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Distribution and localization Patterns of Estrogen Receptor- β and Insulin-Like Growth Factor-1 Receptors in Neurons and Glial Cells of the Female Rat Substantia Nigra

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Abstract

Although many studies have focused on the neuroprotective effects of estrogen (E2) on stroke, there have been tantalizing reports that E2 may be neuroprotective in degenerative neuronal diseases such as Alzheimer's and Parkinson's disease. In animal models of Parkinson's disease, E2 protects the nigrostriatal dopaminergic (DA) system against neurological toxins. However, little is known about the cellular and molecular mechanism(s) involved by which E2 elicits its neuroprotective effects on the nigrostriatal DA system. A preferred mechanism for neuroprotection is interaction of E2 with specific neuroprotective growth factors and receptors. One of such neuroprotective factor/receptor system is insulin-like growth factor-1 (IGF-1). E2 neuroprotective effects in the SN DA system have been shown to be dependent on IGF-1, a growth factor released by both astrocytes and neurons. To determine whether E2 also interacts with the insulin-like growth factor-1 receptor (IGF-1R) and to determine the cellular localization of estrogen receptor (ER) and IGF-1R we compared the distribution of ER and IGF-1R in the substantia nigra (SN). Stereological measurements revealed that a subpopulation (39.8%) of tyrosine hydroxylase-immunoreactive (TH-ir) SNpc DA neurons are immunoreactive to estrogen receptor- β (ER β). No immunolabeling for ER α was observed. In situ hybridization confirmed the expression of IGF-1R mRNA in the SNpc and immunohistochemistry revealed that almost all TH-ir SNpc DA neurons were IGF-1R-ir (98.5%). Moreover, 33% of glial fibrillary acidic protein (GFAP-ir) cells in the SN were ER β -ir and 67% of GFAP-ir cells displayed IGF-1R-ir. Thus, the localization of ER β and IGF-1R on SNpc DA neurons and astrocytes suggests a modulatory role of E2 on IGF-1R and this modulation may impact neuroprotection.

Keywords

Stereology; Confocal microscopy; substantia nigra

INTRODUCTION

The ovarian steroid hormone estrogen (E2) can act on the central nervous system throughout an organism's lifespan. The lipophilic nature of steroids allows them widespread access to cells both outside and inside the blood-brain-barrier, coordinating the functions of multiple tissues. Although, the most known effects of E2 are on the reproductive system and on parts of the brain regulating reproduction, recently, it has also become evident that E2 exerts a powerful protective role in the CNS (Chowen et al., 2000; Garcia-Segura et al., 2001; Stein,

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2001; Wise et al., 2001; McEwen, 2002). Many studies have focused on E2 protection in brain injuries such as stroke (Barrett-Connor and Bush, 1991; Zhang et al., 1998) and traumatic brain injury (Roof and Hall, 2000a; Roof and Hall, 2000b; Wagner et al., 2004), but there are also suggestive reports that E2 may protect against neurodegeneration in diseases such as Alzheimer's (Sherwin, 1997) and Parkinson's disease (Kuppers et al., 2000). The mechanism(s) of E2 neuroprotection has been difficult to elucidate. An important reason for this is that a plethora of E2 effects appears to mediate neuroprotection. For instance, E2 can act via classical genomic mechanisms (Hall et al., 2001) and membrane associated receptors that alter G-protein signaling which activate anti-apoptotic signaling pathways (for review see (Kelly et al., 2003)). In addition, E2 interacts with trophic factors, such as insulin-like growth factor-1 (IGF-1) to prevent neurodegeneration (for review see (Mendez et al., 2005)).

In previous studies using unilateral medial forebrain bundle 6-hydroxydopamine (6-OHDA) lesions as model of Parkinson's disease in rats, we demonstrated that E2 reduced the loss of substantia nigra pars compacta (SNpc) dopamine (DA) neurons and prevented motor deficits produced by 6-OHDA lesions (Quesada and Micevych, 2004). Interestingly, IGF-1 administered centrally or peripherally displays the same effect as E2. Most importantly, *in vivo* blockaded of IGF-1 receptors (IGF-1R) completely abolished E2 and IGF-1 neuroprotection of SNpc DA neurons. In the 6-OHDA lesion models, these data indicate that E2 protects SNpc DA neurons, and the IGF-1 system has a central role in the mechanism through which E2 exerts its neuroprotective effect on the nigrostriatal DA system. However, an underpinning question is which cell populations, are targeted by E2 and IGF-1.

Within the central nervous system, there is an overlapping expression of estrogen receptor- α (ER α) and estrogen receptor- β (ER β) mRNA where in some cases the two receptor subtypes are expressed in the same cell (Shughrue et al., 1997). ER α appears to be the dominant receptor mediating E2 actions in many neuronal circuits (McEwen, 2002; Micevych et al., 2003). In the mesencephalon region where nigral DA neurons are located, the ER β mRNA is the predominant receptor subtype expressed (Shughrue et al., 1997). Immunohistochemical studies confirmed the presence of ER β -ir in the substantia nigra pars compacta (SNpc) (Shughrue and Merchenthaler, 2001). Although, neurons were thought to be the predominant cell-type expressing estrogen receptor (ER), recent studies in our laboratory have demonstrated that astrocytes also express functional ERs (Sinchak et al., 2003; Chaban et al., 2004).

Neurons and glia cells not only express ERs but are also immunopositive for IGF-1 and IGF-1R (Garcia-Segura et al., 1991; Cardona-Gomez et al., 2000). In addition, E2 can regulate the expression of IGF-1 and IGF-1R in neural tissue (Cardona-Gomez et al., 2001; Cheng et al., 2001; El-Bakri et al., 2004). As previously demonstrated, E2-induced neuroprotective effects on the nigrostriatal DA system are dependent on the activation of the IGF-1R (Quesada and Micevych, 2004). Therefore, besides being extremely important in female reproductive function (Quesada and Etgen, 2001; Quesada and Etgen, 2002), E2/IGF-1 interactions on neurons and glial cells seem also to be critical for neuroprotection during brain injury (Garcia-Segura et al., 2000; Mendez et al., 2005).

To elucidate the anatomical support for the proposed neuroprotective actions of E2 and IGF-1 on SNpc DA neurons we examined whether ER α , ER β and IGF-1R are expressed on SNpc DA neurons and/or glial cells. Confocal immunofluorescence microscopy was employed to localize ER β -immunoreactive (ir) and IGF-1R-ir with tyrosine hydroxylase immunoreactive (TH-ir) DA neurons in the SNpc. Moreover, retrograde fluorogold labeled SNpc DA neurons were investigated by examining double-immunofluorescence for ER β and IGF-1R to verify whether these SNpc DA neurons project to the striatum. We have also

compared the patterns of IGF-1R-ir with the expression of IGF-1R mRNA in the SN. Finally, due to the fact that glial cells play a critical role in IGF-1 synthesis (Ye et al., 2004), we examined whether ER β -ir and IGF-1R-ir were localized in glial fibrillary acidic protein (GFAP-ir) glial cells of the SN.

MATERIAL and METHODS

Animals

Adult female (225–250 grams) Long-Evans rats bilaterally ovariectomized (OVX) by the provider, were purchased from (Charles River, Wilmington, MA). OVX animals were used in order to eliminate differences over the cycle and avoid possible differences in ER β mRNA expression and translation by E2 treatment (Shughrue and Merchenthaler, personal communication). At arrival at UCLA, all animals were housed two per cage in a partially reversed, 12/12 hr light/dark cycle (lights on at 12 midnight) and provided with food and water *ad libitum*. All of the experimental procedures were approved by the Chancellor's Animal Research Committee at the University of California, Los Angeles.

Tissue processing

Immunohistochemistry—rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and transcardially perfused with physiological saline (4°C) followed by 4% paraformaldehyde in 0.1M Sorensen's phosphate buffer (4°C). Brains were removed, postfixed for 4–24 hrs in 4% paraformaldehyde solution and then transferred to 15% sucrose in 0.1 M phosphate buffer (pH 7.5), for cryoprotection. Coronal 30 μ m sections through the ventral midbrain from four ovariectomized rats were processed simultaneously (Paxinos et al., 1985). Every fourth section was taken through the substantia nigra in a systematic-random manner. Free-floating sections of the ventral midbrain were collected in 0.01 M phosphate buffered saline (PBS, pH 7.4). To quench endogenous peroxidase, all sections were incubated with 10% methanol containing 1% H₂O₂ for 30 min.

In situ Hybridization—rats were deeply anesthetized with sodium pentobarbital (100 mg/kg) and killed by decapitation. Brains were removed, immediately frozen in 2-methylbutane (Sigma) at a temperature of –50 to –60°C and stored at –80°C. Coronal frozen sections of the brain were serially cut at 20 μ m on a cryostat, mounted on Superfrost/plus slides and stored at –70°C until processing. Slides containing fresh-frozen sections were thawed for 10 min, fixed in 4% paraformaldehyde in PBS at 4 °C for 1 hr, washed in PBS (10 mM pH 7.4) 3 $\times$ for 10 min, penetrated with 0.4% Triton X-100 for 10 min and acetylated for 10 min in 100 mM triethanolamine pH 8.0 containing 0.25% (v/v) of acetic acid. The slides were rinsed in PBS (3 $\times$ for 8 min) and distilled water, dehydrated in 50% and 70% ethanol and air-dried.

Antibodies used—Tyrosine hydroxylase (TH) is an affinity purified sheep polyclonal antibody (Chemicon International, Temecula, CA). Insulin-like growth factor-1 receptor (IGF-1R), a mouse monoclonal antibody (Chemicon Research Products, San Diego, CA) was used. For ER β immunocytochemistry; Z8P, an affinity purified rabbit polyclonal antiserum raised against amino acid 468–485 of the C-terminal domain of the mouse ER β (Zymed laboratories, San Francisco, CA). The Z8P antibody was previously characterized (Shughrue and Merchenthaler, 2001) and ER β -ir is present in cells that express ER β mRNA throughout the brain of female rats including the SNpc. Another ER β antibody used was, Ab-1, a rabbit polyclonal antiserum raised against amino acids 467–485 of the C-terminal domain of the Rat ER β (Oncogene Research Products, San Diego, CA), a region with no homology to ER α , and recognizes ER β of rat origin by Western blotting (Li et al., 1997). For ER α , a rabbit polyclonal antibody raised against a peptide corresponding to amino acid

580–599 of the C-terminal domain of the mouse ER α (MC-20, Santa Cruz Biotechnology, Santa Cruz, CA). Glial fibrillary acidic protein (GFAP) immunoreactivity was detected using a rabbit polyclonal antibody raised against human GFAP (G9269; Sigma, St. Louis, MO).

Dual Color Streptavidin-Biotin-Peroxidase Immunocytochemistry

For stereologic analysis of ER β -ir, we employed the Z8P antibody, because it predominately labeled the nucleus of TH-ir SNpc DA neurons. To quantify the number of ER β -ir, IGF-1R-ir in TH-ir SNpc DA neurons, sections were incubated in blocking solution (5% normal donkey serum (NDS), 0.3% Triton X-100 in PBS) for 1 hr in order to block non-specific binding. Then, sections were treated with 0.2% Triton X-100 (3 $\times$) for 10 minutes followed by 0.1 M glycine for 30 min. Sections were incubated with anti-TH (1:6000; Chemicon, Temecula, CA) overnight at 4°C, followed by biotinylated anti-sheep IgG and avidin-biotin-peroxidase complex (Vector Laboratories) for 1 hr at RT. TH immunoreactivity was visualized by 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma). Sections were incubated for 48–72 hr at 4 °C with Z8P (2.5 μ g/ml in 1% NDS, 0.3% Triton X-100 in PBS) or anti-IGF-1R (1:600 in 1% NDS, 0.3% Triton X-100 in PBS). After incubation with primary antibody, sections were incubated with biotinylated anti-rabbit IgG or biotinylated anti-mouse IgG (Vector Laboratories) for ER β and IGF-1R, respectively for 1h at RT. Sections were then incubated in avidin-biotin-peroxidase complex (Vector Laboratories) for 1 hr. After series of washes, ER β and/or IGF-1R were visualized by DAB-nickel peroxidase reaction (Vector DAB substrate kit Cat # SK-4100, Vector Laboratories). Sections were mounted on Superfrost Plus slides, dehydrated through a series of graded alcohols cleared in xylene, and coverslipped with Permount mounting medium (Fisher Scientific).

Stereology

Total numbers of ER β and/or IGF-1R that are expressed in TH immunopositive neurons of the SNpc were estimated using the optical fractionator method (West et al., 1991) with stereoinvestigator[®] software (MicroBright-Field, Colchester, VT). The precision of the serial analysis was assessed by the coefficient of error ($p < 0.1$) (West and Gundersen, 1990; West et al., 1996). Briefly, each subfield of the SNpc was manually outlined at low magnification (5 $\times$ objective) and a grid of 150 $\times$ 150 μ m was superimposed by the software. The SNpc was delimited rostrally by the optic tract, caudally by the level of decussation of the superior cerebellar peduncle, ventrally by the pars reticulata subdivision of substantia nigra, and medially by the third cranial nerve rootlets. A 63 $\times$ oil immersion objective (1.4 NA) was used to achieve optimal optical sectioning during disector analysis. Quantitative analysis was performed by placing an unbiased optical disector frame (50 $\times$ 50 μ m = 2,500 μ m² in the x-y-axis) over the contour outlining the SNpc. The final post-processing thickness of the sections was measured by the microcator, resulting in a 15 μ m averaged mounted section thickness. A 1.5 – 2 μ m guard zone was used to exclude artifacts at tissue surface. For colocalization quantitative analysis, only those TH-ir cells that clearly demonstrated nuclear ER β were counted.

Single Label Immunofluorescence

To enhance the sensitivity of the immunocytochemical reaction the tyramide signal amplification method (TSA-Direct, Perkin Elmer Life and Analytical Sciences, Boston, MA) was employed. Sections were incubated 48–72 hr at 4°C with anti-TH (1/500 in 1% NDS, 0.3% Triton X-100 in PBS); anti-IGF-1R (1:600 in 1% NDS, 0.3% Triton X-100 in PBS); Z8P (1/400 in 1% NDS, 0.3% Triton X-100 in PBS) or Ab-1 (1/200 in 1% NDS, 0.3% Triton X-100 in PBS). After incubation with primary antibodies, sections were washed in PBS (3 $\times$) for 10 min and incubated for 1 hr at RT with the secondary antibodies. To visualize TH-ir, rodamine (RITC)-conjugated Donkey Anti-Sheep (1:100; Jackson

ImmunoResearch lab, INC; Baltimore, PA) secondary antibody was used. To visualize ER β -ir, sections were incubated with biotinylated donkey anti-rabbit serum (1:1000; Jackson ImmunoResearch Lab, West Grove, PA). These sections were then incubated with HRP-conjugated streptavidin (1:100; TSA kit in TNB buffer) for 30 min at RT, rinsed in PBS (3 $\times$) for 5 min, and then incubated with fluoroscein conjugated tyramide (FITC-conjugated tyramide; 1:100 in 1 $\times$ amplification diluent; TSA kit) for 10 min at RT. To visualize IGF-1R, sections were incubated with biotinylated donkey anti-mouse serum (Vector Labs; 1:200 in PBS) for 1 hr. Sections were then washed in PBS (3 $\times$) for 10 min and incubated with HRP-conjugated streptavidin (1:100; TSA kit in TNB Buffer) for 30 min at RT, rinsed in PBS (3 $\times$) for 5 min, incubated with fluoroscein conjugated tyramide (FITC-conjugated tyramide; 1:100 in 1 $\times$ amplification diluent; TSA kit) for 10 min at RT. Tissue sections were washed in PBS (3 $\times$) for 10 min, mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), air-dried and coverslipped using Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

Double Immunofluorescence

For double immunofluorescence for ER α / β or IGF-1R in TH-ir nigral DA neurons, sections were incubated for 48–72 hr at 4°C with a cocktail of antisera raised in different species containing anti-TH with either IGF-1R or Ab-1 or Z8P. Double immunofluorescences for ER β or IGF-1R in GFAP-ir glial cells, sections were processed as mentioned above. Following amplification of either IGF-1R and/or ER β with the TSA kit as previously described above, sections were incubated in blocking solution (5% NGS in PBS for 1 hr) and incubated for 1 hr at 4 °C with rabbit anti-GFAP (G9269; Sigma, St. Louis, MO; diluted 1:50) in 1% NGS and 0.3% Triton X-100 in PBS. GFAP-ir was detected by incubating the sections for 1 hr in Texas Red –conjugated goat anti-rabbit antibody (Molecular Probes; diluted 1:1000; 1% NGS). Colocalization for ER β or IGF-1R in GFAP-ir cells in the SN was analyzed by the (Carl Zeiss Inc, Thornwood NY) LSM 3.2 software. Z-stacks of images were taken using a 40 $\times$ oil immersion objective. An area of 1000 mm² was delineated from each Z-stack. Quantitative data is expressed as an area of a colocalized number of pixels.

Controls

The following controls were performed: omitting the primary or secondary antibodies, dilution series and nonimmune rabbit or mouse immunoglobulin G (IgG; 50 μ g/ μ l). The following peptides were used for preabsorbance: the epitope peptide to anti-ER α (MC20, Santa Cruz Biotechnology); and peptide to block the anti-ER β Z8P antibody and Ab-1 antibody was preabsorbed with the 19 –amino acid sequence of ER β (amino acids 467–485) (PA1-310B; Affinity Bioreagents). All staining was blocked by preabsorption. The pattern of staining for each antibody was the same in double or single labeled sections. For double labeling, the order of primary antibodies was reversed and homologous and heterologous absorption controls were done. Nonspecific immunofluorescent staining, cross-immunostaining, or bleeding-through were not observed.

Fluorescence microscopy

Immunofluorescence double-labeled profiles were scanned with a Zeiss Axioskop LSM 510 laser scanning confocal microscope system equipped with an Axiocam CCD camera and a digital image analysis system (Zeiss USA Inc.). The excitation source was a krypton-argon laser (Coherent, Santa Clara, CA) with output at 488,568 and 633 nm. FITC was visualized with a 488 nm excitation and a 515–540 nm bandpass emission filter. Rhodamine was visualized with a 530 nm excitation and 560–610 nm bandpass emission filter. Z-stacks were obtained by using identical settings for laser intensity, confocal aperture, scan speed and Z-stack step size (0.5 – 1.0 μ m). All final images were adjusted for contrast and background using Adobe Photoshop 5.0 (Mountain View, CA).

Retrograde Tract Tracing Experiments

OVX rats were implanted with a guide cannula aimed at the striatum (coordinates: antero-posterior, bregma +0.48 mm; lateral, $\pm$ 3.0 mm; and dorso-ventral, -5.0 mm, (Swanson, 1992). A 29 gauge cannula containing the retrograde tracer Fluorogold™ (FG; 5% w/v dissolved in 0.9% saline; Fluorochrome, Denver, CO) was used to slowly eject 1 μ l of FG over 5 min (Iravani et al., 2002). The cannula was connected to a 25 μ l Hamilton syringe via a plastic tube. The cannula was allowed to remain in place an additional 5 min to allow diffusion of drug away from the cannula tip. Seventh day after FG injection, animals were perfused.

Fluorogold detection

Immunocytochemical labeled sections from FG injected animals were visualized with a Axiofluoroskop 2 equipped with epifluorescent illumination and *Axiovision 3.1* software. Digital pictures were taken with an Axiocam CCD camera mounted on the microscope (Zeiss Inc. USA). To detect the immunolabeling of either ER β -ir and/or IGF-1R-ir in striatal projecting SNpc DA neurons, SN sections from rats injected with FG into the striatum were processed for immunofluorescence for either ER β and/or IGF-1R immunohistochemistry as described above. The number of FG SNpc cells immunolabeled for either ER β and/or IGF-1R were averaged at 4 different levels corresponding to -4.8 mm, -5.1 mm, -5.4 mm and -5.7 mm with respect to bregma (Paxinos et al., 1985) and expressed as a percentage. FG was visualized with a 333 nm excitation and wideband ultraviolet emission filter at 408 nm. FITC was visualized with a 488 nm excitation and a 515–540 nm bandpass emission filter.

In-Situ Hybridization for IGF-I Receptor

The IGF-1R probe was complementary to 15 bases of 5' untranslated region encoding the signal peptide, which correspond to the first 53 amino acids of the alpha subunit (Werner et al., 1989). The cDNA EcoR I/Sma I fragment (265 bp) was subcloned into a pGem-3Z transcription vector (gift from Dr. D. LeRoith; National Institutes of Health, Bethesda, MD, USA), and linearized with *EcoRI* (antisense) or *BamHI* (sense) and isotopically labeled riboprobes were generated by *in vitro* transcription using SP6 (antisense) and T7 (sense) polymerases in the presence of 35 S-UTP and 35 S-CTP (Amersham Biosciences Corp. Piscataway, NJ). Riboprobes were purified through NucTrap purification columns (Stratagene), diluted in hybridization buffer (100 mM Tris pH 7.5, 600 mM NaCl, 1 mM EDTA, 0.1 mg/ml sonicated salmon sperm, 0.5 mg/ml t-RNA, 1 $\times$ Denhardt's, 10% dextrane sulfate, 50% formamide) to 100,000 cpm/ μ l and used within 48 hr.

Tissue sections were covered with hybridization buffer (80 μ l) containing antisense or the sense riboprobes, coverslipped to prevent evaporation and hybridized for 16–18 hr at 58°C in a moist chamber containing 50% v/v formamide. Following hybridization, coverslips were carefully removed, sections were washed with 2 $\times$ sodium citrate (SSC) and 1 $\times$ SSC for 15 min each at room temperature and exposed to RNase buffer (20 μ g/ml RNase A, 1 U/ml RNase T1, 10 mM Tris-HCl, 0.5 M NaCl, 1 mM EDTA) at 37°C for 40 min. Afterwards, slides were washed in decreasing concentrations of SSC to a final stringency of 0.1 $\times$ SSC at 62 °C for 1 hr. Finally slides were washed in double distilled water, dehydrated through graded ethanols and air dried. Slides were dipped in K5 photoemulsion (Ilford, Paramus, NJ), diluted 1:1 with double-distilled water. Emulsion coated slides were air-dried in the dark for 12 hr, packed into light-tight boxes and exposed at 4°C for 21–28 days, developed (D-19 developer) and fixed (Kodak fixer) both products from (Kodak Co, Rochester, NY). The slides were counterstained with Toluidine blue, dehydrated and coverslipped with DPX (BDH Chemical Ltd. London, UK).

RESULTS

ER immunoreactivity in the midbrain of female rats

The distribution of ER β in the SNpc was examined using two distinct antisera (Z8P and Ab-1). Single immunolabeling with these ER β antibodies displayed similar immunostaining patterns for ER β , especially in the SNpc (Fig 1E and 1F). Throughout the mesencephalon, the ventral tegmental area (VTA) had the densest number of ER β -ir cells, which expanded laterally to the SNpc, substantia nigra pars reticularis and substantia nigra pars lateralis. Within the SN, both ER β antibodies labeled a high density of cells in the SNpc compared to the pars reticularis or pars lateralis (Fig 1E and 1F). Moreover, scattered ER β -ir were also noted in the interpeduncular nucleus, periaqueductal gray, mesencephalic reticular nucleus, medial lamniscus, raphe nuclei and optic tectum as previous confirmed by (Shughrue and Merchenthaler, 2001)(data not shown). For ER α -ir, the rabbit polyclonal antibody to ER α did labeled an abundant amount of ER α in the mediobasal hypothalamus, a region abundant with ER α ; however, only a scattered number of ER α -ir cells were detected in the VTA, but none were observed in the SN (data not shown).

In situ hybridization and immunoreactivity of IGF-1R in the midbrain of female rats

IGF-1R mRNA and protein were demonstrated by in situ hybridization and immunocytochemistry in the mesencephalon region of OVX rat (Fig 1C and 2B). Within the ventral midbrain, abundant hybridization signal for IGF-1R was observed in the VTA and SN (Fig 2B). Within the SN, the SNpc had the highest density of IGF-1R-ir cells followed by the substantia nigra pars reticularis (Fig 1C and Table 1). Other regions that had strong IGF-1R mRNA expression and protein immunoreactivity, include the interpeduncular nucleus, and the superior and inferior colliculus, with moderate expression in the medial geniculate (data not shown).

Coexpression of ER β and IGF-1R on TH-ir neurons in the SN

The present double immunolabeling studies revealed that in the midbrain, the VTA had the majority of TH-ir cells that express ER β , followed by the SNpc with a few scattered double-labeled cells in the SN pars reticulata and pars lateralis (Table 1). However, the cellular localization was significantly different between Ab-1 and Z8P ER β antibodies. The Ab-1 ER β antibody showed ER β -ir predominantly in the cytoplasm of TH-ir SNpc DA neurons with sparse nuclear labeling (Fig 3A), whereas the Z8P ER β antibody predominately labeled the nucleus with low extra-nuclear staining in TH-ir DA SNpc neurons (Fig 3B). Unbiased stereologic analysis revealed that in the SNpc, $39.8\% \pm 4.7$ of TH-ir cells were double-labeled with ER β -ir (Figure 4A & 4B; Table 2). A majority of double-labeled neuronal somata were oval in shape, with an average neuronal diameter of $14.8 \pm 0.7 \mu\text{m}$ and nuclear diameter of $8.3 \pm 0.4 \mu\text{m}$. Confocal microscopy revealed a significant colocalization of ER β - and TH-ir in SNpc DA neurons (Fig 5A–C).

Virtually all of TH-ir cells were immunopositive for IGF-1R within the mesencephalon. The VTA demonstrated an abundant colocalization (Table 1). Within the SN, the SNpc demonstrated a complete colocalization $98.5\% \pm 0.5$, which expanded laterally to the SN pars lateralis and reticularis (Table 1; Fig 4C & 4D; Table 2). Confocal microscopy revealed that IGF-1R-ir was predominantly localized to the membrane in TH-ir cells although some cytoplasmic labeling was also observed (Fig 6A–C).

Coexpression of ER β and IGF-1R in fluorogold neurons in the SN

To verify that TH-ir DA neurons in the SNpc that are ER β -ir and/or IGF-1R-ir project to the striatum, FG was injected into the striatum to retrogradely label SNpc DA neurons (Fig 1B). $43 \pm 8.9\%$ of these FG striatal projecting neurons were ER β -ir (Fig 5D–F). Likewise, the

vast majority of FG labeled neurons were immunopositive for IGF-1R ($97.8 \pm 9.8 \%$) (Fig 6D–F).

Localization of ER β and IGF-1R in astrocytes of the midbrain

The morphology of GFAP-ir glial cells in the SN of OVX rats were stellate, hypertrophic, thorn-shape and coiled (Fig 7A and 7D)(Lu et al., 2003). Analysis of colocalization revealed that ER β -ir with Z8P antibody labeled $33 \pm 8.9 \%$ GFAP-ir astrocytes. ER β -ir was distributed in the nucleus, somal cytoplasm and in the processes of GFAP-ir cells (Fig 7C). $67 \pm 10.5 \%$ of the GFAP-ir cells contained IGF-1R-ir. The IGF-1R was primarily localized in the perikarya and processes of GFAP-ir astrocytes (Fig 7F).

DISCUSSION

In these studies we demonstrate that SNpc DA neurons and GFAP-ir astrocytes in the SN express ER β and IGF-1R in OVX rats. These results provide anatomical basis for the proposed mediated neuroprotection of E2 and IGF-1 in SNpc DA neurons. The present immunohistochemical study demonstrates the localization of ER β in SNpc DA neurons without a parallel localization of ER α in the SN and supporting the role for ER β in neuronal survival of SNpc DA neurons (Sawada et al., 2000; Wang et al., 2001). In addition, since both glial cells and neurons were immunoreactive for ER β and IGF-1R, suggest a complex interaction in which paracrine and/or autocrine functional regulation of SNpc DA neurons.

E2 and IGF-1 interactions have been shown to be important in physiological regulation of CNS activity such as regulation of growth, sexual maturation and adult neuroendocrine function (Fernandez-Galaz et al., 1997; Miller and Gore, 2001; Quesada and Etgen, 2001; Quesada and Etgen, 2002). Furthermore, E2 and IGF-1 interactions have also implemented in protecting the CNS in different experimental models of neurodegenerative diseases (Azcoitia et al., 1999b; Garcia-Segura et al., 2001; Carro et al., 2003). For instance, our previous studies demonstrated that E2 acting through activation of IGF-1R produced an 80% survival of SNpc DA neurons treated in vivo with 6-OHDA, compared to 40% survival after 6-OHDA lesion in the midbrain (Quesada and Micevych, 2004). In the present study, stereologic analysis reveals that 39.8% of SNpc DA neurons have ER β -ir, however 33% of GFAP-ir glial cells in the SN also displayed ER β -ir. E2 has been shown to increase the expression of growth factors, such as IGF-1 in glial cells (Fernandez-Galaz et al., 1997; Zhang et al., 2004) suggesting that astrocytic IGF-1 may act to spare DA neurons from neurodegenerative processes. Through these actions, E2 may protect the remaining non-expressing ER β SNpc DA neurons indirectly by the release of IGF-1 from astrocytes. A hypothetical model for these interactions is presented in Figure 8. The localization of ER β on GFAP-ir cells in the SN provide cellular support for E2 actions on these glial cells to express and secrete beneficial anti-neurotoxic signals to SNpc DA neurons. Therefore, ER β and IGF-1R may be relevant for the coordinated actions of E2 and IGF-1 on both SNpc DA neurons and glial cells.

Our experiments employed *in situ* hybridization and immunocytochemistry to reveal the presence of IGF-1R in the SNpc of OVX female rats. Almost all of SNpc DA neurons (98.5%) and a vast majority of GFAP-ir glial cells in the SN are IGF-1R-ir (67.0%). These results are consistent with the idea of IGF-1 playing a major neuroprotective action on the nigrostriatal DA system, which accounts for the protection of SNpc DA neurons against 6-OHDA lesions, when animals receive centrally and peripherally administered IGF-1 (Quesada and Micevych, 2004). Therefore, if the IGF-1 system is activated by neuronal injury, then antagonism of IGF-1R would exacerbate the neuronal injury as we and others previously shown (Quesada and Micevych, 2004).

Although there is some controversy about the distribution of ER β -ir in the SN (Creutz and Kritzer, 2002) our data support the presence of ER β -ir, in agreement with others (Shughrue and Merchenthaler, 2001; Zhang et al., 2002; Mitra et al., 2003). In contrast, a recent report using the Z8P antibody failed to detect ER β -ir in the SNpc or in GFAP-ir cells of the SN (Creutz and Kritzer, 2002). These authors found colocalization of ER β -ir in TH-ir cells located in a region immediately dorsal to the SNpc, but not below. To examine this possibility, the present study-combined tract tracing with ER β immunohistochemistry and clearly demonstrates ER β -ir in SNpc DA neurons that project to the striatum, confirming the previous report (Shughrue and Merchenthaler, 2001).

The cellular distribution of ER β with the Z8P antibody in SNpc DA neurons and glial cells is in agreement with previous reports demonstrating nuclear ER β -ir and cytoplasmic labeling in GFAP-ir astrocytes in the interpeduncular nucleus of female rats (Zsarnovszky et al., 2002) and in cultured cortical astrocytes (Chaban et al., 2004). In addition, the nuclear and cytoplasmic distribution of ER β in SNpc DA neurons and in glial cells throughout the brain has been observed with the Ab-1 antibody (Cardona-Gomez et al., 2000; Ravizza et al., 2002). Our present results are in agreement with others demonstrating differences in cellular localization of ER β by Z8P (Shughrue and Merchenthaler, 2001; Blurton-Jones and Tuszyński, 2002; Ravizza et al., 2002; Chakraborty et al., 2003) and Ab-1 (Azcoitia et al., 1999a; Ravizza et al., 2002). As discussed by Shughrue and Merchenthaler (2001) the difference in staining between these two antibodies (Z8P and Ab-1) may be a difference between rat vs. mouse peptide (4aa), peptide conjugation, inoculation, and purification resulting in antisera that are vastly different in both their sensitivity and specificity for rodent ER β . Moreover, using different ER β antibodies than in the present study, cytoplasmic staining on SNpc DA neurons has been demonstrated (Ravizza et al., 2002; Zhang et al., 2002; Mitra et al., 2003). Recently, there has been an increasing body of evidence suggesting that membrane-bound ERs are present in the brain (Nishio et al., 2004) and mediate neuroprotection as demonstrated in cultured mesencephalic DA neurons (Bains, 2005) and cultured cortical astrocytes (Bains, 2005; Dhandapani et al., 2005). Despite the subcellular distribution of ER β -ir in the SNpc, the present results suggest that the neuroprotective effects of E2 are mediated by ER β which may act through transcriptional regulation or rapid signaling cascades which can lead to signaling transcription regulation (Dubal et al., 1998; Garcia-Segura et al., 2001; Wise et al., 2001; Ivanova et al., 2002; Boulware et al., 2005).

E2 and IGF-1 can mutually regulate each others receptor expression (Westley and May, 1994; Cardona-Gomez et al., 2001). For example, IGF-1 can activate ER, in the absence of E2, suggesting that ER may play an important regulating role in IGF-1R signaling (Ma et al., 1994; Cardona-Gomez et al., 2001). The localization of ER β - and IGF-1R-ir on nigral DA neurons and GFAP-ir glial cells in the SN may provide the anatomical support for these interactions between E2 and IGF-1 (Mendez et al., 2003). In this context, E2 may upregulate the expression of IGF-1/IGF-1R or ER β may be important for IGF-1 neuroprotection by which IGF-1 regulates the expression of ER β in SNpc DA neurons. Alternatively, MAPK and PI3K, two rapid intracellular kinases activated by E2 and IGF-1 have been shown to protect neurons against toxic effects in vitro (Singer et al., 1999; Singh et al., 2000). Therefore, coexpression of ER β and IGF-1R in the same cell may allow a cross-talk between ER β and IGF-1R signaling pathways mediating neuroprotection (Mendez et al., 2005). Indeed, future experiments are needed to examine the intracellular signaling pathways used by E2 and IGF-1 in the neuroprotection of nigral DA neurons.

In addition to genetic and environmental factors that contribute to vulnerability for neurodegeneration, age is one of the most important risk-factor. As women age and become menopausal, systemic E2 levels drop to undetectable levels and IGF-1 levels are

significantly reduced (Lamberts et al., 1997), suggesting that the interaction of E2 and IGF-1 signaling pathways in neuroprotection may be relevant concerning the increased neurodegenerative incidence at old age. Moreover, in human brains, the SN has an abundant expression of IGF-1R (De Keyser et al., 1990). These results along with past studies suggest that decreased IGF-1R expression levels and/or IGF-1R signaling capacity may be underlie susceptibility for deficient in neurodegenerative diseases such as Parkinson's disease. Within the context of this working hypothesis, the survival of SNpc DA neurons is dependent in a trophic influence of E2 and IGF-1. Therefore, the IGF regulatory system will be a prime target for future studies into the pathogenesis of several age and sex hormone related degenerative diseases providing important clues as to how E2 is protective.

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Fig 1. Immunofluorescent photomicrographs from substantia nigra pars compacta sections. (A) TH (DAB), black dashed lines depicts SNpc. (B) TH (Texas Red), white dashed lined depicts SNpc. (C) Fluorogold retrograde staining. (D) Immunofluorescent product for IGF-1R (green). (E) Immunofluorescent product for Z8P ERβ (Green). (F) Ab-1 ERβ (Green). (A) Scale bar = 200 μm. (B–F) Scale bar = 50 μm.

Fig 2.

Darkfield photomicrographs of the substantia nigra of the adult OVX female rat showing hybridization signal for IGF-1R mRNA. (A) Panel represents sense probes. (B) Panel is the result with an antisense probe. (C) Representative photomicrograph depicting brightfield localization of IGF-1R mRNA (silver grains) counterstained with thioinin. Arrows point to spots of dense signal corresponding to the positive cells. Scale bar = 200 μm (A & B). Scale bar = 50 μm (C). Ventral Tegmental Area (VTA), red nucleus (R), medial lemniscus (ML), substantia nigra pars compacta (SNpc), pars lateralis (SNpl), pars reticularis (SNpr).

Fig 3.

Immunofluorescent photomicrographs demonstrating differences in localization of ER β -ir between Z8P antibody (A) and Ab-1 (E) on TH-ir SNpc DA neurons. (B–D) Enhanced magnification of ER β and TH colocalization. TH (red, B), Z8P (green, C). Overlay (D) of B and C; arrow demonstrates nuclear labeling. (F–H) Enhanced magnification of ER β and TH colocalization. TH (red, F), and Ab-1 antibody (green, G). Overlay (H) of F and G; arrow demonstrates cytoplasmic labeling. Scale bar = 10 μ m.

Fig 4.

Low (A) and high-power (B) photomicrographs illustrating the distribution of nickel DAB ERβ-ir on TH-ir SNpc DA neurons. (B) Arrowheads point to TH-ir neurons displaying nuclear ERβ-ir, arrows demonstrate no nuclear ERβ-ir. Low (C) and high-power (D) photomicrographs illustrating the distribution of nickel DAB IGF-1R-ir in TH-ir SNpc DA neurons. (D) Arrowheads, demonstrate IGF-1R-ir in TH-ir SNpc DA neurons, arrows demonstrate no IGF-1R-ir. Scale bar = 50 μm (A and C). Scale bar = 10 μm (B and D).

Fig 5. Colocalization of ER β and FG labeled SNpc DA neurons. Confocal microscopy showing the fluorescent immunoreaction product for Fluorogold (red color substituted with Axivision 3.1 software, A). Immunofluorescent product for Z8P ER β (green, B). 25 Overlay images (C) from A and B; Arrows indicate ER β immunolabeling (yellow) in FG retrograde labeled DA neurons of the SNpc (Red). Scale bar = 200 μ m (A–C).

Fig 6. Immunolocalization of IGF-1R in SNpc DA neurons. Confocal microscopy displaying fluorescent immunoreactivity for TH (red, A), and IGF-1R (green, B and E). Overlay images (C) from A and B; arrow indicates co-localization (yellow) of IGF-1R and TH in DA neurons of the SNpc. Arrowhead indicates TH-immunoreactivity alone. Fluorogold (D) (red color substituted with Axivision 3.1 software). Overlay images (F) from D and E, co-localization (yellow) in fluorogold containing neurons in the SNpc (Red). Scale bar = 50 μ m (A–C) and 200 μ m (D–F).

Fig 7.
Confocal photomicrographs from substantia nigra sections showing immunofluorescence for GFAP (red, A and D), ER β (green, B) or IGF-1R (green, E), and their colocalization (yellow, C and F). Magnified image in (C) displays nuclear, cytoplasmic and processes immunostaining for ER β . Scale bar = 50 μ m.

Fig 8.

Proposed model for the neuroprotective interactions between E2 and IGF-1 on SNpc DA neurons. A) E2 and IGF-1 can directly protect the subset of SNpc DA neurons that express ERβ (green labeled nucleus) or IGF-1R (red dots). B) E2 and IGF-1 can stimulate secretion of neuroprotective factors (e.g. IGF-1; yellow filled circles) in glial cells that express ERβ, resulting in indirect neuroprotection of SNpc DA neurons that are ERβ negative.

TABLE 1Distribution of ER β and IGF-1R immunoreactivity in the mesencephalon region of ovariectomized female rats

	ER β	IGF-1R
Substantia nigra		
Compacta	N++, A++	N+++, A+++
Lateral	N+, A++	N++, A+++
Reticular	N+, A++	N+, A+++
Ventral tegmental area	N+++, A+++	N+++, A+++

+, scarce; ++, frequent; +++, very frequent. N, neurons; A, astrocytes. Examination of the sections and evaluation of the immunostained profiles were performed by two investigators independently.

TABLE 2Summary of Estrogen Receptor- β and IGF-1R Localization and TH-*ir* SNpc DA Neurons

No. of TH cells	ER β /TH cells	Colocal (%)	IGF-1R/TH cells	Colocal (%)
12988 $\pm$ 2148	4910 $\pm$ 440	39.8 $\pm$ 4.7	12818 $\pm$ 2165	98.5 $\pm$ 0.5

All values are shown as mean $\pm$ SEM, Number of cells are counted within 30 μ m coronal sections (n = 4).