The oxysterol 27-hydroxycholesterol regulates α-synuclein and tyrosine hydroxylase expression levels in human neuroblastoma cells through modulation of liver X receptors and estrogen receptors-Relevance to Parkinson’s disease

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Abstract
Loss of dopaminergic neurons and α-synuclein accumulation are the two major pathological hallmarks of Parkinson’s disease (PD). Currently, the mechanisms governing depletion of dopamine content and α-synuclein accumulation are not well understood. We showed that the oxysterol 27-hydroxycholesterol (27-OHC) reduces the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis, and increases α-synuclein levels in SH-SY5Y cells. However, the cellular mechanisms involved in 27-OHC effects were not elucidated. Here, we demonstrate that 27-OHC regulates TH and α-synuclein expression levels through the estrogen receptors (ER) and liver X receptors (LXR). We specifically show that inhibition of ERβ mediates 27-OHC-induced decrease in TH expression, an effect reversed by the ER agonist estradiol. We also show that 27-OHC and the LXR agonist GW3965 increase α-synuclein while the LXR antagonist ECHS significantly attenuated the 27-OHC-induced increase in α-synuclein expression. We further demonstrate that LXRβ positively regulates α-synuclein expression and 27-OHC increases LXRβ-mediated α-synuclein transcription. Our results demonstrate the involvement of two distinct pathways that are involved in the 27-OHC regulation of TH and α-synuclein levels. Concomitant activation of ERβ and inhibition of LXRβ prevent 27-OHC effects and may therefore reduce the progression of PD by precluding TH reduction and α-synuclein accumulation.

Keywords
Parkinson’s disease; α-synuclein; Tyrosine Hydroxylase; estrogen receptors; Liver X receptor; 27-hydroxycholesterol

Introduction
Parkinson disease (PD) is neuropathologically characterized by loss of dopaminergic neurons and the presence of intracellular inclusion known as Lewy bodies. Reduction in levels of tyrosine hydroxylase (TH), the rate limiting enzyme in dopamine biosynthesis, and the accumulation of α-synuclein protein, the major constituent of Lewy body inclusions, are a consistent observation in PD brains (Spillantini et al. 1997; Crowther et al. 2000). Several
animal and *in vitro* biochemical studies have also established a causal role of α-synuclein in dopaminergic cell loss (Zhou et al. 2000; Xu et al. 2002; Zhou et al. 2002; Zhou & Freed, 2005) as well as decreased TH expression (Yu et al. 2004; Gao et al. 2007) and activity (Perez et al. 2002; Peng et al. 2005). The causes of PD are likely multi-factorial with several factors including environmental agents and genetic susceptibility participating in the pathogenesis of the disease. A recent large prospective study has suggested that total serum cholesterol at baseline is associated with an increased risk of PD (Hu et al. 2008). In other studies however, elevated serum levels of cholesterol were either not associated with PD risk (de Lau et al. 2005) or were related to a decreased PD risk (Scigliano et al. 2006; Powers et al. 2009). The discrepancies in these studies may be explained by the following: *First*, it may be possible that abnormalities in cholesterol metabolism take part in PD pathogenesis at mid-age (at late-age, when PD progresses, the correlation between PD and cholesterol levels is no longer consistent). *Second*, fluctuations in the cholesterol oxidation products (oxysterols) but not in cholesterol *per se* may correlate better with the onset of PD. 27-OHC, the major oxidized cholesterol metabolite in the circulation, has the ability to cross into and out of the brain (Lutjohann et al. 1996; Bjorkhem et al. 2002). 27-OHC, the major cholesterol metabolite in the circulation, is synthesized by almost all peripheral cells from cholesterol by the enzyme CYP27A1. 27-OHC is an endogenous ligand of the Liver X receptors α/β (LXRA/β) and therefore may possess essential physiological function in the brain such as regulation of cholesterol biosynthesis, modulation of brain lipid metabolism and inflammatory signaling pathways. The daily influx of 27-OHC from the peripheral circulation into the brain is ~5mg (Heverin et al., 2005). While there exists a normal physiological flux of 27-OHC into the brain, increased influx due to hypercholesterolemia or a lack of blood-brain-barrier integrity may have widespread pathological ramifications. 27-OHC and other oxysterols are widely implicated in the pathogenesis of a multitude of neurodegenerative diseases including Alzheimer’s disease, Huntington disease and multiple sclerosis (Leoni & Caccia, 2011). A recent study showed increased levels of several oxysterols including 27-OHC in cortex of PD brains (Cheng et al. 2011), suggesting a potential role of cholesterol homeostasis in PD.

We have recently shown that the oxysterol 27-hydroxycholesterol (27-OHC) can simultaneously increase levels of α-synuclein and reduce TH availability for dopamine synthesis in human neuroblastoma cells (Rantham Prabhakara et al. 2008). Although, a direct causal role of increased oxysterol levels in PD is still to be demonstrated, our studies are of extreme relevance to identifying cellular mechanisms that regulate expression levels of TH and α-synuclein that play a key role in the pathogenesis of PD. Identifying signaling pathways that modulate levels of these two proteins is important to better understand the pathophysiology of PD. Emerging data suggests that 27-OHC exhibits selective estrogen receptor modulator (SERM) properties (Umetani et al. 2007; DuSell et al. 2008). Estradiol, the most prominent endogenous estrogen and an agonist of the estrogen receptors (ERs), has been shown to regulate TH expression *in vivo* (Thanky et al. 2002) and *in vitro* (Maharjan et al. 2005; Maharjān et al. 2010). However, the extent to which 27-OHC can regulate TH levels by modulating ER has not been determined. With regard to α-synuclein, although the signal transduction mechanisms involved in α-synuclein expression are not well characterized, involvement of LXR signaling pathway has been recently suggested in the regulation of α-synuclein expression (Cheng et al. 2008). The results by Cheng and colleagues are in accordance with our published data showing that 27-OHC, which is a ligand of LXR, increases α-synuclein levels. However, it is not clear whether LXRA or LXRB is involved in the regulation of α-synuclein expression. Here we explore the role of ERα/β and LXR α/β in the regulation of TH and α-synuclein expression, and characterize the extent to which the oxysterol 27-OHC utilizes these pathways to modulate expression levels of these two proteins.
Materials and methods

Materials

27-OHC was purchased from Medical Isotopes (Pelham, NH). Estradiol (E2) and the LXR agonist GW3965 were purchased from Sigma Aldrich (Saint Louis, MO). The LXR antagonist 5α-6α-epoxycholesterol-3-sulfate (ECHS) was purchased from Steraloids Inc. (Newport, RI). ERE-luciferase and LXRE-luciferase DNA constructs were purchased from SA Biosciences (Frederick, MD). The human TH-Luciferase promoter construct was purchased from SwitchGear Genomics (Menlo Park, CA). All cell culture reagents, with the exception of fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA) and antibiotic/antimycotic mix (Sigma Aldrich, Saint Louis, MO) were purchased from Invitrogen (Carlsbad, CA). Human SH-SY5Y neuroblastoma cells were purchased from ATCC (Manassas, VA).

Cell Culture and Treatments

SH-SY5Y cells were grown in DMEM/F12 medium containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic mix. Cells were maintained at 37°C in a saturated humidity atmosphere containing 95% air and 5% CO2. After reaching 80% confluence, cells were incubated with vehicle (control), 10μM 27-OHC, 1nM estradiol, 10μM 27-OHC + 1nM estradiol, 10μM GW3965, 10μM 27-OHC + 10μM GW3965, 10μM ECHS, and 10μM 27-OHC + 10μM ECHS, for 24 hours in cell medium.

siRNA for ERβ and LXRβ and the respective scrambled non-silencing control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The transfection of siRNA was performed in the cells with siRNA transfection reagent (Santa Cruz Biotechnology) and siRNA transfection medium (Santa Cruz Biotechnology) according to the manufacturer’s recommendation. The siRNAs stock solution (10μM) was prepared by dissolving 3 nmol of siRNAs in 330μL of RNAse free water. The 10μM siRNA stock solution was further diluted 1:10 using transfection reagent and subsequently 1:100 in transfection medium following manufacturer’s protocol to yield a final concentration of 10nM. The cells were transfected for 16 hours followed by 24 hour incubation in normal media before being subjected to respective treatments.

To overexpress ERβ in SH-SY5Y neuroblastoma cell line, cells grown to 70% confluence were transfected with Adenoviral Vector containing ERβ expression cassette (Ad-CMV-C/EBPα) driven by the CMV promoter, custom designed by Vector Labs (Philadelphia, PA). The Ad-CMV-GFP vector system (empty vector) was used as a control vector as well as to determine transfection efficiency. Cells were grown to 80% confluence in 6-well plates and transfected with 2×10⁵ viral particles (PFU)/ml of media with either Ad-CMV-ERβ expression cassette or the Ad-CMV-GFP empty vector. The cells were transfected for 16 hours followed by 24 hour incubation in normal media before being subjected to respective treatments.

Western blot analysis

Treated SH-SY5Y cells were washed with PBS, trypsinized and centrifuged at 5000g. The pellet was washed again with PBS and homogenized in NE-PER tissue protein extraction reagent (Thermo Scientific, Rockford, IL) supplemented with protease and phosphatase inhibitors. Protein concentrations from the cytosolic and nuclear homogenates were determined with BCA protein assay. Proteins (10 μg) were separated on SDS-PAGE gels, transferred to a polyvinylidene difluoride membrane (BioRad, Hercules, CA), and incubated with the following monoclonal antibodies: anti-TH mouse antibody (1:1000; Sigma Aldrich, Saint Louis, MO), anti-α-synuclein mouse antibody (1:500; Chemicon, Temecula, CA), anti-
ERα rabbit antibody (1:500; Abcam, Cambridge, MA), anti-ERβ rabbit antibody (1:200; Upstate, Lake Placid, NY), anti-LXRα rabbit antibody (1:500; Abcam, Cambridge, MA), and anti-LXRβ mouse antibody (1:500; Abcam, Cambridge, MA). β-actin and Lamin A were used as a gel loading control for cytosolic homogenates and nuclear homogenates respectively. The blots were developed with enhanced chemiluminescence (Immun-star HRP chemiluminescent kit, Bio-Rad, Hercules, CA). Bands were visualized on a polyvinylidene difluoride membrane and analyzed by LabWorks 4.5 software on a UVP Bioimaging System (Upland, CA). Quantification of results was performed by densitometry and the results analyzed as total integrated densitometric values (arbitrary units).

Quantitative Real time RT-PCR analysis

Total RNA was isolated and extracted from treated cells using the 5 prime “PerfectPure RNA tissue kit” (5 Prime, Inc., Gaithersburg, MD). RNA estimation was performed using “Quant-iT RNA Assay Kit” using a Qubit fluorometer according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). cDNA was synthesized by reverse transcribing 1 μg of extracted RNA using an iScript cDNA synthesis kit” (BioRad, Hercules, CA). The oligomeric primers (Sigma, St Louis, MO) used to amplify the TH mRNA and α-synuclein mRNA are enumerated in Table 1. The cDNA amplification was performed using an iQ SYBR Green Supermix kit following the manufacturer’s instructions (BioRad, Hercules, CA). The amplification was performed using an iCycler iQ Multicolor Real Time PCR Detection System (BioRad, Hercules, CA). The expression of specific TH and α-synuclein transcripts amplified were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Electrophoretic Mobility Shift Assay (EMSA)

The Electrophoretic Mobility Shift Assay (EMSA) was performed using a kit from Active Motif (Carlsbad, CA) following manufacturer’s protocol. Nuclear extract was prepared using NE-PER protein extraction reagent following the manufacturer’s instructions (Thermo Scientific, Rockford, IL). The 5′-biotin labeled and unlabeled oligonucleotide probes that correspond to the ERE binding site (−51 to −20 of the TH promoter) in the TH promoter region and LXRE binding sites (−13796 to −13767 of the α-synuclein promoter) in the α-synuclein promoter region were purchased from Sigma Aldrich (St Louis, MO). 10μg of nuclear proteins were incubated with either 20 femto moles of biotin labeled oligonucleotide probes or 4 pico moles of unlabelled oligonucleotide probes. To exhibit specificity of the biotin labeled oligonucleotide probes, unlabelled oligonucleotide probes were used as specific competitors for binding reactions at 200-fold greater concentration than that of the biotin labeled probes. 1μg of Poly d (I-C) was used as a non-specific competitor for binding reactions. The resulting binding reaction mix was loaded and resolved on a 5% TBE gel (Bio Rad, Hercules, CA) followed by transfer onto a nylon membrane. The bands were visualized using the HRP-Streptavidin-Chemiluminescent reaction mix provided with the kit on a UVP Bioimaging System (Upland, CA).

Chromatin Immunoprecipitation (ChIP) Analysis

ChIP analysis was performed to evaluate the extent of ERα/ERβ and LXRα/LXRβ binding to the DNA elements in the TH promoter and α-synuclein promoter regions respectively using “SimpleChIP™ Enzymatic Chromatic IP kit” from Cell Signaling (Boston, MA). Briefly, cells from each treatment group (3 × 10^6 cells) were washed with PBS, trypsinized, and centrifuged at 5000g. The pellet containing the cells was further washed with PBS and cross-linked using 37% formaldehyde for 15 min followed by the addition of glycine solution to cease the cross-linking reaction. The cells were washed with 4x volumes of 1× PBS and centrifuged at ~220g for 5 min. The pellet was resuspended and incubated for 10 min in 5ml of tissue lysis buffer containing DTT and protease and phosphatase inhibitors.

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The subsequent steps to isolate the cross-linked chromatin were performed according to the manufacturer’s protocol. The cross-linked chromatin from each sample was apportioned into four equal parts. One quarter of the cross-linked chromatin was set aside as “input”. One quarter of the cross-linked chromatin from each sample was incubated with 5 μg of anti-ERα mouse antibody (Cell Signaling, Boston, MA) and 5 μg of anti-ERβ rabbit antibody (Upstate, Bedford, MA), or 5 μg of anti-LXRα rabbit antibody (Abcam, Cambridge, MA) and 5 μg of anti-LXRβ mouse antibody (Abcam, Cambridge, MA), while the remaining one quarter of the cross-linked chromatin from each sample was incubated with 5 μg of normal Rabbit IgG to serve as negative control. The cross-linked chromatin samples were incubated overnight at 4°C with their respective antibodies. The DNA-protein complexes were collected with Protein G agarose beads and washed to remove non-specific antibody binding. The DNA from the DNA-protein complexes from all the samples including the input and negative control was reverse cross-linked by incubation with 2 μL of Proteinase K for 2 hours at 65°C. The crude DNA extract was eluted and then washed several times with wash buffer containing ethanol (provided with the kit) followed by purification with the use of DNA spin columns provided by the manufacturer. The pure DNA was eluted out of the DNA spin columns using 50 μL of the DNA elution buffer provided in the kit. 1 μL of the purified DNA was used for DNA concentration analysis using the “Quant-iT™ dsDNA Assay kit from Invitrogen (Carlsbad, CA). The DNA fragment size was determined by electrophoresis on a 1.2% agarose FlashGel® system (Lonza, Rockland, ME). The relative abundance of the ERα or ERβ antibody precipitated chromatin containing the ERE in the TH promoter region and LXRα or LXRβ antibody precipitated chromatin containing the LXRE in the α-synuclein promoter region was determined by qPCR using an iQ SYBR Green Supermix kit following the manufacturer’s instructions (BioRad, Hercules, CA) and sequence specific primers (Table 1). The amplification was performed using an iCycler iQ Multicolor Real Time PCR Detection System (BioRad, Hercules, CA). The fold enrichment of the ERE in the TH promoter region and LXRE in the α-synuclein promoter region was calculated using the ΔΔ Ct method (Livak & Schmittgen, 2001) which normalizes ChIP Ct values of each sample to the % input and background.

Luciferase Reporter Assays

Constructs encoding ERE and TH promoter conjugated to the firefly luciferase gene were used in the study. Human neuroblastoma SH-SY5Y cells were plated in 96-well plates at a density of 2×10⁴ cells/well. The cells were transfected when 80% confluent with 0.25 μg of either ERE-firefly luciferase reporter construct, LXRE-firefly luciferase reporter construct, or TH-firefly luciferase promoter construct. Respective non-inducible reporter constructs containing constitutively expressing Renilla luciferase were used as negative internal controls. Constitutively expressing GFP constructs were used as positive control to determine transfection efficiency. Cells were incubated for 24 hours with Opti-MEM serum free medium (Invitrogen, Carlsbad, CA) containing the reporter constructs dissolved in transfection reagent. After 24 hours the medium was changed and the cells were incubated in normal DMEM/F12 medium containing 10% FBS and cells were treated with the different treatment regimens. The cells were treated in triplicate and harvested 24 hours later and subjected to dual-luciferases assay. The dual-luciferase assay was performed using a “Dual-Luciferase Reporter Assay System” from Promega (Madison, WI). The luminescence recorded is expressed as Relative Luminescence Units (RLU) and normalized to per mg protein. Unit value was assigned to control and the magnitude of differences among the samples is expressed relative to the unit value of control cells.

Dopamine measurement by HPLC

Dopamine levels were determined by a specific HPLC assay utilizing an Antec Decade II (oxidation: 0.5) electrochemical detector operated at 33°C at the Vanderbilt University
CMN/KC Neurochemistry Core Lab. Cell homogenates were spun in a microcentrifuge at 10000g for 20 min (Lindley et al. 1998); 20 μL samples of the supernatant were injected using a Water 717+ autosampler onto a Phenomenex Nucleosil (5 u, 100 Å) C18 HPLC column (150 × 4.60 mm) (Phenomenex, Torrance, CA, USA). Samples were eluted with a mobile phase consisting of 89.5% 0.1 M trichloroacetic acid, 10^{-2} M sodium acetate, 10^{-4} M EDTA, and 10.5% methanol, pH 3.8. Solvent was delivered at 0.8 mL/min using a Waters 515 HPLC pump (Waters, Milford, MA, USA). HPLC control and data acquisition were managed by Waters Empower software. The values were normalized to mg protein content of respective cell homogenates and are reported as ng of Dopamine/mg protein.

**Statistical analysis**

The significance of differences among the samples was assessed by One Way Analysis of Variance (One Way ANOVA) followed by Tukey’s post-hoc test. Statistical analysis was performed with GraphPad Prism software 4.01. Quantitative data for Western blotting analysis are presented as mean values ± S.E.M with unit value assigned to control and the magnitude of differences among the samples being expressed relative to the unit value of control. Quantitative data for RT-PCR analysis are presented as mean values ± S.E.M, with reported values being the product of absolute value of the ratio of TH and α-synuclein mRNA to GAPDH mRNA multiplied by 1000000.

**Results**

**27-OHC reduces TH expression and estradiol reverses the effects of 27-OHC and increases basal expression levels of TH**

We determined the involvement of ER in TH expression and tested the effects of estradiol treatment on the 27-OHC-induced attenuation of TH expression levels. In accordance with our previous study, we found that 27-OHC elicits a 26% decrease in TH protein levels (p<0.05) (Fig 1a, b). Interestingly, estradiol treatment resulted in a profound increase in TH protein levels by 37% (p<0.01). Furthermore, concomitant treatment of SH-SY5Y neuroblastoma cells with 27-OHC and estradiol resulted in a significant increase in TH protein levels compared to control (p<0.05) or 27-OHC treated samples (p<0.01) (Fig 1a,b). The complete reversal of 27-OHC-induced attenuation of TH expression by estradiol suggests that 27-OHC and estradiol may utilize a common downstream effector to modulate TH expression. To correlate the changes on expression of TH with its enzymatic activity, we determined the effects of 27-OHC and concomitant estradiol treatment on the levels of TH phosphorylated at the Ser^{40} residue. Although, unphosphorylated TH exhibits intrinsic enzymatic activity, phosphorylation of TH at the Ser^{40} profoundly increases the enzymatic activity and the levels of p-Ser^{40} TH can be considered as a surrogate marker of TH activity (Anagnoste et al. 1974; Morgenroth et al. 1975; Haycock, 1990; Haycock & Haycock, 1991). We found that while 27-OHC induces a 3-fold decrease in phosphorylation of TH at Ser^{40} (p<0.001), estradiol elicits a 2.7-fold increase in p-Ser^{40} TH levels (p<0.001) (Fig 1c). Estradiol treatment also completely precludes the 27-OHC-induced reduction in p-Ser^{40} TH levels and results in a 2-fold increase in levels of p-Ser^{40} TH compared to basal levels (p<0.001) (Fig 1c). We subsequently investigated whether the effects of 27-OHC and estradiol on TH expression were transcriptional in nature. Real Time RT-PCR analysis demonstrates that 27-OHC reduces the TH mRNA (p<0.05) and this transcriptional attenuation is completely reversed by estradiol treatment (p<0.01) (Fig 1d). Furthermore, estradiol treatment elicits an increase in basal mRNA expression of TH (p<0.05) (Fig 1d).
27-OHC reduces the nuclear translocation of ERα and ERβ and estradiol reverses the effects of 27-OHC and increases basal levels of ERα and ERβ in the nucleus

We investigated the potential of 27-OHC to modulate ER activity and subsequently regulate ER mediated TH expression. We found that 27-OHC elicits a 33% and 36% decrease in nuclear translocation of ERα (p<0.05) (Fig 2c,d) and ERβ (p<0.05) (Fig 2g,h) respectively, while increasing the retention of ERα (Fig 2a,b) and ERβ (2e,f) in the cytosol by 45% (p<0.05) and 35% (p<0.05) respectively. These results suggest that 27-OHC possesses ER antagonist properties in the SH-SY5Y neuroblastoma cell line paradigm. Furthermore, concomitant treatment of cells with estradiol completely reduced the 27-OHC-induced attenuation in the nuclear translocation of ERα (p<0.01) (Fig 2c,d) and ERβ (p<0.01) (Fig 2g,h) resulting in a 65% (p<0.05) and 56% (p<0.01) increase in nuclear translocation of ERα and ERβ respectively compared to control. Estradiol treatment alone also caused a similar 60% (p<0.01) and 57% (p<0.01) increase in nuclear translocation of ERα and ERβ respectively compared to basal levels.

27-OHC reduces TH expression by attenuating the binding of ER to ERE half site in the promoter region of TH. Treatment with estradiol reverses the effects of 27-OHC

As we found an attenuation in the nuclear translocation of ER in response to 27-OHC treatment, we next investigated the binding of ER to the exogenous consensus sequence corresponding to the ERE half site in the TH promoter region. To this end, Electrophoretic Mobility Shift Assay (EMSA) was performed using nuclear extracts from treated samples (Fig 3a). We found that there was a decrease binding of ER to the exogenous double stranded DNA probe representing the ERE half site on the TH promoter (Fig 3a). This could be a direct ramification of decreased ERα/β translocation to the nucleus, or decreased binding affinity of the translocated ERα/β to the ERE, or a combination of the two aforementioned processes. Concomitant treatment with estradiol completely reverses the inhibitory effects of 27-OHC on binding of ER to the exogenous DNA probe (Fig 3a). The EMSA shows that there is “mobility shift” in each of the treated samples. However, the EMSA clearly depicts that the optical density of the shifted ER-DNA complex is of lower intensity in the 27-OHC treated cells compared to control. On the contrary, cells treated with a combination of 27-OHC and estradiol exhibit a profoundly greater intensity of the shifted ER-DNA complex. This clearly suggests that 27-OHC evokes a lower binding of nuclear ER to the exogenous double stranded DNA probe corresponding to the ERE-half site on the TH promoter, while concomitant treatment with estradiol not only reverses, but profoundly increases binding of nuclear ER to the exogenous probe double stranded DNA probe corresponding to the ERE-half site on the TH promoter.

27-OHC-induced reduction in TH expression is mediated via the attenuation of ERβ binding to ERE half site in the promoter region of TH and subsequent attenuation in ERβ mediated TH transcription

The EMSA analysis clearly revealed decreased binding of ER to the exogenous double stranded DNA probe corresponding to the ERE in the TH promoter. However, binding of ER to the ERE in the DNA is regulated by a host of epigenetic factors. EMSA does not account for other epigenetic modifications which occur concurrently and the presence of co-activators/co-repressors which regulate transcription. Furthermore, these epigenetic factors may regulate specific co-activators/co-repressors involved in TH expression and may also dictate the subtype of ER (ERα or ERβ) involved in the regulation of TH expression. Therefore, to further characterize the effects of 27-OHC on the ERα/β binding to ERE on TH promoter and to elucidate the subtype of ER involved in the regulation of TH expression, we performed Chromatin Immunoprecipitation (ChIP) analysis (Fig 3b). We found that binding of ERβ to the ERE in the TH promoter is about 10 times greater than binding of ERα to the ERE in TH promoter region in control samples (Fig 3b). This suggests...
that in the basal state, the expression of TH is primarily responsive to ERβ. Also, 27-OHC treatment induced no significant attenuation of ERα binding to the ERE on the TH promoter (Fig 3b) despite attenuating TH expression. 27-OHC treatment however did profoundly attenuate ERβ binding to the ERE on the TH promoter by 4-fold (p<0.001) (Fig 3b). Estradiol treatment, on the other hand, elicited a 4.5-fold increase in ERβ binding to the ERE half-site on the TH promoter (p<0.001), while producing no effect on ERα binding to the same site (Fig 3b). This suggests that the 27-OHC-induced attenuation in TH expression is primarily mediated via the inhibition of ERβ binding to the ERE in the TH promoter region. Furthermore, treatment with estradiol concomitant to 27-OHC treatment increases ERβ (p<0.001), but not ERα, binding to the ERE on the TH promoter. These results are in support of ERβ regulating TH transcription and 27-OHC attenuating TH expression by reducing ERβ binding to TH promoter. To further demonstrate that the changes in ERβ binding to the TH promoter indeed result in changes in transcription of TH, we performed ER reporter assay and TH promoter analysis using a dual-luciferase assay system (Fig 4a,b). We found that 27-OHC decreases ER-mediated transcription by ~5-fold (p<0.001) (Fig 4a). Furthermore, 27-OHC reduced TH promoter activity by ~4-fold (p<0.001) and this effect was completely reversed by estradiol (p<0.001) (Fig 4b). Estradiol treatment also evoked a 2.5-fold increase in basal TH promoter activity (p<0.001) (Fig 4b).

To further implicate the inhibition of ERβ as the mediator of 27-OHC-induced attenuation in TH expression, we systematically silenced ERβ expression using siRNA and overexpressed ERβ using AAV-ERβ (adeno-associated viral) vector system. Estradiol treatment failed to evoke an increase in basal protein levels of TH (Fig 5a,b) and mRNA expression of TH in ERβ-silenced cells (Fig 5d), suggesting that ERβ mediates the estradiol-induced increase in TH. However estradiol induced a significant increase in levels of p-Ser40 TH in ERβ-silenced cells (p<0.05) (Fig 5c), indicating that estradiol-regulated post-translational modification of TH may be independent of ERβ which mediates most of the genomic effects of estradiol. As 27-OHC induced a reduction in nuclear translocation of ERβ and a subsequent decrease in the binding of ERβ to the TH promoter, we determined the effect of overexpressing ERβ in native SH-SY5Y cells. ERβ overexpressing SH-SY5Y cells (AAV-ERβ transfected cells) exhibit a profound increase in basal protein levels (p<0.01), mRNA expression of TH (p<0.01), and levels of p-Ser40 TH (p<0.01) (5e–h). Moreover, AAV-ERβ transfected SH-SY5Y cells treated with 27-OHC demonstrated no attenuation in TH expression and p-Ser40 TH levels, but rather exhibited a significant increase in the expression of TH (p<0.01) and p-Ser40 TH levels (p<0.01) compared to control levels (Fig 5e–h). This suggests that ERβ overexpression is sufficient to preclude the 27-OHC-induced mitigation in TH expression. All together, these results point to ERβ as the subunit receptor that regulates TH expression.

To correlate the expression of TH and p-Ser40 TH with the activity of TH and role of ERβ, we measured dopamine levels in estradiol treated samples in native SH-SY5Y cells and ERβ-silenced SH-SY5Y cells. While it produced a 3-fold increase in dopamine levels compared to control untreated cells (0.08 ng/mg protein in control and 0.25 ng/mg protein in estradiol-treated cells), estradiol treatment in ERβ-silenced cells didn’t affect dopamine levels (0.08 ng/mg protein in control and 0.08 ng/mg protein in estradiol + siRNA to ERβ). These results further implicate ERβ in the regulation of TH expression, activity, and Dopamine biosynthesis. As dopamine content in SH-SY5Y cells is very low, the dopamine concentrations presented here are the average of four individual samples that were grouped together in each treatment group (Table II).
27-OHC and the LXR agonist GW3965 increase α-synuclein expression and treatment with the LXR antagonist ECHS reverses the 27-OHC effects

To investigate the involvement of LXR signaling in α-synuclein expression, we probed into the effects of 27-OHC in addition to a well characterized LXR agonist GW3965 (Collins et al. 2002; Joseph et al. 2002; Mitro et al. 2007) and the LXR antagonist ECHS (Song et al. 2007). We found that 27-OHC and GW3965 increase α-synuclein expression levels by 2.5-fold (p<0.001) and 3-fold (p<0.001) respectively, whereas the LXR antagonist ECHS does not produce any effect on basal α-synuclein expression levels (Fig 6a,b). However, the LXR antagonist ECHS significantly attenuated the 27-OHC-induced increase in α-synuclein levels (p<0.01) (Fig 6a,b), thereby implicating LXR pathway in the 27-OHC-induced upregulation of α-synuclein expression. To test the hypothesis that the effects of 27-OHC, GW3965, and ECHS are transcriptional in nature, we performed a Real Time RT-PCR analysis. We found an increase in α-synuclein mRNA expression with 27-OHC (9-fold) (p<0.001) as well as GW3965 (10-fold) (p<0.001) treatments and a marked attenuation in the 27-OHC-induced increase in α-synuclein mRNA with ECHS treatment (p<0.001) (Fig 6c). These results suggest that LXR pathway regulates the basal as well as the 27-OHC-induced increase in α-synuclein expression at the level of transcription.

27-OHC and GW3965 increase LXRα/β expression levels in the cytosol as well as the nucleus and ECHS reverses the effects of 27-OHC

We investigated the effects of 27-OHC treatment on the expression levels of LXRα/β in the cytosol and the nucleus and the extent to which this may have an impact on LXR binding to the α-synuclein promoter. We also determined LXRα/β expression levels in the cytosol and the nucleus in response to the synthetic agonist GW3965 and the antagonist ECHS. We found that 27-OHC increases LXRα protein levels both in the cytosol (p<0.01) (Fig 7a,b) and the nucleus (p<0.001) (Fig 7c,d), as well as LXRβ protein levels in the nucleus (p<0.01) (Fig 7g,h). The synthetic LXRα/β agonist GW3965 exerted an analogous, but more pronounced increase in LXRα and LXRβ levels in the cytosol (p<0.01 and p<0.05 respectively) (Fig 7a,b,e,f) and the nucleus (p<0.001 and p<0.01 respectively) (Fig 7c,d,g,h). On the other hand, treatment with the LXRα/β antagonist ECHS reduced levels of LXRα and LXRβ in the cytosol (p<0.05) as well as the nucleus (p<0.05) (Fig 7a–h). Furthermore, ECHS significantly attenuated the 27-OHC-induced increase in LXRα and LXRβ in the nucleus (p<0.05 and p<0.01 respectively) (Fig 7c,d,g,h). Our results show that 27-OHC and the LXR agonist GW3965 not only increase the nuclear expression of LXRα/β, but also produce an increase in LXRA/β in the cytosolic extracts as well, thereby suggesting that 27-OHC and the synthetic LXR agonist increases the global expression of LXRα/β nuclear receptors. Our results are in accordance with and corroborate other studies demonstrating that LXRα/β genes are autoregulated by LXRA/β itself (Laffitte et al. 2001; Whitney et al. 2001). On the other hand, the LXR antagonist ECHS decreased the basal expression of LXRA and LXRB in the cytosol and the nucleus (Fig 7a–h), further implicating LXRA/β in the autoregulatory loop. Furthermore, ECHS also completely reversed the 27-OHC-induced increase in LXRA/β expression in the nucleus (Fig 7c,d,g,h).

27-OHC and GW3965 increase α-synuclein expression by increasing the binding of LXRA/β to LXRE in the promoter region of α-synuclein, effects reversed by ECHS

To correlate the increased or decreased nuclear levels of LXRA/β with their transcriptional effects on target genes such as α-synuclein, we performed an EMSA to determine the extent to which treatments with 27-OHC, GW3965, or ECHS modulate LXRA/β binding to the exogenous double stranded DNA sequence corresponding to the LXRE consensus motif on the α-synuclein promoter. Two functional LXRE – SITE1 located 13788 nucleotides upstream (~13788) and the other SITE2 located 67043 nucleotides downstream (+67043) of the transcription start site have been identified in the α-synuclein promoter region (Cheng et al. 2002; Joseph et al. 2002; Mitro et al. 2007). We found that 27-OHC and GW3965 increase α-synuclein expression levels by 2.5-fold (p<0.001) and 3-fold (p<0.001) respectively, whereas the LXR antagonist ECHS does not produce any effect on basal α-synuclein expression levels (Fig 6a,b). However, the LXR antagonist ECHS significantly attenuated the 27-OHC-induced increase in α-synuclein levels (p<0.01) (Fig 6a,b), thereby implicating LXR pathway in the 27-OHC-induced upregulation of α-synuclein expression. To test the hypothesis that the effects of 27-OHC, GW3965, and ECHS are transcriptional in nature, we performed a Real Time RT-PCR analysis. We found an increase in α-synuclein mRNA expression with 27-OHC (9-fold) (p<0.001) as well as GW3965 (10-fold) (p<0.001) treatments and a marked attenuation in the 27-OHC-induced increase in α-synuclein mRNA with ECHS treatment (p<0.001) (Fig 6c). These results suggest that LXR pathway regulates the basal as well as the 27-OHC-induced increase in α-synuclein expression at the level of transcription.
27-OHC and GW3965 increase α-synuclein expression by increasing the binding of LXRβ to LXRE in the α-synuclein promoter region. ECHS reverses the effects of 27-OHC and GW3965

EMSA clearly depicts increased binding of LXRα/β to the exogenous DNA sequences representing the two LXRE consensus motifs in the α-synuclein promoter. However, LXRα/β mediated transcription is contingent on concomitant epigenetic modifications and the presence of co-activators/co-repressors that modulate LXRα/β binding to the LXRE on target genes. We therefore, performed a ChIP assay to determine the effects of 27-OHC, GW3965, and ECHS on LXRα/β binding to SITE1 LXRE in the α-synuclein promoter. ChIP analysis not only concurred and corroborated EMSA analysis data but also unveiled the LXR subtype involved in the α-synuclein promoter regulation. We found that the binding of LXRβ to the LXRE in the α-synuclein promoter is about 5 times higher than the binding of LXRα (Fig 8b). This suggests that in the basal state, the expression of α-synuclein is primarily responsive to LXRβ relative to LXRα. Furthermore, 27-OHC treatment induced no significant augmentation of LXRα binding to the LXRE on the α-synuclein promoter (Fig 8b) despite augmenting α-synuclein expression. 27-OHC treatment however did profoundly elevate LXRβ binding to the LXRE (~3 fold) in the α-synuclein promoter (p<0.001) (Fig 8b). This suggests that the 27-OHC-induced increase in α-synuclein expression may be primarily mediated via the activation of LXRβ binding to the LXRE in the α-synuclein promoter region. Treatment with the LXR agonist GW3965, either alone or in combination with 27-OHC, increases LXRβ binding to the LXRE on the α-synuclein promoter by 3.5-fold (p<0.001) and 4-fold (p<0.001) respectively (Fig 8b). However, the LXR agonist GW3965, either alone or in combination with 27-OHC, did not elicit any increase in LXRα binding to the LXRE in the α-synuclein promoter (Fig 8b). This further implicates LXRβ in the regulation of α-synuclein expression. Furthermore, treatment with the LXR antagonist ECHS resulted in a ~2.8 fold decrease in LXRβ binding to the LXRE on the α-synuclein promoter (p<0.001) (Fig 8b). However, ECHS did not significantly attenuate LXRα binding to the LXRE on the α-synuclein promoter (Fig 8b). This further corroborates the fact that LXRβ mediates basal as well as 27-OHC-induced increase in α-synuclein expression. Furthermore, ECHS significantly reversed the 27-OHC-induced increase in LXRβ binding to the LXRE in the α-synuclein promoter (p<0.01) (Fig 8b).

To further implicate LXRβ in 27-OHC and GW3965-induced increase in α-synuclein expression, we silenced LXRβ expression using RNAi and subsequently determined the effects of 27-OHC and GW3965. In accordance with the ChIP data implicating LXRβ as the mediator of 27-OHC and GW3965-induced increase in α-synuclein expression, 27-OHC and GW3965 failed to evoke an increase in α-synuclein expression in LXRβ-silenced cells (Fig 9a–f). This data suggests that LXRβ is necessary for 27-OHC and GW3965-induced increase in α-synuclein expression.
Discussion

We have shown previously that the oxysterol, 27-hydroxycholesterol (27-OHC) reduces TH expression and induces an elevation in α-synuclein expression (Rantham Prabhakara et al. 2008). However the molecular mechanisms by which 27-OHC elicited these effects were not known. Reduction in TH and elevation in α-synuclein expression are important biochemical events in the pathogenesis of PD. We demonstrate in this study that ERα, but not ERβ, signaling positively regulates TH expression. 27-OHC reduces TH expression by exhibiting SERM activity and attenuating ERβ-induced TH expression and this effect of 27-OHC is reversed by concomitant estradiol treatment. We further demonstrate that LXRβ, and not LXRα, signaling positively regulates α-synuclein expression. 27-OHC increases α-synuclein expression via the activation of LXRβ and this effect is reversed by concomitant treatment with the LXR antagonist ECHS.

Estrogens elicit their majority of effects by activating the cognate nuclear receptors ERα and ERβ. Estradiol is the most abundant and potent endogenous estrogen and activates both ERα and ERβ. Estradiol binding to the ER results in a conformational change that allows receptor dimerization (Beato et al. 1995; Mangelsdorf et al. 1995). ER dimerization results in nuclear translocation, binding to the response element in the DNA, recruitment of co-activators or co-repressors and culminates into modulation of target gene expression (Beato et al. 1995; Mangelsdorf et al. 1995). 27-OHC has been characterized as a selective estrogen receptor modulator (SERM) (Umetani et al. 2007; DuSell et al. 2008). Some studies have reported that 27-OHC activates ER, while others studies have found that 27-OHC acts as an ER antagonist (Umetani et al. 2007; DuSell et al. 2008). The general consensus is that 27-OHC and several other SERM (4-hydroxy tamoxifen) have tissue specific effects contingent on a multitude of coincident stimuli. There is a plethora of evidence suggesting that estradiol induces the expression of TH (Liaw et al. 1992; Gayrard et al. 1994; Serova et al. 2002; Curran-Rauhut & Petersen, 2003; Serova et al. 2004). Furthermore, several studies have shown that the effects of estradiol on TH expression are mediated by ERα and ERβ (Ivanova & Beyer, 2003; Maharjan et al. 2005). Several studies have characterized the neuroprotective role of estrogens in the preservation of dopaminergic cells in both in vivo (Sawada et al. 1998; Sawada et al. 2002) and in vitro (Callier et al. 2000; Grandbois et al. 2000; Callier et al. 2001) models of PD. Estradiol, the most prominent endogenous estrogen and an agonist of ERα and ERβ, has been shown to increase TH immunoreactivity in dopaminergic neurons (Gayrard et al. 1994) and TH mRNA in gonadectomized rats (Liaw et al. 1994; Serova et al. 2002; Curran-Rauhut & Petersen, 2003; Serova et al. 2004). Estradiol increases TH promoter activity in ovariectomized TH9-LacZ transgenic mice (Thanky et al. 2002), suggesting that the positive effect of estradiol on TH promoter activity is transcriptional in nature. The TH promoter contains a multitude of response elements for transcription factors that may regulate TH promoter activity in response to estradiol including AP1, Sp1 (Fung et al. 1992; Nakashima et al. 2003), and CREB (Kilbourne et al. 1992; Kim et al. 1993; Lewis-Tuffin et al. 2004). Furthermore, the TH promoter contains an Estrogen Response Element (ERE) half site to which activated estrogen receptors (ERα and ERβ) can bind and induce TH promoter activity (Serova et al. 2002). We hypothesized that 27-OHC may regulate TH expression via the modulation of ER. Indeed, we found that 27-OHC decreases ERα and ERβ protein levels in the nucleus and concomitant treatment with estradiol precludes this 27-OHC-induced decrease in nuclear ERα and ERβ protein levels. ERα and ERβ expression levels increased significantly in the cytosolic extracts of 27-OHC-treated cells and decreased in the estradiol-treated cells, a reflection of the phenomenon that 27-OHC decreases the nuclear translocation of ERα and ERβ whereas estradiol increases the nuclear translocation of ERα and ERβ. Furthermore, to correlate the decreased ERα/β levels with lower transcriptional activity, EMSA, ChIP and TH promoter analyses were performed to elucidate the role of ERα/β in the transcriptional regulation of TH expression and
determine the extent to which 27-OHC affects TH transcription via the modulation of ERα/β.

The ERα and ERβ exhibit high homology and belong to the steroid receptor superfamily. However ERα and ERβ possess different transactivation domains that confer unique properties with respect to each other. The respective activation function (AF) motifs in ERα and ERβ can differentially interact with transcriptional co-activators, co-repressors, and co-integrators thereby bestowing ERα and ERβ with disparate biological activities and functions (McKenna et al. 1999a; McKenna et al. 1999b). EMSA analysis clearly showed that 27-OHC decreases ERα/β binding to the double stranded oligonucleotide probe that corresponds to the ERE-half site in the TH promoter. We aimed to determine the subtype of ER specifically involved in the regulation of basal and 27-OHC-induced attenuation in TH expression. ChIP analyses shows that, in the basal state, ERβ bound to the ERE-half site in the TH promoter is 10 times higher than ERα bound to the same site. Furthermore, 27-OHC attenuated the ERβ binding to the ERE half site in the TH promoter while having no effect on ERα binding to the same site. This suggests that ERβ predominantly regulates basal expression of TH in SH-SY5Y cells and 27-OHC-induced inhibition of TH expression is mediated via ERβ. To further implicate ERβ in the regulation of TH expression, we silenced ERβ expression using siRNA approach and also overexpressed ERβ using an AAV-ERβ vector system in SH-SY5Y cells. We found that estradiol fails to evoke an increase in basal TH expression levels and 27-OHC-induced decrease in TH expression levels in ERβ-silenced cells, suggesting that ERβ mediates the 27-OHC-induced increase in TH expression levels. Furthermore, overexpression of ERβ increases the basal expression levels of TH and also completely reverses the 27-OHC-induced reduction in TH expression. Maharjan and colleagues have demonstrated in PC12 cells that estradiol treatment caused an increase in TH expression via ERα while eliciting attenuation in TH expression through ERβ (Maharjan et al. 2005). In this light, our results are novel and further substantiate a critical role of ERβ in the brain. ERβ knock-out mice have markedly diminished cognitive function (Krezel et al. 2001). It is the general consensus that ERβ mediates the non-reproductive biological functions of estradiol in the brain (Weiser et al. 2008). ERα and ERβ are pervasively expressed throughout the brain and spinal cord. However, in certain brain regions such as hypothalamic nuclei ERα expression predominates whereas in the CA1 and CA3 regions of the hippocampus ERβ expression predominates (Laflamme et al. 1998). Of relevance to PD, the substantia nigra almost exclusively expresses ERβ receptors (Quesada et al. 2007).

The two LXR receptor subtypes (namely LXRα and LXRβ) exhibit 77% homology (Alberti et al. 2000), but have significantly different expression profiles. 27-OHC, and other oxysterols such as 24,25-epoxycholesterol, are potent endogenous ligands and activators of LXRs (Janowski et al. 1996; Lehmann et al. 1997). Ligand-activated LXRs form heterodimers with Retinoid X receptors (RXR) and modulate the expression of target genes by binding to the LXRE in the promoters of target genes. Furthermore, LXRα regulates its own transcription thereby suggesting an autoregulatory loop (Laffitte et al. 2001; Whitney et al. 2001). Endogenous LXRa/β ligands such as 20(S) hydroxycholesterol, 22(R) hydroxycholesterol and synthetic LXRa/β ligands -GW3965 & TO901317 have been demonstrated to increase LXRe expression levels (Laffitte et al. 2001a; Whitney et al. 2001). LXRa is abundantly expressed in peripheral visceral organs such as liver, kidney, intestine, and spleen (Willy et al. 1995), whereas LXRB exhibits constitutive low-level expression in all tissues (Teboul et al. 1995; Repa et al. 2000; Repa & Mangelsdorf, 2000) including brain (Kainu et al. 1996). LXRa (NR1H3) and LXRβ (NR1H2) are members of the Type II family of nuclear receptors that function as ligand-induced transcription factors that regulate the expression of genes involved in de novo synthesis, transport and metabolism of cholesterol (Peet et al. 1998b) and lipogenesis (Schultz et al. 2000; Stulnig et al. 2002). Janowski and colleagues have demonstrated that oxysterols such as 27-OHC bind

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to LXR with an EC$_{50}$ of 2–10µM (Janowski et al. 1999). LXR$\alpha$ and LXR$\beta$ modulate transcription of target genes by forming heterodimers with Retinoid X Receptor $\alpha$ (RXR$\alpha$) and subsequently binding to LXRE in the promoter regions of target genes. Using in silico analysis, Cheng and colleagues (Cheng et al. 2008) have identified functional LXRE in the human $\alpha$-synuclein promoter region. In this study we found that 27-OHC increases $\alpha$-synuclein expression levels. We hypothesized that 27-OHC might increase $\alpha$-synuclein expression by activating LXR$\alpha$ and subsequently binding to LXRE in the promoter regions of target genes. Using in silico analysis, Cheng and colleagues (Cheng et al. 2008) have identified functional LXRE in the human $\alpha$-synuclein promoter region. We found increased binding of LXR$\alpha$ to this oligomeric probe comprising of the LXRE in the $\alpha$-synuclein promoter. To this end, we first performed an EMSA to determine the LXR$\alpha$/LXR$\beta$ interaction with an exogenous consensus sequence corresponding to the LXRE in the $\alpha$-synuclein promoter region. We found increased binding of LXR$\alpha$/LXR$\beta$ to this oligomeric probe comprising of the LXRE in the $\alpha$-synuclein promoter. We next performed a ChIP assay to further characterize the involvement of LXR and elucidate the subtype of LXR that mediates 27-OHC-induced increase in $\alpha$-synuclein expression. We found that, in the basal state, LXR$\beta$ bound to the LXRE in the $\alpha$-synuclein promoter region is 5.5-fold higher compared to LXR$\alpha$. 27-OHC increased LXR$\beta$ binding to the LXRE in the $\alpha$-synuclein promoter region, but produced no effect on LXR$\alpha$ binding to the same site. Furthermore, the LXR agonist GW3965 also increased LXR$\beta$ binding to the LXRE on the $\alpha$-synuclein promoter without eliciting any increase in LXR$\alpha$ binding to the same site. The LXR antagonist ECHS on the other hand, decreased both the basal LXR$\beta$ binding as well as 27-OHC-induced increase in LXR$\beta$ binding to the LXRE on the $\alpha$-synuclein promoter, but produced no effect on LXR$\alpha$ binding to the same site. To further confirm the role of LXR$\beta$ in the regulation of $\alpha$-synuclein expression, we silenced the expression of LXR$\beta$ using RNAi approach. We found that both, 27-OHC and the LXR agonist GW3965 fail to evoke an increase in $\alpha$-synuclein levels. Our data implicates LXR$\beta$ in the regulation of basal expression of $\alpha$-synuclein as well as 27-OHC-induced increase in $\alpha$-synuclein expression. Interestingly, LXR$\beta$ is the main LXR subtype in the brain (Kainu et al. 1996). Furthermore, in addition to regulating $\alpha$-synuclein expression via LXR receptors, 27-OHC-mediated LXR signaling has also been implicated in neuroinflammatory and neurodegenerative changes associated with PD and PD-related spectrum of disorders (Wang et al. 2002; Kim et al. 2008). However these results did not demonstrate direct evidence for the subtype of LXR expressed in the dopaminergic neurons of the substantia nigra. Although, LXR$\alpha$ and LXR$\beta$ exhibit 77% homology and bind their endogenous ligands with relative similar affinity, their endogenous physiological functions, target genes and the effects on target genes may vary (Prüfer & Boudreaux, 2007). Recent evidence indeed suggests that LXR$\alpha$ and LXR$\beta$ differentially regulate target genes involved in lipid metabolism (Prüfer & Boudreaux, 2007). It is now the consensus that the canonical effects of LXRs such as upregulation of ATP-binding cassette transporters ABCA1, ABCG1, ABCG5, ABCG8 (Costet et al. 2000; Schwartz et al. 2000), upregulation of the expression of proteins such as ApoE (Laffitte et al. 2001b) and lipoprotein lipase (Zhang et al. 2001) involved in lipoprotein metabolism, and upregulation of the transcription factor SREBP-1c involved in the expression of lipogenic enzymes are mediated by LXR$\alpha$, and not LXR$\beta$ (Repa et al. 2000; Schultz et al. 2000, Venkateswaran et al. 2000). Further corroboration of the differential effects of LXR$\alpha$ and LXR$\beta$ has surfaced from LXR$\alpha$ and LXR$\beta$ knock-out mice. Knock-out of LXR$\alpha$ in mice results in lower expression of lipogenic genes such as SCD-1 and FAS, while knock-out of LXR$\beta$ elicited no effect (Alberti et al. 2001). Instead, LXR$\beta$ knock-out mice exhibited augmented expression of lipogenic enzyme ACC (Alberti et al. 2001) and the pivotal transcription factor SREBP-1c involved in the expression of lipogenic genes (Schuster et al. 2002). Therefore, inference can be drawn from aforementioned studies that LXR$\alpha$ and LXR$\beta$ have diverse and opposite roles in lipid metabolism. LXR$\beta$ represses the expression of lipogenic genes, while LXR$\alpha$ induces their expression. In light of this fact, lipogenic organs such as the liver and adipose tissue express LXR$\alpha$ more abundantly than LXR$\beta$ (Peet et al. 1998a). Therefore the relative expression of LXR$\alpha$ and LXR$\beta$ in a given cell-type or tissue will determine the effects of endogenous LXR ligands (oxysterols) and synthetic LXR agonists (GW3965). Consistent with this

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notion, LXR agonists designed pharmacologically to induce the beneficial effects of LXR on cholesterol metabolism also induce hepatic steatosis by increasing lipogenesis in the liver because of predominance of LXRα over LXRβ expression in the liver (Repa et al. 2000; Schultz et al. 2000, Yoshikawa et al. 2001). In this study we have demonstrated that the oxysterol 27-OHC and the synthetic LXR agonist increased the nuclear levels of both LXRα and LXRβ. However, both 27-OHC and GW3965 increased the binding of LXRβ, and not LXRα, to the α-synuclein promoter. This may be primarily attributed to the fact that α-synuclein may be an LXRβ regulated gene and not because LXRβ is more abundantly expressed in SH-SY5Y cells.

The involvement of α-synuclein as one of the prime proteins in the neuropathogenesis of PD (Ueda et al. 1993) has been the focal point of numerous studies in the last decade. Duplication, triplication or overexpression of the of the SNCA (α-synuclein) gene causes autosomal dominant PD (Masliah et al. 2000; Singleton et al. 2003; Chartier-Harlin et al. 2003; Farrer et al. 2004). Furthermore, point mutations (A30P, E46K, A53T) in the SNCA gene and overexpression of the mutant α-synuclein protein also produces autosomal dominant PD (Polymeropoulos et al. 1997; Kruger et al. 1998; Masliah et al. 2000).

Paradoxically, knocking-down or silencing of the SNCA gene also causes stress and dysfunction of the dopaminergic neurons in the substantia nigra (Abeliovich et al. 2000). Therefore, α-synuclein, although being involved in the neuropathogenesis of PD, yet serves an essential indispensable role in the physiology of the dopaminergic neurons of the nigrostriatal system. Recent evidence has indeed demonstrated that wild-type (wt) α-synuclein protects neuronal cells from caspase3-mediated apoptosis by downregulating the p53 pathway (da Costa et al. 2000). However, mutant α-synuclein does not elicit a neuroprotective response in a multitude of cell lines (Alves da Costa et al. 2002). Moreover, the dopaminergic toxin 6-hydroxydopamine (6-OHDA) produces toxicity by abolishing the anti-apoptotic effect of α-synuclein by inhibiting the ubiquitin-mediated catabolism of α-synuclein and augmenting its aggregation (Alves da Costa et al. 2006). Therefore, further studies are warranted to determine the effects of 27-OHC and LXR pathway on the increased production, accumulation and aggregation kinetics of α-synuclein.

In summary, our results demonstrate that the oxysterol 27-OHC modulates expression of TH and α-synuclein via two distinct pathways. 27-OHC decreases TH expression by attenuating ERβ-mediated transcription of TH and increases α-synuclein by augmenting LXRβ-mediated transcription of α-synuclein. Attenuation of TH expression and elevation of α-synuclein expression are important biochemical events implicated in PD. Our results are seminal and of high relevance to the pathogenesis of sporadic PD as high levels of 27-OHC were found in the cortices of patients with PD and Lewy body dementia (Bosco et al. 2006). A recent study also found an increase in 27-OHC levels in the cortex of PD brains (Cheng et al. 2011). Furthermore, 27-OHC levels are also significantly increased in the plasma of PD patients (Lee et al. 2009; Seet et al. 2010). Therefore, regulation of 27-OHC levels as well as concomitant activation of ER pathway and inhibition of LXR pathway may represent a potential target for the design of therapeutic interventions to reduce the progression of PD.

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Figure 1.
Effects of 27-OHC and concomitant estradiol treatment on TH expression in SH-SY5Y neuroblastoma cells. 27-OHC attenuates TH expression levels and concomitant treatment with estradiol reverses the deleterious effects of 27-OHC on TH expression in SH-SY 5Y neuroblastoma cells. (a) Representative Western blot, (b) densitometric analysis, and (d) real time RT-PCR analysis demonstrate that 27-OHC decreases TH protein and mRNA levels. Co-treatment with estradiol precludes the attenuation imposed by 27-OHC on TH protein and mRNA levels. Estradiol increases the expression of TH protein and mRNA. (a,c) Representative Western blot and densitometric analysis demonstrates that 27-OHC reduces the levels of p-Ser^{40} TH while co-treatment with estradiol completely precludes the 27-OHC-induced reduction in the levels of p-Ser^{40} TH. Estradiol treatment alone also induces a significant increase in p-Ser^{40} TH levels. Data is expressed as Mean ± S.E.M and includes determinations made in four separate cell culture experiments (n=4). *p<0.05, **p<0.01, and ***p<0.001 versus control, ††p<0.01 and †††p<0.001 versus 27-OHC.
Figure 2.
Effects of 27-OHC and concomitant estradiol treatment on ERα and ERβ translocation to the nucleus. (a,c) Representative Western blot and (b,d) densitometric analysis demonstrate that 27-OHC reduces the levels of ERα in the nucleus while increasing the retention of ERα in the cytosol. Concomitant estradiol treatment completely overrides the 27-OHC-induced inhibition in nuclear translocation of ERα. Estradiol treatment alone also significantly increases the nuclear translocation of ERα. *p<0.05 and **p<0.01 versus control, †p<0.05 and ††p<0.01 versus 27-OHC.

(b,f) Representative Western blot and (g,h) densitometric analysis clearly demonstrate that 27-OHC reduces the translocation of ERβ to the nucleus. Co-treatment with estradiol completely reverses the 27-OHC-induced inhibition in nuclear translocation of ERβ. Estradiol treatment alone also significantly increases nuclear translocation of ERβ. Data is expressed as Mean ± S.E.M and includes determinations made in four separate cell culture experiments (n=4).
Figure 3. Effects of 27-OHC and concomitant estradiol treatments on the binding of ERα and ERβ to the ERE in TH promoter. (a) EMSA was performed with double stranded biotin labeled oligonucleotide probe (20 fmole) corresponding to the ERE-half site in the TH promoter region. EMSA shows that 27-OHC decreases the binding of ERα/β to the exogenous oligonucleotide probe. Concomitant treatment of cells with 27-OHC and estradiol or estradiol alone significantly increases the binding of ERα/β to the exogenous oligonucleotide probe that corresponds to the ERE-half site in the TH promoter. (b) Chromatin Immunoprecipitation (ChIP) assay was performed by precipitating one quarter of the chromatin each with 5μg of ERα or ERβ antibody. ChIP analysis demonstrates that there are no significant differences in ERα binding to the ERE-half site in the TH promoter in
response to treatments with 27-OHC, estradiol, or both. Moreover, ChIP assay shows that in the basal state ERβ bound to the ERE-half site is 10-fold higher than ERα bound to the same ERE-half site in the TH promoter. 27-OHC significantly attenuates the binding of ERβ to the ERE-half site in the TH promoter. Concomitant treatment with 27-OHC and estradiol or estradiol alone significantly increases the binding of ERβ to the ERE-half site in the TH promoter. Data is expressed as Mean ± S.E.M and includes determinations made in four separate cell culture experiments (n=4). ***p<0.001 versus control, †††p<0.001 versus 27-OHC.
Figure 4.
(a) Dual-luciferase assay demonstrating the effect of 27-OHC on ER-mediated transcription. Cells were transfected with reporter construct comprising of ERE coupled upstream of the firefly luciferase gene. 27-OHC significantly attenuates the ERE-mediated transcription. (b) Dual-luciferase assay demonstrating the effects of 27-OHC and concomitant estradiol treatments on the TH promoter activity. Cells were transfected with a reporter construct containing the TH promoter fused upstream of the firefly luciferase gene. 27-OHC significantly reduces TH promoter activity, while concomitant estradiol treatment completely precludes the inhibition imposed by 27-OHC on TH promoter activity. Data is expressed as Mean ± S.E.M and includes determinations made in four separate cell culture experiments (n=4). ***p<0.001 versus control, †††p<0.001 versus 27-OHC.
Figure 5.
Effects of silencing and overexpression of ERβ on TH expression and p-Ser^{40} TH levels
(a,b) Representative Western blot and densitometric analysis demonstrate that estradiol
treatment fails to evoke an increase in TH expression levels in ERβ-silenced cells. (c)
Estradiol treatment increases the levels of p-Ser^{40} TH in ERβ-silenced cells suggesting that
ERβ does not regulate the phosphorylation of TH. (d) Estradiol treatment also fails to evoke
an increase in TH mRNA expression in ERβ-silenced cells suggesting the requisite nature of
ERβ in TH expression. (e,f) Representative Western blot and densitometric analysis
demonstrate that overexpression of ERβ precludes the 27-OHC-induced attenuation in TH
protein levels. Overexpression of ERβ also significantly increases the basal protein levels of
TH (g) Overexpression of ERβ also precludes the 27-OHC-induced attenuation in p-Ser^{40}
TH protein levels while also increasing the basal levels of p-Ser^{40} TH. (h) ERβ
overexpression in cells also results in a significant increase in basal levels of TH mRNA
expression and also precludes the 27-OHC-induced reduction in TH mRNA expression.
Data is expressed as Mean ± S.E.M and includes determinations made in four separate cell
culture experiments (n=4). *p<0.05, **p<0.01, and ***p<0.001 versus
control, †p<0.05, ††p<0.01, and †††p<0.001 versus 27-OHC.
Figure 6.
Effects of 27-OHC and concomitant treatment with LXR agonist GW3965 and LXR antagonist ECHS on α-synuclein expression in SH-SY 5Y neuroblastoma cells. (a) Representative Western blot (b) densitometric analysis, and (c) real time RT-PCR analysis demonstrate that 27-OHC significantly increases α-synuclein protein and mRNA levels. Treatment with the LXR agonist GW3965 also significantly increases α-synuclein protein and mRNA levels. Co-treatment with 27-OHC and the LXR agonist GW3965 accentuates the increase induced by 27-OHC on α-synuclein protein levels, but not mRNA levels. Treatment with the LXR antagonist ECHS produces no effect on the levels of α-synuclein protein and mRNA. However, the LXR antagonist ECHS significantly reverses the 27-OHC-induced increase in α-synuclein protein and mRNA levels. Data is expressed as Mean ± S.E.M and includes determinations made in four separate cell culture experiments (n=4). *p<0.05, **p<0.01and ***p<0.001 versus control, ††p<0.01 and †††p<0.001 versus 27-OHC; △△p<0.01 versus ECHS.
Figure 7.
Effects of 27-OHC and concomitant treatment with LXR agonist GW3965 and LXR antagonist ECHS on LXRα and LXRβ levels in the cytosol and the nucleus. Representative Western blot and densitometric analysis demonstrate that 27-OHC increases levels of LXRα (a–d) and LXRβ (e–h) both in cytosol (a,b,e,f) and the nucleus (c,d,g,h). Treatment with the LXR agonist GW3965 also significantly increases LXRα and LXRβ levels both in the cytosol and the nucleus (a–h). Co-treatment with 27-OHC and the LXR agonist GW3965 increases the LXRα and LXRβ levels in the nucleus to the same extent as treatment with 27-OHC or GW3965 alone. Treatment with the LXR antagonist ECHS markedly reduces the cytosolic as well as the nuclear levels of LXRα and LXRβ. ECHS also significantly reverses the 27-OHC-induced increase in the cytosolic and the nuclear levels of LXRα and LXRβ. Data is expressed as Mean ± S.E.M and includes determinations made in four separate cell culture experiments (n=4). *p<0.05, **p<0.01 and ***p<0.001 versus control, †p<0.05 and ††p<0.01 versus 27-OHC, △p<0.05 and △△p<0.01 versus ECHS.
Figure 8.
Effects of 27-OHC and concomitant treatment with LXR agonist GW3965 and LXR antagonist ECHS on the binding of LXRα/β to the LXRE in the α-synuclein promoter. (a) EMSA was performed with a double stranded biotin labeled oligonucleotide probe (20 fmoles) corresponding to the LXRE sites in the α-synuclein promoter region – SITE1: 13788 nucleotides upstream of transcription start site (−13796 to −13767). The EMSA shows that LXR agonist GW3965 and 27-OHC increase binding of LXRα/β to the exogenous oligonucleotide probe corresponding to the α-synuclein promoter region. The LXR antagonist, on the other hand, significantly reduces the 27-OHC-induced increase in LXR binding to the oligonucleotide probe. (b) Chromatin Immunoprecipitation (ChIP) assay was performed by precipitating one quarter of the chromatin each with 5μg of LXRα or LXRβ antibody. ChIP analysis demonstrates that there are no significant differences in LXRα binding to the LXRE in the α-synuclein promoter. However, ChIP assay shows that in the basal state LXRβ bound to the LXRE is 5.5-fold higher than LXRα bound to the same LXRE in the α-synuclein promoter. The LXR agonist GW3965 and 27-OHC significantly augment the binding of LXRβ to the LXRE in the α-synuclein promoter. The LXR antagonist ECHS significantly reduces the binding of LXRβ to the LXRE compared to control. Furthermore, ECHS significantly inhibits the 27-OHC-induced increase in binding of LXRβ to the LXRE in the α-synuclein promoter. Data is expressed as Mean ± S.E.M and
includes determinations made in four separate cell culture experiments (n=4). *p<0.05 and ***p<0.001 versus control, ††p<0.01 versus 27-OHC, ΔΔΔΔΔp<0.001 versus ECHS.
Figure 9.
Effects of silencing LXRβ expression on the α-synuclein expression levels. (a,b) Representative Western blot and (c,d) densitometric analysis clearly show that silencing LXRβ expression precludes the 27-OHC and GW3965-induced increase in α-synuclein protein levels. (e,f) Real-Time RT-PCR analysis clearly demonstrates that 27-OHC and GW3965 fail to elicit an increase in α-synuclein mRNA expression in LXRβ-silenced cells. Data is expressed as Mean ± S.E.M and includes determinations made in four separate cell culture experiments (n=4). ***p<0.001 versus control and †††p<0.001 versus 27-OHC.
### Table I

Primers designed and used for TH, α-synuclein, TH promoter and α-synuclein promoter

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Table II
Effect of estradiol treatment on Dopamine levels in native and ERβ-silenced SH-SY5Y cells

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Values are expressed as ng of dopamine/mg protein with a single determination drawn from four individual experiments combined.