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Caffeine Stimulates Cytochrome Oxidase Expression and Activity in the Striatum in a Sexually Dimorphic Manner

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ABSTRACT

Epidemiological studies indicate that caffeine consumption reduces the risk of Parkinson's disease (PD) in men, and antagonists of the adenosine 2A receptor ameliorate the motor symptoms of PD. These findings motivated us to identify proteins whose expression is regulated by caffeine in a sexually dimorphic manner. Using mass spectroscopy, we found that Cox7c, a nuclear-encoded subunit of the mitochondrial enzyme cytochrome oxidase, is up-regulated in the striatum of male but not female mice after receiving a single dose of caffeine. The expression of two other Cox subunits, Cox1 and Cox4, was also stimulated by caffeine in a male-specific fashion. This up-regulation of Cox subunits by caffeine was accompanied by an increase in Cox enzyme activity in the male striatum. Caffeine-induced stimulation of Cox expression and activity were reproduced using the adenosine 2A receptor (A2AR)-specific antagonist 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-ε]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261), and coadministration of the A2AR-specific agonist 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680) counteracted the elevation of Cox expression and activity by caffeine. Caffeine also increased Cox activity in PC-12 cells. In contrast, small interfering RNA (siRNA) knockdown of Cox7c expression in PC-12 cells blunted Cox activity, and this was counteracted by caffeine treatment. Caffeine was also found to increase Cox7c mRNA expression in the striatum and in PC-12 cells. This occurred at the level of transcription and was mediated by a segment of the Cox7c promoter. Overall, these findings indicate that cytochrome oxidase is a metabolic target of caffeine and that stimulation of Cox activity by caffeine via blockade of A2AR signaling may be an important mechanism underlying the therapeutic benefits of caffeine in PD.

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Caffeine is the most widely used psychoactive drug in the world and has been used for centuries to increase alertness and energy. Several of caffeine's psychostimulant properties are attributed to its ability to modulate the physiology of basal ganglia (BG), which control voluntary movement (Fredholm et al., 1999). Caffeine is an antagonist of adenosine receptors, and it regulates the physiology of BG by binding to these receptors on neurons, particularly those of the striatum—the major input locus of BG (Schiffmann et al., 2003).

Although caffeine has little or no selectivity for adenosine receptor subtypes, its ability to stimulate movement has been shown to be mediated by the adenosine 2A receptor (A2AR), inasmuch as enhanced locomotion produced by caffeine treatment is lost in A2AR knockout mice (Chen et al., 2001). A2AR forms heterodimeric complexes with the dopamine 2 receptor (D2R) on striatal GABAergic projection neurons (Canals et al., 2003; Ciruela et al., 2004). The binding of caffeine and agonists such as CGS21680 to A2AR in the striatum alters both the signaling by D2R and the affinity of D2R for dopamine (Ferre et al., 1992). The locomotor effects of caffeine have also been correlated with the phosphorylation state of DARRP-32, a protein that mediates intracellular signaling by dopamine receptors (Lindskog et al., 2002).

Several epidemiological studies have concluded that caffeine consumption reduces the risk of Parkinson's disease

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ABBREVIATIONS: BG, basal ganglia; A2AR, adenosine 2A receptor; D2R, dopamine 2 receptor; Cox, cytochrome *c* oxidase; Cox7c, cytochrome *c* oxidase subunit VIIc; CGS21680, 2-[*p*-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine; PD, Parkinson's disease; siRNA, small interfering RNA; KF17837, (*E*)1,3-dipropyl7-methyl-8-(3,4-dimethoxystyryl)xanthine; KW6002, istradefylline; SCH58261, 5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-ε]-1,2,4-triazolo[1,5-*c*]pyrimidine; PCR, polymerase chair reaction; PBS, phosphate-buffered saline; TFA, trifluoroacetic acid; SELDI-TOF, surface laser-enhanced desorption/ionization time-of-flight mass spectrometry; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; DAB, 3,3′-diaminobenzidene-tetrahydrochloride; Cox, cytochrome *c* oxidase.

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(PD) in men (Benedetti et al., 2000; Ross et al., 2000; Ascherio et al., 2001, 2004). Moreover, a single dose of caffeine was shown to attenuate MPTP-induced striatal dopamine loss in male mice but not in female mice. This effect of caffeine was abolished when male mice were treated with estrogen (Xu et al., 2002, 2006). Despite evidence for the strong inverse correlation between caffeine consumption and the incidence of PD in male mice, the molecular mechanisms underlying the therapeutic benefits of caffeine in PD are unknown.

In light of the findings that the A2AR is critical for stimulation of motor activity by caffeine, several therapeutic A2AR-specific antagonists have been developed. These include xanthine derivatives such as KF17837 and KW6002, and nonxanthine compounds such as SCH58261 (Shimada et al., 1992; Nonaka et al., 1994; Aoyama et al., 2002). In recent years, these therapeutic A2AR antagonists have received considerable attention as a promising nondopaminergic therapy for Parkinson's disease (Cacciari et al., 2003). A2AR antagonists can rescue Parkinsonian-like motor impairments in dopamine 2 receptor knockout (D2R-KO) mice (Aoyama et al., 2000). Moreover, these drugs have been used successfully to treat Parkinsonian symptoms such as akinesia and to reduce L-DOPA-related motor complications (Chen et al., 2003; Xiao et al., 2006).

To date, few studies have analyzed sexually dimorphic changes in gene expression that occur in response to caffeine administration in mammals. In previous work, we showed that caffeine increased dopamine 2 receptor expression in the striatum of female mice but not in male mice (Stonehouse et al., 2003). Based on these findings, we set out to identify proteins in addition to D2R whose expression is regulated by caffeine in a sexually dimorphic manner.

In the present study, we used a proteomic approach and identified a particular protein—cytochrome oxidase VIIc (Cox7c)—whose expression in the mouse striatum shows a sexually dimorphic response to caffeine. Cox7c is one of 10 nuclear-encoded subunits of cytochrome oxidase (Cox), an enzyme of the mitochondrial electron transport chain that is essential for oxidative metabolism. Caffeine also stimulated the expression of two other important Cox subunits (Cox1 and Cox4). Induction of Cox7c, Cox1, and Cox4 expression by caffeine was accompanied by a male-specific increase in cytochrome oxidase activity in the striatum. These male-specific responses were mediated by the A2AR, inasmuch as coadministration of the A2AR agonist CGS21680 counteracted the increase in Cox expression and activity by caffeine, and treatment of male mice with the A2AR antagonist SCH58261 also stimulated Cox expression and activity. Additional experiments revealed that siRNA-mediated inhibition of Cox7c expression in PC-12 cells decreased Cox activity, and caffeine counteracted this effect. Caffeine also increased Cox7c mRNA expression in the male striatum, as well as in PC-12 cells. This regulation occurred at the level of transcription, and was mediated by a segment of the proximal promoter of the Cox7c gene.

Overall, these findings suggest that cytochrome oxidase is a metabolic target of caffeine. They also prompt further investigations to determine whether up-regulation of Cox expression and activity is a fundamental mechanism by which caffeine ameliorates the motor symptoms and protects the brain from Parkinson's disease.

Materials and Methods

Animals, Cell Culture, and Drug Treatments. Male and female C57B6/S129 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). All of the mice used were between 3 and 5 months of age. Mice received intraperitoneal injections of 400 μl of either vehicle (0.5% methyl cellulose), caffeine (7.5 mg/kg), or the A2AR agonist CGS21680 (0.5 mg/kg). Caffeine and CGS21680 were obtained from Sigma-Aldrich (St. Louis, MO). Animals were sacrificed 12 h after drug treatment, and striata were dissected. Protein and RNA were then extracted for Cox assays, quantitative Western blotting, and real-time quantitative PCR as described below. All animal procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rat PC-12 cells obtained from the American Type Culture Collection (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% horse serum, 5% fetal calf serum, and 1× penicillin/streptomycin/glutamine (Invitrogen).

Preparation and Fractionation of Mouse Striatal Proteins and Proteomic Analyses Using Mass Spectrometry. Striata from sham- and caffeine-injected mice were individually weighed and homogenized in a Dounce homogenizer (Glas-Col, Terre Haute, IN) in 10× (vol/mass) volume of ice-cold PBS with protease inhibitors (Sigma Chemicals, St. Louis, MO). The homogenizer was washed with an additional 10× volume of cold PBS that was added to the initial extract. An aliquot (20% vol/mass) of cold, precondensed Triton X-114 was added to the extract and incubated for 30 min on ice. After solids were removed by centrifugation at 4°C, followed by heating, the cleared extract was warmed to 37°C for phase separation. Detergent-rich and -poor phases were separated by centrifugation at room temperature. The detergent-rich phase was washed 1× with an equal volume of cold PBS and reseparated as described above.

Two different protein chips were used to fractionate the striatal proteins: Q10 (a strong anion exchanger) and H50 (a hydrophobic matrix). All protein samples were diluted 1:10 (200 μl total) in the appropriate binding buffer and incubated for 1 h with vigorous shaking. The binding buffer for Q10 chips was 50 mM Tris-HCl, pH 9.0, and 0.1% Triton X-100. The binding buffer for H50 chips was 5% acetonitrile and 0.1% trifluoroacetic acid (TFA). The proteins bound to the protein chip matrices were then washed 3 times for 5 min in 200 μl of binding buffer followed by a final wash with de-ionized water. Chips were then dried for 10 min followed by application of an energy-absorbing matrix (2 × 1 μl of sinapinic acid in 50% acetonitrile/0.5% TFA).

Samples were then subjected to surface laser-enhanced desorption/ionization time-of-flight mass spectrometry (SELDI-TOF) on a PBSIIc mass spectrometer (Bio-Rad Laboratories/Ciphergen Biosciences, Inc. Hercules, CA). Spectra were acquired using ProteinChip software (Bio-Rad/Ciphergen) and were subjected to differential expression analyses using the Ciphergen Express 2.1 program (Bio-Rad/Ciphergen). Spectra were baseline corrected, normalized for protein amount, and aligned (mass calibrated).

The spectra derived from SELDI analyses showed peaks with mass-to-charge (m/z) ratios corresponding to the masses of full-length protein species. The spectra were imported into a software program that automatically measured and compared the intensities of these peaks in untreated versus caffeine-treated mice, thereby identifying striatal proteins whose expression is regulated by caffeine treatment. Clusters of data representing differential expression values for each protein showing peaks in the range of 2 and 30 kDa were automatically compared using this software; only significant changes in the intensity of peak heights ($P < 0.05$) were scored. One of the proteins showing differential expression in caffeine-treated versus untreated mice with a mass of 5444 Da was subjected to further analysis.

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Identification of the 5444-Da Protein as Cox7c. Using the proteomics tool TAGIDENT (Expasy Proteomics Webserver), the only protein that matched the intact mass of the 5444-Da protein in the mouse database (SwissProt accession number P17665) was Cox7c (search tolerance of ± 5 Da, within the accuracy of the PBSIIc calibrated from 1 to 7 kDa). The 5444-Da form of Cox7c is a mature, processed form of Cox7c that is found in the inner membrane of mitochondria. To validate the identity of the protein as Cox7c, a partial purification and tryptic digestion of the protein was performed. For these experiments, striata from six C57B6/S129 mice were homogenized, and proteins were extracted as described above. The detergent-poor phase was adjusted to 10% acetonitrile/0.5% TFA and allowed to bind to reversed-phase beads (equilibrated in 10% acetonitrile/0.5% TFA) for 30 min. The beads were eluted with a step gradient of acetonitrile/0.1% TFA. All fractions were monitored using SELDI-TOF mass spectrometry and indicated the 5444 *m/z* species eluted in the 50 and 60% acetonitrile fraction. These two fractions were pooled, neutralized and dried. The sample was then diluted in nonreducing tricine sample buffer (Invitrogen, Carlsbad, CA) and analyzed by polyacrylamide gel electrophoresis on a 16% Tricine gel. The protein/peptide bands were visualized by colloidal Coomassie staining (Invitrogen). Bands were excised and washed with 200 μl of 50% methanol/10% acetic acid for 30 min, dehydrated in 100 μl of acetonitrile for 15 min, and extracted with 70 μl of 50% formic acid/25% acetonitrile/15% isopropanol/10% water for 2 h at room temperature with vigorous shaking. Extractions were spotted on normal phase protein chips (NP20; Bio-Rad/Ciphergen) and analyzed using SELDI-TOF mass spectrometry. Gel extractions containing only the 5444-Da protein were pooled, dried, and digested with 10 ng/μl of modified trypsin (Roche, Indianapolis, IN) in 20 μl of 50 mM ammonium bicarbonate, pH 8.0, for 3 to 4 h at 37°C. Endoproteolytic cleavage of the 5444 Da protein was performed using 1 μl of trypsin (Sigma). Samples and gel blanks (negative controls) were spotted on NP20 chips with 1 μl of saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.5% TFA and analyzed using SELDI-TOF mass spectrometry. These analyses detected a tryptic fragment of 2372 Da, which corresponds to residues 37 to 57 (LLAMMTVYFGSGFAAPFFIVR) of Cox7c (0 miscuts, both methionine residues oxidized).

Comparisons of Cox7c Protein Expression in Male and Female Mice. Six male and six female C57B6/S129 mice injected with either vehicle or caffeine (7.5 mg/kg); 12 h later, striata were harvested. Then, mitochondrial fractions of striatal proteins were prepared, bound to protein chips, and analyzed for relative levels of Cox7c expression using SELDI-TOF. To prepare mitochondrial fractions, the striata were homogenized in 2.5 ml of mitochondrial isolation buffer (10 mM Tris-HCl, pH 7.8, 0.2 mM EDTA, and 0.25 M sucrose) with 20 strokes in a Dounce homogenizer (Glas-Col, Terre Haute, IN). Extracts were centrifuged for 5 min at 2500 rpm at 4°C. The supernatant was collected, and then centrifuged for 15 min at 13,000 rpm to obtain a crude mitochondrial fraction. The pellet was then resuspended in 200 μl of mitochondrial isolation buffer. Ten microliters was removed to determine the protein concentration in each of the samples (Bio-Rad Laboratories). Protein concentrations between 0.3 and 0.5 μg/μl were routinely obtained. Triton X-100 was added to samples to a final concentration of 1%, and then samples were shaken vigorously for 30 min at 4°C. Approximately 20 μg of protein from each sample were resuspended in Q10 binding buffer (50 mM Tris-HCl, pH 9.0 and 0.1% Triton X-100) and loaded onto Q10 protein chips (in duplicate). SELDI-TOF and Cox7c protein expression analyses were performed as described above.

Quantitative Western Blotting. Striata were dissected from male and female mice and homogenized with 15 to 20 strokes in a Dounce homogenizer in 2 ml of radioimmunoprecipitation assay buffer [150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0 containing complete protease inhibitor (Roche)], and shock-frozen in liquid nitrogen. Samples were quickly thawed at 55°C and centrifuged twice at 13,000 rpm for 10 min at 4°C to obtain a clean supernatant. Ten microliters of lysate were removed and assayed for protein concentration using Bio-Rad reagent. Then, 20 μg of protein was taken from each sample and electrophoresed on 4 to 12% Bis-Tris Nu-PAGE gels (Invitrogen) and then electro-transferred to polyvinylidene difluoride membranes (Invitrogen). The membranes were incubated with a 1:1000 dilution of the appropriate primary antibody. The antibodies used were monoclonal anti-mouse Cox1 and Cox4 antibodies (Mitosciences, Inc., Eugene, OR) or a monoclonal anti-mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody (Assay Designs/Stressgen Inc., Ann Arbor, MI). Primary antibody treatments were followed by addition of an alkaline phosphatase-conjugated anti-mouse IgG (Western Breeze; Invitrogen). Enhanced chemiluminescent detection of bands corresponding to Cox1, Cox4, and GAPDH was carried out using CDP-Star. Immunoblot analyses revealed predominant bands at approximately 35 kDa (for Cox1), 18 kDa (for Cox4), and 36 kDa for GAPDH. The intensities of bands were quantified using the ImageJ image processing and analysis program (http://rsbweb.nih.gov/ij/). Relative levels of expression for Cox1 and Cox4 proteins were determined after normalizing to the levels of GAPDH. The expression data were derived from lysates that were prepared from six independent male or female mice for each drug treatment and time point that was analyzed.

Cytochrome Oxidase Activity Assays. Cytochrome oxidase assays were performed on samples obtained from either mouse striatum or rat PC-12 cells using a modification of the 3,3′-diaminobenzidene-tetrahydrochloride (DAB) method in microtiter plate format (Chrzanowska-Lightowlers et al., 1993). In brief, striata were harvested and suspended in 2.5 ml of mitochondrial isolation buffer and homogenized with seven strokes in a dounce homogenizer with a Teflon pestle (Glas-Col). Samples were subjected to centrifugation at 13,000*g* or 5 min. Cell pellets were then resuspended in 150 μl of mitochondrial isolation buffer with 1 mM *n*-dodecyl-β-D-maltoside (Biochemika/Sigma-Aldrich, St. Louis, MO). Triplicate samples of 10 μl were removed to determine the protein concentration using Bio-Rad reagent, and triplicate samples of 35 μl were removed to assay for Cox activity in 96-well format. For Cox assays involving PC-12 cells, the cells were cultured in six-well plates to 80% confluence, harvested by scraping without trypsin, and then washed in ice-cold phosphate-buffered saline, pH 7.4, and centrifuged for 5 min at 13,000*g*. Cell pellets were then resuspended in 150 μl of 1 mM *n*-dodecyl-β-D-maltoside, and processed as described above. Striatal or PC-12 cell samples (35 μl) were mixed with 100 μl of DAB assay cocktail (Chrzanowska-Lightowlers et al., 1993) and assayed for Cox activity in 96-well plate format. Cox activity in samples was detected by monitoring production of the oxidized polymer of DAB, which is proportional to Cox activity and is detected spectrophotometrically at 450 nm using a microplate reader (model 680; Bio-Rad). The final concentration of the assay medium was 4 mM DAB (Sigma-Aldrich), 150 μM reduced ferrocytochrome *c* (Sigma-Aldrich), 4 μg/ml catalase (Worthington, Inc. Lakewood, NJ) in 0.15 M sodium phosphate buffer, pH 7.0. The A450 measurements were taken at 5, 10, 15, 20, 25, and 30 min to establish the linear range for the production of the DAB, and measurements of DAB (Cox activity) were routinely performed at 20 min. The measurements of Cox activity for the experiments shown in Fig. 3 were performed on six untreated and six caffeine-treated male or female mice per time point (45 min, 4 h, and 12 h). Cox activity assays in PC-12 cells in the presence of caffeine or Cox7c siRNA were derived from at least four independent experiments in which at least three wells of PC-12 cells for each condition were examined. Statistical comparisons were performed between treated and untreated groups using a two-tailed Student's *t* test.

Quantitative RT-PCR Experiments. Total RNA was extracted from mouse striata or PC-12 cells using TRIzol reagent (Invitrogen). Ten micrograms of RNA were reverse transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen). PCR was performed using Cox7c and β-actin-specific primers. Cox7c has many pseudogenes in mammalian genomes that can produce spurious signals and confound the detection of Cox7c mRNA in PCR

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reactions. We therefore identified a single of PCR primers that produced only one band corresponding to the true Cox7c message when RNAs from both the mouse striatum and rat PC-12 cells were assayed. The primers were derived from a 5′ region of the Cox7c gene, spanned the first intron, and were degenerate (i.e., were derived by comparing sequences of mouse, human, and rat Cox7c cDNA sequences). The forward primer was 5′-AGCATGTTGGGCCA-GAGT-3′, and the reverse primer was 5′-ACTGAAAACGGCAAAT-TCTT-3′. These primers gave a 94-base pair PCR product, which was confirmed as a true Cox7c amplification product by automated DNA sequencing. In addition, PCR reactions were performed using β-actin gene-specific primers (Adachi et al., 2005) or GAPDH gene-specific primers (Stonehouse et al., 2003) to normalize the expression levels of Cox7c mRNA. PCR products were labeled by incorporating fluorescent dNTPs using the SYBR green PCR master mix (Applied Biosystems, Chatsworth, CA). Quantification of fluorescent PCR products was performed using the GeneAMP 5700 sequence detector (Applied Biosystems). β-Actin PCR products (generated after 17 to 18 cycles) and GAPDH PCR products (generated after 15 to 18 cycles) were used to normalize quantities of cDNA, allowing quantification of Cox7c PCR products generated after 20 to 21 cycles. Cox7c, β-actin, and GAPDH expression levels were derived from at least three independent experiments in which nine separate PCR reactions were performed on each of the cDNA samples.

SiRNA Experiments. SiRNA experiments in PC-12 cells were performed using the Stealth system (Invitrogen) after transfection with Lipofectamine 2000 (Invitrogen). The Cox7c and control siRNAs had the following sequences: Cox7c: sense, 5′-GAUGAC-CGUGUACUUUGGAUCUGGA-3′; antisense, 5′-UCCAGAUCCA-AAGUACACGGUCAUC-3′; control: sense, 5′-GAUGCCUGUUC-AGUUAUAGUCGAGGA-3′; antisense, 5′-UCCUCGACUAAAC-UGAACAGGCAUC-3′. Nucleotides in the control siRNAs that are underlined indicate base substitutions which prevented complementary base pairing to Cox7c mRNA. PC-12 cells in six-well plates (50–70% confluence) were transfected with 100 pmol of the appropriate siRNA duplex in media and incubated for 4 h. The media were then changed and cells were incubated for an additional 24 h in normal media, or media containing 100 μM caffeine. Cells were harvested and assayed either for Cox7c mRNA expression (using Cox7c and β-actin primers) or for Cox activity using the DAB method, as described above.

Preparation of Mouse Cox7c Reporter Constructs and Luciferase Assays of Cox7c Promoter Activity in PC-12 Cells. Two mouse Cox7c promoter/luciferase reporter constructs containing −2063/+74 and −93/+74 regions of the mouse Cox7c gene (Genbank accession number NT039589.4) were generated by 30 cycles of PCR amplification using mouse genomic DNA as template (Clontech, Mountain View, CA). The following primers were used to amplify the two Cox7c promoter segments. For the −2063/+74 construct, the 5′ primer was 5′-ga*tagCTA*GCAGTGAGATATCTTAAACAAAGAAG-GCAT-3′ and the 3′ primer was 5′ ta*ctcgaG*GAAGGAGGAAGAAATG-GCCGTACCACC-3′.

For the −93/+74 construct, the 5′ primer was: 5′-ga*tagctag*-CCATTGAATCACTAAAGGTAAGTTATCA-3′. The 3′ primer for the −93/+74 construct was the same one used to make the −2063/+74 construct. The bases indicated in lower case represent extra bases added to make NheI and XhoI restriction sites (which are underlined). PCR products were inserted into the promoterless pGL3 basic vector (Promega, Madison, WI). Sequences of both constructs were verified by automated DNA sequencing. Each construct was transfected into PC-12 cells and assayed for luciferase activity. For transfection, 6×10^5 PC-12 cells were seeded in each well of a six-well plate (Costar; Corning Life Sciences, Acton, MA). Twenty-four hours later, cells were transfected with 1ug of either pGL3 basic, −2063/+74 construct, or −93/+74 construct as described previously (Stonehouse et al., 2003). In all experiments, cells were cotransfected with 100 ng of the plasmid pCMV-β (Clontech) to assess transfection efficiency and to normalize luciferase activity values to an internal standard of β-galactosidase activity. PC-12 cells were transfected using FuGENE6 (Roche) and Opti-MEM 1 media (Invitrogen). After 24 h, media were changed and treated with the concentrations of caffeine or CGS21680 that are indicated in each of the figures. CGS21680 was dissolved in DMSO before delivery. After incubating for various times (between 4 and 24 h), cells were harvested, washed in phosphate-buffered saline, resuspended in 150 μl of 1× reporter lysis buffer (Promega), assayed for β-galactosidase activity using the FluoReporter kit (Invitrogen) and assayed for luciferase activity using a Microlumat LB968 luminometer (Berthold Technologies, Bad Wildbad, Germany) The data presented were derived from at least four independent experiments, performed in triplicate.

Results

Cox7c Protein Expression Is up-Regulated in the Striatum by Short-Term Caffeine Treatment in Male but Not Female Mice. To identify striatal proteins whose expression was altered by a single dose of caffeine, we first performed a proteomic expression difference mapping analysis of striatal protein extracts from 12 4-month old male C57B6/S129 mice. Six mice were sham-injected with vehicle, whereas the other six mice received an injection of caffeine (7.5 mg/kg). This dose of caffeine is on the low end of the physiologically relevant range (which is between 2 and 50 mg/kg) and is approximately equivalent to the concentration of caffeine (35 μM) found in the human brain after consuming two cups of brewed coffee (Fredholm et al., 1999; Xu et al., 2006).

The injections of mice were performed in the evening (10 PM) and the animals were sacrificed 12 h later. Extracts of striatal proteins were fractionated on protein chips and subjected to SELDI-TOF in a PBSIIc mass spectrometer. The resulting spectra were then imported into a software program that automatically quantified the intensities of protein peaks in the 2- to 30-kDa range. This method enabled us to visualize and compare the expression of several hundred proteins in caffeine-treated versus untreated animals. Comparisons of the spectra revealed that a striatal protein having a mass of 5444 Da showed a significant increase in peak intensity in caffeine-treated male C57B6/S129 mice versus controls (Fig. 1A). When the 5444-Da peak intensities from all six of the extracts from caffeine-treated animals were compared with those from the six untreated animals in a scatter plot (Fig. 1B), it was evident that caffeine-treatment resulted in a 2-fold increase in the expression of the 5444-Da protein versus control mice. The 5444-Da protein was also identified in a separate proteomic experiment as one of four proteins showing differential expression in male dopamine 2 receptor knockout mice versus C57B6/S129 control mice (data not shown). The convergence of these two data sets prompted us to establish the identity of the 5444 Da protein and characterize its activity and expression in greater detail.

The only protein matching the 5444-Da protein in the mouse database was cytochrome *c* oxidase VIIc (Cox7c), a nuclear-encoded subunit of the mitochondrial enzyme cytochrome *c* oxidase (Cox). Therefore, to validate the identity of the 5444 Da protein as Cox7c, we partially purified the protein from striatal extracts and digested it with trypsin (see *Materials and Methods*). SELDI analyses of tryptic digests revealed a major fragment with a mass of 2372 Da, corresponding to an internal fragment (residues 37–57) of Cox7c.

The 5444-Da form of Cox7c is the mature, fully processed form of Cox7c that is found in the inner membrane of mito-

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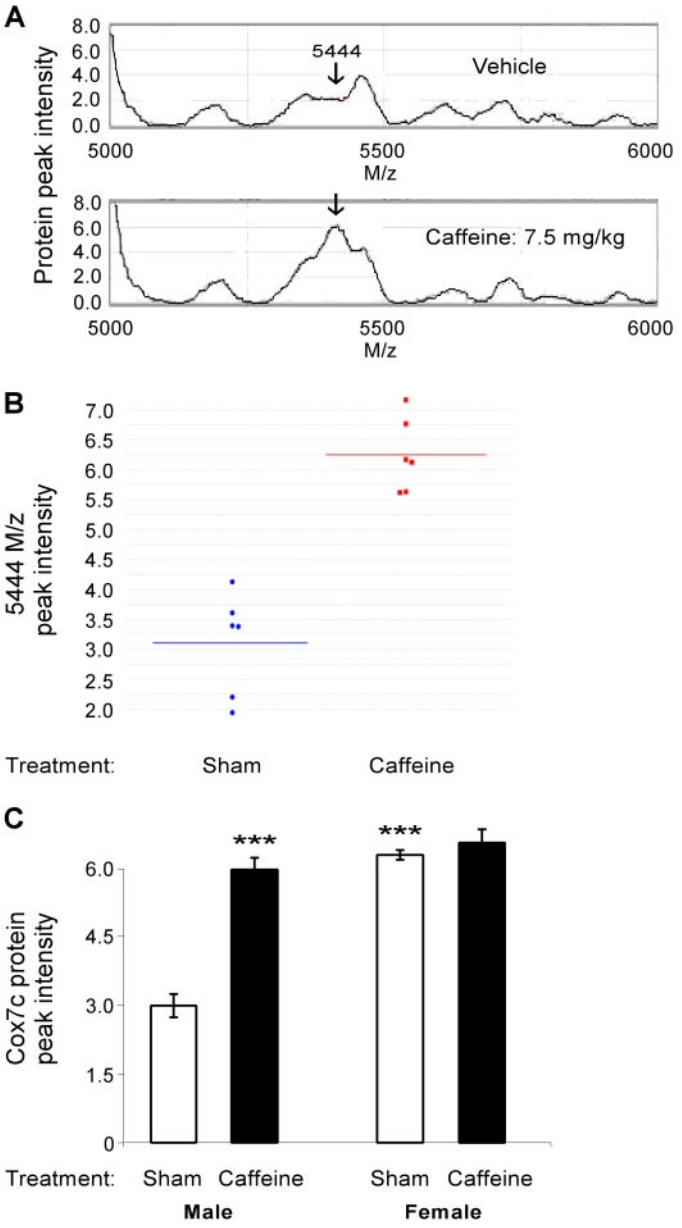

Fig. 1. Identification of Cox7c as a protein in the striatum that is up-regulated in a sexually dimorphic manner by caffeine. Cox7c protein levels were determined using SELDI on proteins prepared from striata isolated from male and female C57B6/129 mice. A, two representative spectra from SELDI analyses of striatal proteins showing differences in the intensity (expression) of a protein peak corresponding to a mass (*m/z*) of 5444 Da in control (top) versus caffeine-treated (bottom) male mice. The identity of the 5444-Da protein was subsequently identified as Cox7c. B, scatter plot generated by the Ciphergen Express 2.1 program showing the relative intensities of the 5444 Da peak (Cox7c) in striatal protein samples prepared from six sham-injected male mice versus samples prepared from six caffeine-treated male mice. The lines within the scatter plot indicate the mean peak intensities of the 5444-Da protein (Cox7c). The intensity of the 5444 *m/z* peak corresponding to Cox7c is increased an average of 2-fold in caffeine-treated versus sham-injected mice. C, expression of Cox7c protein in mitochondrial extracts of striatal proteins prepared from sham-injected or caffeine-treated male and female mice. The values of Cox7c protein peak intensities were derived from protein extracts prepared the striatum of six different animals in each of the four categories shown in the figure. Expression was quantified using SELDI analyses of Q10 protein chips. Results are expressed as mean protein peak intensity ± S.E.M. The two-tailed Student's *t* test was used to analyze differences in expression between samples from caffeine-treated and untreated animals in C. ***, $P < 0.001$.

chondria. Therefore, we examined whether caffeine treatment increased the levels of mature Cox7c protein in mitochondrial fractions prepared from mouse striatum. At the same time, we examined whether male and female mice showed similar increases in striatal Cox7c protein expression in mitochondria in response to caffeine. SELDI-TOF was used for these experiments because a suitable Cox7c antibody was not available to us to examine the expression of Cox7c via immunoblot analysis. Six male and six female C57B6/S129 mice were injected with either vehicle or caffeine (7.5 mg/kg). Twelve hours later, striata were collected, mitochondrial-enriched striatal protein lysates were prepared and fractionated on protein chips, and the absolute protein peak intensities of Cox7c were determined by SELDI-TOF. As shown in Fig. 1C, samples prepared from male striata showed a 2-fold increase in Cox7c protein peak intensity (expression) in response to caffeine. It is noteworthy that the values of Cox7c protein peak intensities in the striata prepared from sham-injected female mice were approximately 2-fold greater than those found in sham-injected male mice and nearly identical to the Cox7c expression levels that were observed in caffeine-treated male mice. However, compared with the baseline level of Cox7c expression in sham-injected female mice, the expression of Cox7c was not altered by caffeine treatment in female mice (Fig. 1C). These results suggest that levels of Cox7c are repressed approximately 50% in male mice and that caffeine treatment derepresses expression of Cox7c to the level that is normally observed in untreated female mice.

Expression of Cox1 and Cox4 Proteins in the Striatum Is Increased by Caffeine Treatment of Male Mice but Not Female Mice. Inasmuch as SELDI analyses demonstrated that up-regulation of striatal Cox7c protein expression by caffeine was male-specific, we used quantitative Western blotting to determine whether the expression of two other Cox subunits, Cox1 and Cox4, also showed similar sexually dimorphic regulation by caffeine. We also wished to determine whether, like Cox7c, the baseline expression of Cox1 and Cox4 were also different in male and female mice. Striatal protein lysates were prepared from male and female mice that were either sham-injected or injected with a single dose of caffeine (7.5 mg/kg). Three groups of 24 animals (six male and six female mice at the four different experimental conditions) were sacrificed at 45 min, 4 h, and 12 h after injection to examine the expression of Cox1 and Cox4 proteins at each time point. Whole-cell and mitochondrial lysates were prepared from the striata dissected from these animals. The whole-cell lysates were used for Western blot analyses (Fig. 2), and the mitochondrial lysates were used to assay Cox activity in these same animals (Fig. 3A, as described below).

Signals from bands on Western blots corresponding to Cox1 and Cox4 proteins were quantified at each time point, and absolute levels of Cox1 and Cox 4 expression were determined after normalizing to the band intensities of GAPDH in each sample (see *Materials and Methods*). An example of Cox1 and Cox4 band intensities on Western blots in samples from caffeine-treated versus untreated mice at the 12-h time point is shown in Fig. 2A. A qualitative comparison of band signal intensities of Cox1 on Western blots showed that Cox1 protein expression is nearly identical between untreated male and female mice and that caffeine treatment resulted in an increase

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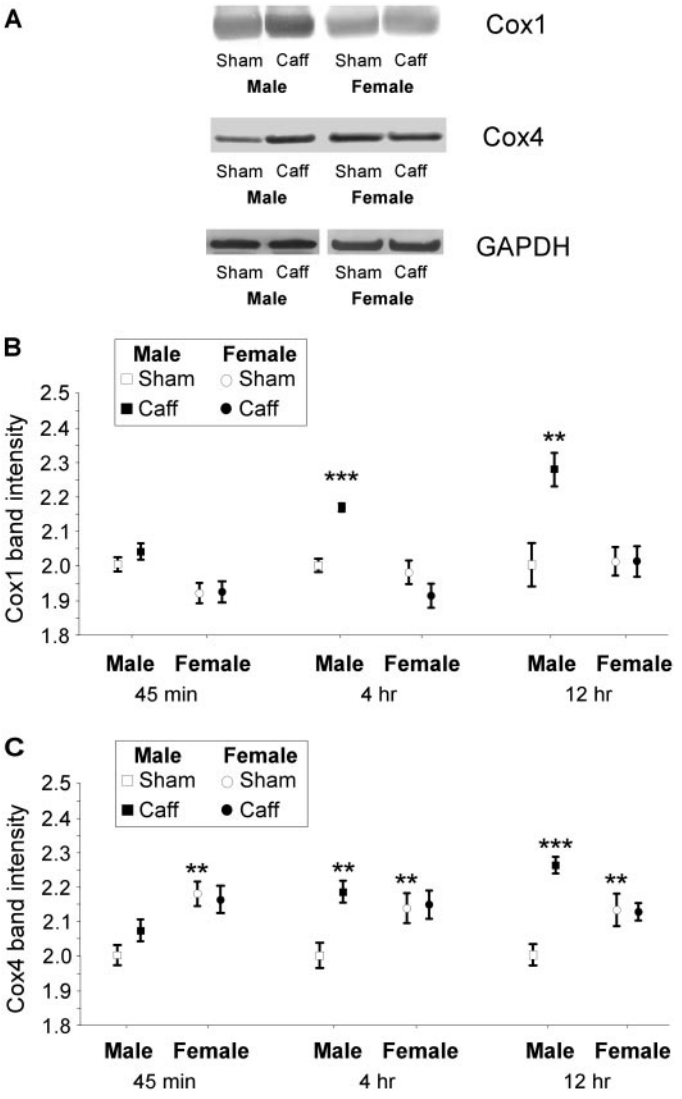

Fig. 2. Caffeine increases the expression of Cox 1 and Cox 4 proteins in the striatum of male mice but not in female mice. A, representative western blots showing the differences in signal intensity from bands corresponding to Cox1 and Cox4 proteins that were typically observed at the 12-h time point from experiments that compared striatal expression of Cox1 and Cox4 in caffeine-injected versus sham-injected male and female mice. The increases in Cox1 and Cox4 band intensities on Western blots in samples from caffeine-treated versus untreated male mice were clearly visible at the 12-h time point, whereas no differences in band intensities were observed in similar comparisons of samples from caffeine-treated versus untreated female mice. Comparisons of the Western blot band signal intensities of Cox1 (B) and Cox 4 (C) protein in the striatum from male and female C57B6/S129 mice 45 min, 4 h, and 12 h after caffeine treatment. The intensities of Cox1 and Cox4 bands on Western blots expression were measured at each time point. For all, experiments, whole-cell lysates were prepared from striata isolated from caffeine-treated or untreated male and female mice (six mice in each of the four groups). Ten micrograms of protein extract were electrophoresed on polyacrylamide gels, electrotransferred to nylon membranes, and the expression of Cox1 and Cox4 proteins was determined by quantitative Western blot analyses using mouse monoclonal Cox1 and Cox4 antibodies. In addition, blots were probed for expression of GAPDH using a GAPDH antibody to normalize levels of Cox1 and Cox4 protein expression within each treatment group (see *Materials and Methods*). Results are expressed as mean band signal intensity ± S.E.M, and statistical significance of the differences in expression between samples from caffeine-treated and untreated animals, and between untreated male and female animals were assessed using a two-tailed Student's t test. **, $P < 0.01$, ***, $P < 0.001$. Sham, mice were sham-injected with the vehicle; Caff, mice received a single 7.5 mg/kg i.p. dose of caffeine.

in Cox1 band signal intensity compared with the sham-injected control mice. However, female mice showed no change in Cox1 expression 12 h after caffeine treatment. A similar qualitative comparison of Cox4 band signal intensities on Western blots showed that caffeine treatment of male mice increased Cox4 band signal intensity in caffeine-treated male mice compared with sham-injected control mice. Similar analyses of female mice showed that caffeine did not increase the intensity of the Cox4 band compared with sham-treated female control mice (Fig. 2A). However, similar to what was observed with Cox7c expression (see Fig. 1C), the intensities of Cox4 bands in caffeine-treated and sham-injected female mice were nearly identical to the those found in caffeine-treated male mice, and these were all more intense than the Cox4 band found in untreated male mice (Fig. 2A).

The Cox1 and Cox4 band signal intensities were then quantitated, and the absolute expression levels of Cox1 and Cox4 are shown in Fig. 2, B and C, respectively. These data show that caffeine treatment increases the striatal expression of Cox1 and Cox4 proteins by approximately 25% in male mice after either 4 or 12 h of caffeine treatment compared with sham-injected control mice. However, caffeine treatment did not increase the level of Cox1 or Cox4 expression in

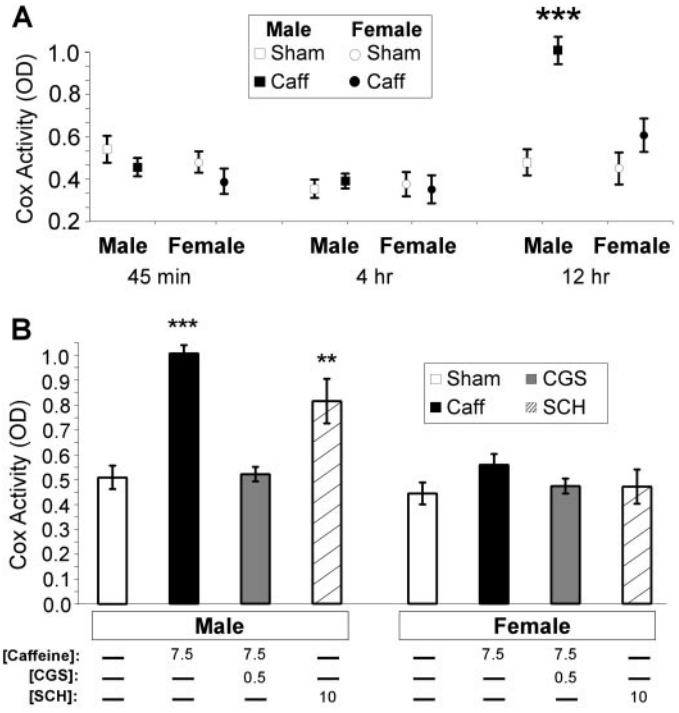

Fig. 3. Caffeine and SCH58261 increase Cox activity in the male striatum; CGS21680 counteracts the increase in Cox activity by caffeine. A, Cox activity levels (OD measurements) in caffeine-treated male and female mice versus injected control mice at 45 min, 4 h, and 12 h after drug treatment or sham injection. Mitochondrial fractions were prepared from the striata that were isolated from the same mice that were examined for Cox1 and Cox4 expression in the Western blot analyses shown in Fig. 2. Extracts were assayed for Cox activity using the DAB method in 96-well format (see *Materials and Methods*). B, Cox activity measurements (OD) were performed 12 h after injection of either male or female mice with either 7.5 mg/kg caffeine (black bars), 7.5 mg/kg caffeine, 0.5 mg/kg CGS21680 (gray bars), or 10 mg/kg SCH58261 (bars with the diagonal line). The values for striatal Cox activities in were derived from six independent animals. The values for Cox activities are expressed as the mean OD ± S.E.M. Statistical significance of data from comparisons between drug-treated mice and sham-injected control mice were derived using a two-tailed Student's t test. **, $P < 0.01$; ***, $P < 0.001$.

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the striatum of female mice at these time points compared with the sham-injected control mice. As suggested by the qualitative assessment of Cox1 and Cox4 band intensities on Western blots (Fig. 2A), quantitative analyses of Cox1 (Fig. 2B) and Cox4 (Fig. 2C) expression indicated that Cox1 expression levels in sham-injected male mice, sham-injected female mice, and in caffeine-treated female mice are roughly equivalent and lower than levels of Cox1 expression in caffeine-treated male mice at all of time points examined. This indicates that up-regulation of Cox1 protein expression by caffeine in male mice may occur by a mechanism that increases Cox4 expression from a baseline level that is common to both male and female mice. However, Cox4 expression levels in caffeine-treated male mice, sham-injected female mice, and caffeine-treated female mice are approximately equivalent and higher than levels of Cox4 expression in sham-injected male mice at 4 h and 12 h after treatment. These data indicate that unlike Cox1 (but similar to Cox7c), up-regulation of Cox4 protein expression in caffeine-treated male mice may occur by a mechanism in which caffeine de-represses Cox4 expression, raising it to a level that is comparable with that found in the striatum of female mice.

Caffeine Increases Cox Activity in the Male Striatum via Blockade of the A2A Receptor. We investigated whether the male-specific increases in the expression of Cox subunits by caffeine were accompanied by a corresponding sexually dimorphic regulation of Cox enzymatic activity itself. In these experiments, the three groups of 24 mice assayed for Cox1 and Cox4 expression in Western blotting experiments (see Fig. 2) were assayed for Cox activity at 45 min, 4 h, and 12 h after treatment, using a modification of the 3,3′-diaminobenzidene-tetrahydrochloride (DAB) method (Chrzanowska-Lightowlers et al., 1993).

As shown in Fig. 3A, the levels of Cox activity in sham-injected male mice, sham-injected female mice, and caffeine-treated female mice were not statistically different over all three of the time points analyzed. However, at 12 h, male mice showed a 2-fold increase in Cox activity, whereas female mice showed only a slight and statistically nonsignificant increase in Cox activity (Fig. 3A). To determine whether the increase in Cox activity by caffeine could be attributed to the blockade of the A2A receptor, we examined whether the induction of Cox activity in the striatum of male mice by caffeine could be counteracted by coadministration of the A2AR-specific antagonist CGS21680. As shown in Fig. 3B, whereas treatment of male mice with a 7.5 mg/kg dose of caffeine led to 2-fold increase in Cox activity 12 h after injection, simultaneous treatment of male mice with 7.5 mg/kg caffeine and 0.5 mg/kg CGS21680 showed no increase in the level of Cox activity compared with sham-injected male mice. These data suggest that caffeine increases Cox activity via blockade of the A2A receptor, because CGS21680 can counteract this effect. To provide more direct evidence that blockade of A2AR in the striatum increases Cox activity in a sexually dimorphic manner, we compared levels of Cox activity in male and female mice 12 h after injection of the A2AR-specific antagonist SCH58261. As shown in Fig. 3B, treatment of male mice with 10 mg/kg SCH58261 resulted in a 1.6-fold increase in Cox activity in the male striatum compared with sham-injected control mice. In contrast, no significant changes in Cox activity were observed in the striatum of female mice that were treated with the drug.

Up-Regulation of Cox7c, Cox1, and Cox4 Expression by Caffeine Occurs by Blockade of the A2A Receptor. Because we showed that stimulation of Cox activity in the male striatum occurred via blockade of the A2A receptor (Fig. 3), we then examined whether the up-regulation of Cox7c, Cox1, and Cox7c protein expression by caffeine could be counteracted by cotreatment with CGS21680 and whether up-regulation of these Cox proteins could be reproduced using the A2AR-specific antagonist SCH58261.

For these experiments, four groups of six male mice were either treated with vehicle (sham-injected), or injected with either 7.5 mg/kg caffeine, 7.5 mg/kg caffeine, and 0.5 mg/kg CGS21680, or 10 mg/kg SHC58261. After 12 h, mice were sacrificed, striata were harvested, mitochondrial extracts were prepared, applied to Q10 protein chips, and analyzed for Cox7c, Cox1, and Cox4 protein peak expression using SELDI mass spectroscopy.

As expected, caffeine treatment led to an increase in the levels of Cox7c, Cox1, and Cox4 protein expression. Coadministration of caffeine and CGS21680 resulted in no significant changes in the expression levels of Cox1 and Cox4 (Figs. 4, B and C), although it led to a significant decrease in Cox7c expression (Fig. 4A). These data indicate that CGS21680 counteracts the stimulation of Cox protein expression by caffeine by relieving the blockade of the A2A receptor. The striatal expression of Cox7c, Cox1, and Cox4 proteins was also stimulated by treatment of male mice with the A2AR-specific antagonist SCH58261. Expression of these proteins increased 2.2-, 3.2-, and 1.33-fold, respectively (Fig. 4). Overall, these data indicate that A2AR antagonists stimulate Cox protein expression, whereas A2AR agonists counteract this stimulation and can even repress Cox expression.

Caffeine Increases and Cox7c siRNA Treatment Decreases Cox Activity in PC-12 Cells. PC-12 cells express the A2AR (Stonehouse et al., 2003) and thus enabled us to study the mechanisms underlying the regulation of Cox7c expression by caffeine in vitro First, we examined doses of caffeine between 10 and 250 μM for their ability to affect Cox activity in PC-12 cells after 12 h. These caffeine concentrations in media are physiologically relevant in that they are approximately equivalent to doses between 2.5 and 50 mg/kg in the animal. As shown in Fig. 5A, a dose of as little as 10 μM caffeine produced a statistically significant increase in Cox activity (25%), and higher doses (between 20 and 250 μM) showed more significant elevations in Cox activity (between 50 and 200%).

We then tested whether blunting the expression of Cox7c mRNA with siRNA was sufficient to reduce Cox activity, and whether caffeine could restore Cox activity levels in siRNA-treated cells. For these experiments, two different siRNAs were synthesized. The siRNA designated "Cox7c" was a double-stranded RNA complementary to bases 184 to 208 of the rat Cox7c coding region. The siRNA designated "control" was a double-stranded RNA derived from the same region of the rat Cox7c gene; however, it contained nine mismatches that prevented complementary base pairing with Cox7c mRNA.

PC-12 cells were transfected with 1 μg of either control or Cox7c siRNA for 3 h. Fresh media were applied, cells were incubated for an additional 12 h, harvested, and examined for levels of Cox7c mRNA expression using RT-PCR. These experiments showed that treatment with Cox7c siRNA reduced Cox7c mRNA expression by 38% versus cells that were

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transfected with the control siRNA (Fig. 5B). To determine whether caffeine could counteract the reduction in Cox7c mRNA expression in Cox7c siRNA-treated cells, PC-12 cells were transfected with 1 μg of Cox7c mRNA for 3 h. Fresh media with 250 μM caffeine were then applied and cells were incubated for an additional 12 h, harvested, and examined for levels of Cox7c mRNA expression using RT-PCR. As shown in Fig. 5B, caffeine treatment resulted in a significant increase (1.4-fold) in Cox7c RNA expression compared with PC-12 cells that were treated with the Cox7c siRNA alone. The level of Cox7c mRNA expression in caffeine- and siRNA-treated cells was nearly equivalent to the level of Cox7c mRNA expression that was found in untreated PC-12 cells, indicating that caffeine, by up-regulating Cox7c mRNA expression counteracts the blunting of Cox7c expression by siRNA treatment. Next, we tested whether treatment of PC-12 cells with Cox7c siRNA resulted in decreased Cox activity and whether caffeine could reverse this effect. As shown in Fig. 5C, PC-12 cells treated with the Cox7c siRNA showed 45% less Cox activity than cells that were treated with the control siRNA. In addition, we observed that 12 h of caffeine treatment after the Cox7c siRNA treatment restored Cox activity to the level observed in PC-12 cells that had been treated with the control siRNA (Fig. 5C), indicating that caffeine, by up-regulating Cox7c mRNA, counteracted the inhibition of Cox activity produced by siRNA knockdown.

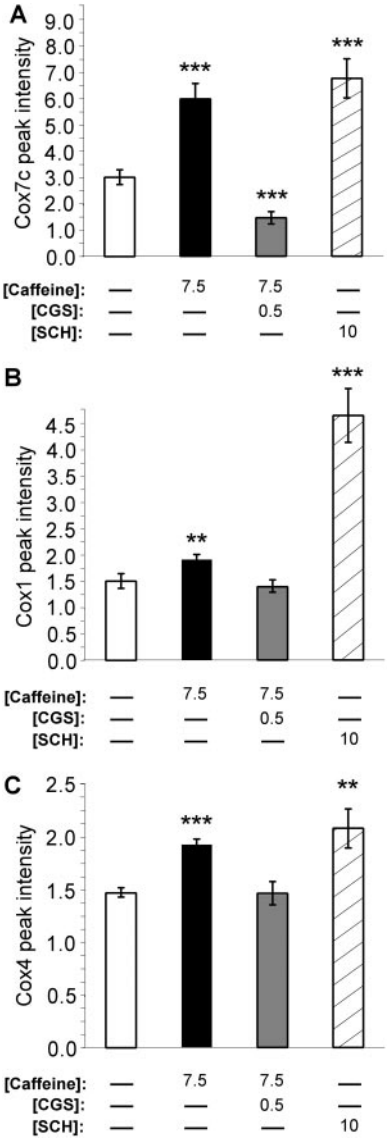

Fig. 4. Caffeine and SCH58261 increase the expression of Cox7c, Cox1, and Cox4 in the male striatum; CGS21680 counteracts the increase in Cox expression by caffeine. Quantitative SELDI analyses of the protein peak intensities (expression levels) of Cox7c (A), Cox1 (B), and Cox4 (C). The intensities of peaks of Cox7c, Cox1, and Cox4 were measured on protein chips from mitochondrial extracts of striatal proteins prepared from male mice 12 h after injection of either vehicle (sham-injected, indicated by open bars), 7.5 mg/kg caffeine (indicated by black bars), 7.5 mg/kg caffeine and 0.5 mg/kg CGS21680 (indicated by gray bars), or 10 mg/kg SCH58261 (indicated by bars with diagonal lines). The values of Cox7c protein peak intensities were derived from protein extracts prepared from six different male mice for each of the four drug treatments shown in the figures. Expression was quantified using SELDI analyses of Q10 protein chips (see *Materials and Methods*). Results are expressed as mean protein peak intensity ± S.E.M. The two-tailed Student's *t* test was used to assess the differences in expression between samples from drug-treated and sham-injected animals. ***, $P < 0.001$; **, $P < 0.01$.

Expression of Cox7c mRNA in the Striatum and in PC-12 Cells Is Increased by Caffeine and Decreased by Treatment with the A2AR Agonist CGS21680. To investigate the mechanism underlying the stimulation of Cox7c expression by caffeine, real-time PCR was used to examine levels of Cox7c mRNA expression in the male striatum (Fig. 6A) and in PC-12 cells (Fig. 4B) after caffeine treatment (see *Materials and Methods*). In these experiments, regulation of Cox7c by CGS21680, an agonist of the adenosine 2A receptor (A2AR), was also examined. As shown in Fig. 6A, a single injection of male mice with caffeine (7.5 mg/kg, i.p.) resulted in a 2-fold increase in Cox7c mRNA expression 12 h later compared with male mice that had received a sham injection. Conversely, male mice injected with CGS21680 (0.5 mg/kg, i.p.) showed a 55% decrease in Cox7c mRNA expression compared with sham-injected control mice. PC-12 cells were exposed to caffeine (250 μM) or CGS21680 (10 μM) for 4, 8, and 24 h and then examined for Cox7c mRNA expression (Fig. 6B). Caffeine treatment of PC-12 cells increased Cox7c mRNA expression between 25% and 90% over a period from 4 to 24 h, whereas treatment of PC-12 cells with CGS21680 led to a 30% decrease in Cox7c mRNA expression over the same time (Fig. 6B). These results indicate that antagonists and agonists of A2AR differentially regulate the expression of Cox7c mRNA expression in PC-12 cells.

Cox7c mRNA Expression Is Regulated by Caffeine and CGS21680 at the Level of Transcription via a 167-Base Pair Region of the Cox7c Promoter. To investigate whether the increase in Cox7 mRNA expression by caffeine and CGS21680 occurs at the level of transcription, two luciferase reporter constructs containing either 2065 or 93 base pairs of DNA from the promoter region and 74 base pairs of the first exon of the mouse Cox7c gene were prepared and examined for their response to 250 μM caffeine and 10 μM CGS21680 at different time points after transfection into PC-12 cells. As shown in Fig. 7A, the luciferase activities produced by these reporters (designated −2065/+74 and −93/+74) showed nearly identical time-dependent increases in response to caffeine. Caffeine treatment resulted in a 2-fold increase in luciferase activities produced by both reporters between 4 and 8 h, and showed maximal activities at 12 h that had not decreased by 24 h. This pattern of Cox7c promoter activation and Cox7c mRNA expression by caffeine

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were very similar (compare data in Fig. 7A with those in Fig. 6B).

We then examined the effect of different doses of caffeine and CGS21680 on the activity of the −93/+74 Cox7c reporter after transfection into PC-12 cells. As shown in Fig. 7B, the minimal dose required for a 50% increase in activity of the −93/+74 Cox7c reporter in PC-12 cells over a 24 h period was approximately 50 μM, and the dose yielding a maximal increase in Cox7c promoter activity (2.7-fold) was 250 μM. In contrast, treatment of PC-12 cells with 0.01, 0.1, and 1 μM CGS21680 resulted in a consistent reduction in the level of Cox7c promoter activity (approximately 30%) at 24 h.

Discussion

The adenosine antagonist caffeine facilitates voluntary movement by altering the physiology of neurons in the striatum of the basal ganglia. Caffeine consumption is also known to lower the incidence of Parkinson's disease (PD) in male mice, and A2AR-specific antagonists such as SCH58261 ameliorate the motor symptoms of PD. We therefore set out to identify genes whose expression in the striatum is regulated by caffeine in a sexually dimorphic manner to reveal novel targets for the action of caffeine.

We found that Cox7c, a nuclear-encoded subunit of cytochrome oxidase, was up-regulated in the striatum of male mice, but not in female mice by caffeine. Cox is an enzyme of the mitochondrial electron transport chain that regulates oxidative metabolism. It is composed of 13 different protein subunits, 10 that are encoded by the nuclear genome, and three that are encoded by the mitochondrial genome (Merle and Kadenbach, 1980; Ludwig et al., 2001).

Caffeine also increased the expression of two other Cox subunits: Cox1, the mitochondrial-encoded catalytic subunit and Cox4, a nuclear-encoded subunit that is the target of allosteric modulation of Cox activity by ATP (Bender and Kadenbach, 2000). Although caffeine increased expression of Cox1, Cox4, and Cox7c, the mechanisms regulating the expression of mitochondrially encoded (such as Cox1) and nuclear-encoded (such as Cox4 and Cox7c) subunits seem to be different. Up-regulation of Cox1 expression by caffeine is straightforward in that the drug-increased expression of Cox1 in male mice and not in female mice, to a level that was above baseline levels of Cox1 expression, which was the same in both male and female mice. However, levels of Cox4 and Cox7c expression were lower in male versus female mice. Therefore, caffeine seemed to de-repress the expression of Cox4 and Cox7c to levels normally observed in untreated female mice. It will be of interest in future experiments to determine whether expression of all 13 Cox subunits is coordinately up-regulated by caffeine in a sexually dimorphic manner and whether there are distinct mechanisms for up-regulating mitochondrial- and nuclear-encoded Cox subunits in response to caffeine.

Consistent with the finding that caffeine stimulates Cox expression, we found that caffeine increased Cox activity in the striatum of male mice. This regulation was dependent on signaling via A2AR, inasmuch as cotreatment with the A2AR agonist CGS21680 counteracted the increase in Cox expression and activity by caffeine. Male-specific increases in the expression of Cox1, Cox4, and Cox7c and Cox activity itself were also reproduced using the A2AR-specific antagonist SCH58261. Overall, these findings indicate that stimulation of Cox expression and activity by caffeine is mediated by the A2AR.

Neurons require large resources of ATP to carry out energetic processes such as membrane depolarization, firing of action potentials, and release of neurotransmitters. It is therefore no surprise that increased Cox expression and activity have been correlated with increased levels of neuronal activity (Hevner and Wong-Riley, 1993; Nie and Wong-Riley, 1996). Conversely, agents that block neuronal activity such as tetrodotoxin decrease Cox expression (Wong-Riley et al., 2001). In previous work, we found that caffeine increased the firing of striatal neurons within minutes (Stonehouse et al., 2003). However, results from the present study show that Cox activity is stimulated 12 h after caffeine treat-

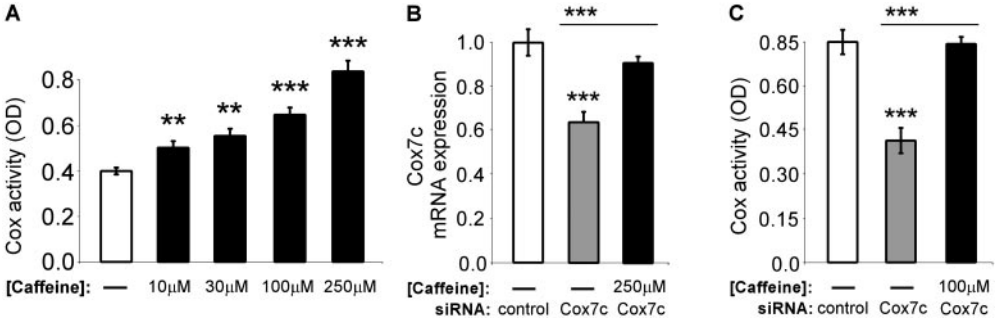

Fig. 5. Caffeine increases Cox activity in PC-12 cells; treatment with Cox7c siRNA decreases cytochrome oxidase activity. A, caffeine treatment of PC-12 cells results in a dose-dependent increase in Cox activity. Protein lysates were prepared from untreated rat PC-12 cells or from PC-12 cells that were treated for 12 h with 10, 30, 100, or 250 μM caffeine. Cells were harvested, normalized for protein content, and assayed for Cox activity using the DAB method (see *Materials and Methods*). Histograms corresponding to caffeine-treated samples appear in black. B, siRNA treatment inhibits Cox7c mRNA expression. PC-12 cells were transfected with either the control siRNA or the Cox7c siRNA. After 3 h, media were changed, and cells were incubated for an additional 12 h and then harvested. RNA was prepared and then analyzed for Cox7c mRNA expression using quantitative real-time PCR (see *Materials and Methods*). Histograms corresponding to values obtained in experiments involving the Cox7c siRNA are shown in gray. C, siRNA treatment inhibits Cox activity; caffeine treatment counteracts this effect. The levels of Cox activity were determined in PC-12 cells in response to siRNA treatment and to caffeine. PC-12 cells were transfected with either the control siRNA or the Cox7c siRNA. After 3 h, cells were incubated in fresh media without caffeine (−) or in media containing 100 μM caffeine for 12 h. Cells were then harvested, normalized for protein content, and assayed for Cox activity. The values for Cox activities in PC-12 cells were derived from three experiments, each performed in triplicate. For all panels, the values are expressed as the mean OD ± S.E.M. Statistical significance of data from comparisons between caffeine-treated mice and sham-injected control mice, between PC-12 cells treated with different doses of caffeine versus control PC-12 cell cultures, and between PC-12 cells treated with the Cox7c siRNA versus the SiRNA control were all derived using a two-tailed Student's *t* test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

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ment. This suggests that up-regulation of Cox activity by caffeine represents a homeostatic response to the drug, inasmuch as elevation of Cox activity might be required for neurons to replenish ATP after sustained periods of membrane depolarization.

To study the effects of caffeine and siRNA treatment on Cox activity in vitro, we performed experiments in PC-12 cells. Caffeine increased Cox activity in a dose-dependent manner. Moreover, blunting Cox7c expression by siRNA treatment inhibited Cox activity, indicating that disruption of the expression of a single nuclear-encoded subunit of Cox is sufficient to decrease Cox activity. It will be revealing to determine whether viral delivery of Cox7c siRNA reduces oxidative metabolism of certain brain regions. Such perturbations may provide a selective means to down-regulate neuronal activity in the striatum.

To further elucidate the mechanism underlying Cox7c gene regulation by caffeine, we examined Cox7c mRNA expression and the activities of Cox7c promoter constructs. In the male striatum and in PC-12 cells, Cox7c mRNA expression was up-regulated by caffeine and down-regulated by the A2AR agonist CGS21680. These results suggest a bidirectional regulation of Cox7c expression by drugs that affect signaling via A2AR, and that this mechanism occurs at the level of transcription. This was confirmed when we found that a segment of the mouse Cox7c promoter (−93/+74) was sufficient for activation of Cox7c expression by caffeine and for repression by the A2AR agonist CGS21680.

In light of these findings, we propose that up-regulation of Cox7c involves a mechanism in which caffeine, by blocking A2AR signaling, de-represses Cox7c gene expression. Activation of A2AR signaling by CGS21680 is likely to stimulate the

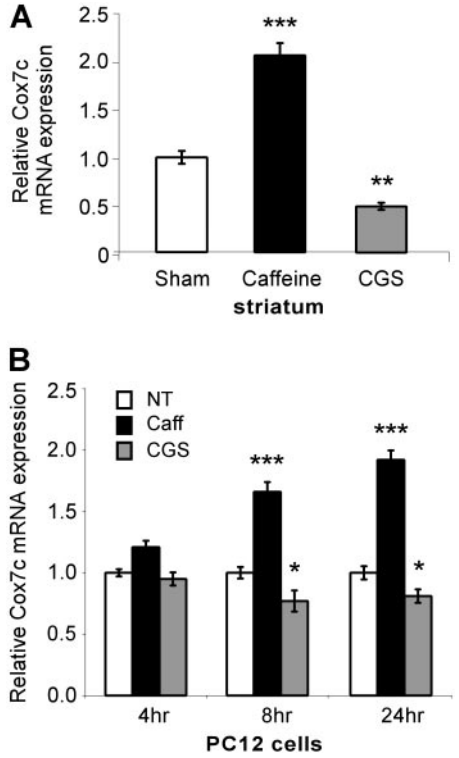

Fig. 6. Expression of Cox7c mRNA in the striatum and in PC-12 cells is increased by caffeine and decreased by CGS21680 treatment. A, levels of Cox7c mRNA expression were determined using quantitative RT-PCR from striatal RNA extracted from either sham-injected, caffeine-treated (7.5 mg/kg), or CGS21680-treated (0.5 mg/kg) male mice 12 h after treatment. The values for Cox7c mRNA expression in the striatum were derived from six different animals for each drug treatment analyzed. B, regulation of Cox7c mRNA expression in PC-12 cells by caffeine and CGS21680. Rat PC-12 cells were either left untreated or treated with 250 μM caffeine or 10 μM CGS21680 for 4, 8, or 24 h. Cells were then harvested, and RNA extracted from these cells was analyzed for Cox7c mRNA expression using quantitative PCR. The values for Cox7c mRNA expression in the mouse striatum and in PC-12 cells were derived from at least three independent experiments. To obtain accurate values for levels of Cox7c mRNA expression, nine separate PCR reactions were performed on each of the cDNA samples that were prepared from mouse striatum as well as from PC-12 cells. The relative expression levels of Cox7c mRNA are shown as a mean ± S.E.M. Histograms corresponding to mRNA expression values obtained in experiments involving caffeine and CGS21680 are indicated in black and gray, respectively. Comparisons of the values for Cox7c mRNA were performed between drug-treated and untreated samples using the two-tailed Student's t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Caff, caffeine-treated; CGS, CGS21680-treated.

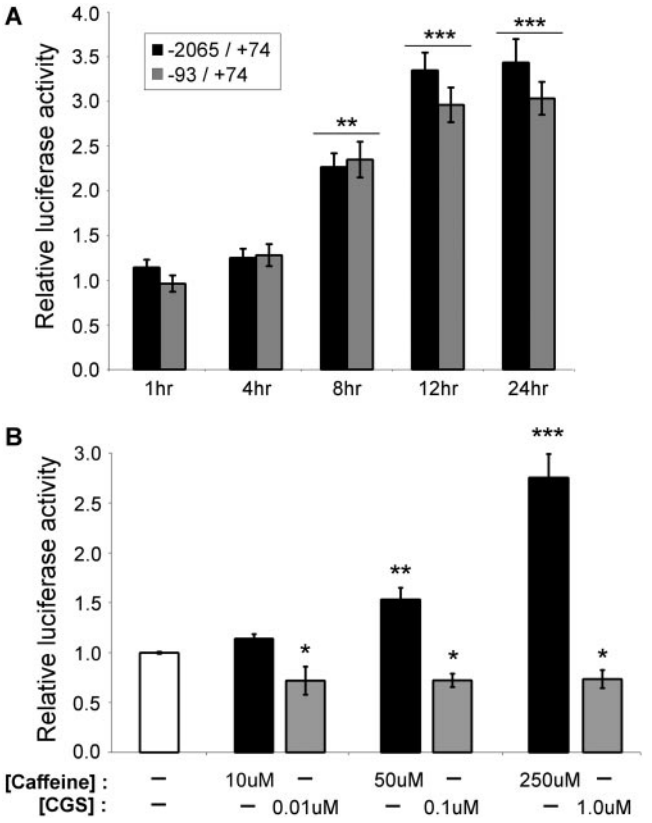

Fig. 7. Regulation of Cox7c promoter activity in PC-12 cells in response to caffeine or CGS21680. A, caffeine treatment increased expression of Cox7c promoter/luciferase reporter constructs in PC-12 cells. Two Cox7c promoter/luciferase reporters were made containing either 2065 or 93 base pairs of DNA from the promoter region and 74 base pairs of the first exon from the mouse Cox7c gene (designated −2065/+74 and −93/+74). PC-12 cells were transfected with 1 μg each reporter along with 100 ng of the plasmid CMV-β, and treated with 250 μM caffeine for either 1, 4, 8, 12, or 24 h. Cells were harvested, extracts were prepared, normalized for β-galactosidase activity and then assayed for luciferase activity. B, Luciferase activity produced by the −93/+74 reporter construct is increased by treatment with caffeine (indicated by the black histograms), and decreased by treatment with CGS21680 indicated by the gray histograms). A Cox7c promoter/luciferase construct (−93/+74) was transfected into PC-12 cells for 24 h, followed by a 24-h treatment with caffeine or CGS21680 at the indicated doses. Cells were then examined for luciferase activity as described in A. Values for luciferase activities are the mean ± S.E.M. and are derived from at least four independent experiments, performed in triplicate. Comparisons of values of luciferase activities from drug-treated samples versus untreated controls were performed and the statistical significance of these comparisons was evaluated using the two-tailed Student's t test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. CGS, CGS21680-treated.

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activity of protein kinases (protein kinase A or mitogen-activated protein kinase), which may increase the binding of nuclear factors that repress Cox7c promoter activity. Antagonists of A2AR signaling such as caffeine would block this effect, thereby relieving the repression of Cox7c transcription.

Which transcription factors might participate in such a mechanism? Previous studies have implicated two binding sites for the transcription factor YY1 in the regulation of the bovine Cox7c promoter (Seelan and Grossman, 1997); both of these YY1 sites are present in the region of the Cox7c promoter that responds to caffeine. YY1 acts as both a repressor and an activator of transcription, depending on the context (He and Casaccia-Bonnefil, 2008). YY1 binding sites are also found in several other genes that encode Cox subunits (Grossman et al., 1998). YY1 is therefore an attractive candidate for mediating repression of Cox7 promoter activity in response to A2AR signaling and its derepression by caffeine. In support of this idea, YY1 and its binding site in the c-*fos* promoter have been shown to repress c-*fos* gene expression via direct interaction with activating transcription factor/cAMP response element-binding proteins, which bind to an adjacent cAMP response element (Zhou et al., 1995). YY1 is also known to mediate glutamate-dependent repression of the EAAT1 promoter (Rosas et al., 2007).

The caffeine-responsive region of the Cox7c promoter also contains a binding site for NRF2, a transcriptional activator that regulates several genes that control oxidative metabolism. NRF-2 has been proposed to be a master regulator of genes encoding Cox subunits (Verbasius et al., 1993), and binding sites for NRF-2 have been identified in all 10 genes encoding the nuclear-encoded Cox subunits (Ongwijitwat and Wong-Riley, 2005). The observation that caffeine regulates transcription of Cox7c via a promoter region containing two of the DNA elements known to regulate the expression of multiple Cox subunits suggests that caffeine may coordinately regulate the expression of several genes encoding Cox subunits.

How is up-regulation of Cox expression and activity related to the neuroprotective and therapeutic properties of caffeine in treating PD? It has been proposed that in the striatum, A2AR-specific antagonists reduce the hyperactivity of striatopallidal neurons that occurs in PD (Kase, 2003). A2ARs are expressed on axon collaterals of striatopallidal neurons that release GABA, as well as on axons of GABAergic interneurons of the striatum. A2AR signaling in these neurons normally suppresses GABA release, resulting in an increase in the overall excitability of striatopallidal neurons. It is believed that A2AR antagonists, by increasing GABA release, may decrease the excitability of striatopallidal neurons (Kase, 2003; Schiffmann et al., 2003).

Alternatively, caffeine's neuroprotective properties may be attributed to its antagonism of A2AR on the presynaptic terminals of dopamine neurons in the striatum. Parkinson's disease is caused by exposure to toxins, some of which target elements of the electron transport chain. It is therefore tenable that up-regulation of Cox activity by caffeine increases energy metabolism in the axon terminals of dopamine neurons, thereby protecting them from oxidative damage from such toxins. In support of this mechanism, caffeine has been shown to stimulate the opening of Kir6.2/SUR1 potassium channels (Mao et al., 2007), which has been shown to prevent neurons from depolarizing and releasing toxic levels of neurotransmitters (Avshalumov et al., 2005; Xu et al., 2006). By altering neuronal metabolism and enhancing the activity of potassium channels within the terminals of dopamine neurons in the striatum, caffeine may inhibit the excitotoxicity of these neurons, protecting them from degeneration.

Up-regulation of Cox activity by caffeine may be only one of several sexually dimorphic mechanisms that protect the brain from PD. Estrogen is known to protect the female brain from dopamine depletion in a rodent model of PD. However, caffeine is neuroprotective for adult male mice, ovariectomized female mice, and retired breeder female mice (all of which have low levels of estrogen), and adding estrogen to these mice interferes with caffeine's neuroprotective properties (Xu et al., 2002, 2006). In this light, it will be revealing in future experiments to examine the influence of sex hormones on the activity of Cox and other enzymes of the mitochondrial electron transport chain. Overall, caffeine and other drugs that modulate the neurophysiology of basal ganglia will be useful tools to discover other gender-specific differences in neuronal biochemistry, particularly those that suggest novel routes toward ameliorating the symptoms and preventing the progression of PD.

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