0–1.25 million cells/ml in Neurobasal medium supplemented with B27, 2 mM glutamine, and antibiotics as...
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Plasmid and Mutagenesis of COX-2 Promoter

Dominant negative CREB (A-CREB) was a gift from Charles Vinson (Addgene plasmid #33371) (44). Mutation of the Sp1 or CRE site in the PTGS2 gene promoter of Plunc371 (pTIS10) was carried out using QuikChange® Site-Directed Mutagenesis kit (Agilent Technologies). The Sp1-binding site was mutated by replacing the GC box sequence GGGCGG with GTTCGG. The CRE binding site was mutated by replacing CTACGTCA with CTGATCAGA.

Transfection of Cortical Neuron

Neuron cultures were transfected 4 days after plating. For each transfection, 4 μl of Lipofectamine 2000 (Invitrogen) were added to 0.7 ml Opti-MEM medium (Invitrogen) and incubated at 25 °C for 10 min. PLuc371 (2 μg) reporter plasmid together with 0.5 μg of GFP reporter plasmid (pGIPZ, Open Biosystems) plus 0.5 μg A-CREB or its empty vector were added to the Lipofectamine mixture, briefly vortexed, and incubated for another 20 min (25 °C). In the mutation studies, 2.5 μg of control (wild type), Sp1 mutant, or CRE mutant COX-2 luciferase reporter DNA constructs plus 0.5 μg of GFP reporter DNA were added to the Lipofectamine mixture, briefly vortexed, and incubated for another 20 min (25 °C).

A portion of neuronal culture medium (250 μl) was removed and immediately replaced with 175 μl of the transfection medium. Neurons were then incubated at 37 °C for 5–6 h, after which they were washed 4× (250 μl) with neuronal plating medium and placed back into a 37 °C, 5.5% CO2 containing normoxic incubator. Transfection efficiency was assessed in live cultures by measurement of GFP fluorescence intensity. Images from four microscopic fields (10× magnification) were acquired by a DP73 high performance Peltier cooled digital color camera mounted on an Olympus IX50 inverted microscope outfitted with epifluorescence controlled by CellSens Standard software (Olympus, Center Valley, PA). GFP intensity was quantified with NIH ImageJ. Luciferase activity in transfected cells was measured 3 days after transfection (DIV 7) using the Dual-Glo® Luciferase Assay Systems according to the manufacturer’s instruction. Plates were freeze-thawed once (−80 °C to 37 °C) after which Dual-Glo® Luciferase Assay Reagent was added to each well. After incubation at 25 °C for 10 min, luciferase activity was determined using a Bio-Tek Synergy2 microplate reader.

Drug Exposure

PNCs were exposed to the NMDA receptor antagonist APV (Tocris) or tetanus toxin (TeNT; Calbiochem) for 2 days (DIV 5–7) at 37 °C in a humidified 5.5% CO2-containing normoxic incubator at a final concentration of 30 μM or 300 ng/ml, respectively. Stock solutions of APV (1.2 mM) and tetanus toxin (12 μg/ml) were prepared in H2O.

qRT-PCR

COX-2 and c-Fos mRNA levels were measured using qRT-PCR. Total RNA was extracted from cells using TRIzol reagent (Invitrogen), and first-strand cDNA synthesis was performed as described previously (17, 76). cDNA was subjected to qRT-PCR in a singleplex reaction containing either mouse Fos (Mm00487425_m1), PTGS2 (Mm00478372_m1), or mouse β-actin (Mm01205647_m1) primer pairs with probes (Assay-On-Demand, Applied Biosystems) along with TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer’s protocol. PCR reactions were performed in duplicate or triplicate using an ABI 7500, an ABI 7900HT Fast Real Time PCR System (Applied Biosystems), or an Eppendorf Realplex2 under the following conditions: 50 °C for 2 min and 95 °C for 10 min followed by 40 amplification cycles (95 °C for 15 s and 60 °C for 1 min). Data analysis was performed using the comparative cycle threshold method (ΔΔCT), where C-T values for COX-2 and c-Fos mRNAs were normalized to β-actin C-T values from the same sample and then compared with a calibrator sample C-T value defined in each figure legend.
after which the beads received a final wash with Tris-HCl (50 mM, pH 7.5). Proteins in the collected supernatant fraction were precipitated by the addition of ice-cold acetone (5 volumes; −20 °C overnight). The resulting pellet was dissolved in 100 μl of a reducing loading buffer. All samples were boiled (100 °C; 2 min) before separation via SDS-PAGE (8% polyacrylamide gel) under reducing conditions. Proteins were then electrotheroically transferred onto nitrocellulose and immunoblotted for NR1 (mouse monoclonal; 1:4500; Novus Biologicals Littleton, CO) and β-tubulin class III (mouse monoclonal; 1:5000; Sigma ) to assess for biotinylation of intracellular proteins should it have occurred. If >15% of the β-tubulin class III was found to be biotinylated, samples were excluded from analysis.

Total Cell Lysates—Purified neuron cultures were washed once with ice-cold PBS and harvested by gentle scraping. Cells were spun (700 × g; 5 min; 4 °C), and the resulting pellet was suspended in lysis buffer containing 20 mM HEPES (pH 7.4), 2 mM EGTA (pH 8.0), 50 mM β-glycerol phosphate (pH 7.2), 1 mM DTT, 1 mM Na2VO4, 5 mM NaF, 1% Triton X-100, 0.2 mM PMSF, and 1× Complete Protease Inhibitor (Roche Applied Science). The resuspended pellet was then incubated on ice (30 min). Cellular debris was removed by centrifugation (12,000 × g; 20 min; 4 °C). 40 μg of protein (BCA assay; Pierce) was separated by 15% SDS-PAGE under reducing conditions and electrotheroically transferred to a PVDF membrane (Bio-Rad). Membranes were blocked (Odyssey® blocking buffer at 25 °C for 1 h) and then probed (4 °C, overnight) with an anti-synaptotobrevin-2 rabbit polyclonal antibody (1:1000 dilution; Synaptic System), and a mouse monoclonal antibody was directed against β-actin (1:4000 dilution; Sigma). Species-specific secondary antibodies labeled with spectrally distinct IRDye® fluorescent dyes (LI-COR Biosciences; Lincoln, NE) were used to detect primary antibodies (1 h at 25 °C). Results were recorded on LI-COR ODYSSEY® Fc Imaging system (LI-COR Biosciences).

Chromatin Immunoprecipitation Assay

Cross-linking of protein-DNA complexes for detection of transcription factor binding to the COX2 promoter was accomplished by either ultraviolet irradiation (SP1; 150 ml/cm2; Stratagene, UV Stratalinker 1800) or 1% formaldehyde fixation (CREB) of purified neuronal cultures at DIV 7 (1.2 × 105 cells/100 mm plate). Subsequently, cells were harvested by gentle scraping and spun at 600 × g for 5 min at 4 °C. The resulting pellet was suspended in ice-cold ChIP lysis buffer (1 ml) containing 50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and 1× Complete Protease Inhibitor (Roche Applied Science). After 10 min on ice, cell lysates were sonicated (Sonics Dismembrator Model 100, Fisher Scientific) 6 times (3 × 15-s each) with 40-s breaks between each and cellular debris pelleted at 8000 × g (1 min, 4 °C). Supernatants were diluted 1:5 in buffer containing 1 mM EDTA (pH 8.0), 1% Triton X-100, 20 mM Tris-HCL (pH 8.0), and 150 mM NaCl and divided equally into two samples. One sample contained the primary antibodies for CREB or Sp1 (5 μg of mouse monoclonal anti-CREB, (SC-271; Santa Cruz, Dallas, TX) or 5 μg rabbit polyclonal anti-Sp1 (07-645; Millipore, Billerica, MA). and the other contained the respective non-immune antibodies (5 μg, mouse IgG; Santa Cruz, Dallas, TX ) and 5 μg of rabbit IgG (Millipore, Billerica, MA). The samples were rotated at 4°C overnight, after which prewashed anti-mouse Ig IP Beads or anti-rabbit Ig IP beads (for CREB and Sp1 pulldown assays, respectively, True Blot®) were added. After rotating for an additional 4 h at 4 °C, beads were pelleted at 10,000 × g (1 min; 4 °C), and the supernatants were discarded. Beads were washed 4× with 1 ml of buffer containing 2 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% SDS, 20 mM Tris-HCL (pH 8.0), and 150 mM NaCl with 1× Complete Protease Inhibitor (Roche Applied Science) then cleared with 1 ml of final wash buffer (2 mM EDTA (pH 8.0), 1% Triton X-100, 0.1% SDS, 20 mM Tris-HCL (pH 8.0) and 500 mM NaCl with 1× Complete Protease Inhibitor (Roche Applied Science)). DNA-protein complexes were eluted from beads by adding 150 μl of warm (30 °C) elution buffer (1% SDS and 100 mM NaHCO3 in H2O) followed by slow vortexing for a total of 15 min. DNA was purified with Promega Wizard SV Gel and PCR clean-up kit (product # A9281).

PCR Amplification

cDNA samples (1 μl) were amplified using MJ Mini Personal Thermocycler for 35 cycles (94 °C/45s, 52 °C/30s, 72 °C/60s) using TaqDNA polymerase (Invitrogen) and target-specific primers in a total volume of 25 μl. Amplifiers for assessment of COX-2 DNA were 5’-GAGGGGAAGCTGTGACACTC-3’ (Sp1 forward) and 5’-ACGCAAATGAGACGAGAAGG-3’ (Sp1 reverse) and 5’-GCGTCTTCCCTGCTCTATT-3’ (CRE forward) and 5’-TGACACCTGGCTGTAATGG-3’ (CRE reverse). PCR products, separated on a 2% agarose gel containing ethidium bromide (200 μg/ml), were visualized with the LI-COR Odyssey Fc Infrared Imaging System.

Statistical Analysis

Data was analyzed using GraphPad Prism software as described in each figure legend. Non-normal data were either transformed before analysis with parametric statistical tests, or non-parametric tests were employed. Significance was assessed at p < 0.05, individual p values are described in the figure legends.

Author Contributions—C. P. provided the transgenic COX-2 reporter mice, initially noting constitutive COX-2 expression in brain. S. J. H. and J. A. H. conceived and coordinated the study and were responsible for the written content. K. D., Y. G. and J. S. contributed equally to the performance and analysis of experiments except for the study presented in Fig. 4, which was performed by J. A. H. Additionally, these authors contributed to the writing of the paper. All authors reviewed the results and approved the final version of the manuscript.

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