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Effect of Ovarian Steroids on Gene Expression Related to Synapse Assembly in Serotonin Neurons of Macaques

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Abstract

Dendritic spines are the elementary structural units of neural plasticity. In a model of hormone replacement therapy (HT), we sought the effect of estradiol (E) and progesterone (P) on gene expression related to synapse assembly in a laser captured preparation enriched for serotonin neurons from rhesus macaques. Microarray analysis was conducted (n=2 animals/treatment) and the results confirmed for pivotal genes with qRT-PCR on additional laser captured material (n=3 animals/treatment). Ovariectomized rhesus macaques were treated with either placebo, E or E+P via Silastic implants for 1 month. The midbrain was obtained, sectioned and immunostained for tryptophan hydroxylase (TPH). TPH-positive neurons were laser captured using an Arcturus Laser Dissection Microscope (Pixel II). RNA from laser captured serotonin neurons was hybridized to Rhesus Affymetrix GeneChips for screening purposes. There was a 2-fold or greater change in the expression of 63 probe sets in the Cell Adhesion Molecule (CAM) category, and 31 probe sets in the Synapse Assembly category were similarly altered in E and E+P treated animals. qRT-PCR assays showed that E-treatment induced a significant increase in Ephrin receptor A4 (EPHA4) and in Integrin A8 (ITGA8), but not Ephrin receptor B4 (EPHB4) or Integrin B8 (ITGB8) expression. E also increased expression of cadherin 11 (CDH11), neuroligin 3 (NLGN3), neuroligin 3 (NRXN3), syndecan 2 (SCD2) and neural cell adhesion molecule (NCAM) compared to placebo. Supplemental P treatment suppressed E-induced gene expression. In summary, ovarian steroids target gene expression of adhesion molecules in serotonin neurons that are important for synapse assembly.

Keywords

serotonin; macaques; estrogen; progesterone; dorsal raphe; Affymetrix array; quantitative PCR; cell adhesion molecules; synapse assembly

Introduction

The serotonin system modulates a wide range of neural outcomes from emotion to intellect to metabolism; it is a target of pharmacotherapies, steroid hormones, cytokines, neuropeptides and trophic factors, all of which impact the generation and efficacy of serotonin neurotransmission. We have reported that ovarian steroids increase serotonin neural function at multiple levels including gene and protein expression, as well as cellular resilience (Bethea et al. 2002; Bethea et al. 2009). In addition, we have shown that ovariectomized macaques in a semi-free ranging troop exhibit an increase in anxiety related behaviors compared to tubal-ligated females, which correlated with a significant decrease in serotonin related gene expression (Bethea et al. 2011; Coleman et al. 2011). In humans, a number of studies indicate that the loss of ovarian steroids, either induced with complete hysterectomy or gradually through menopause, will negatively impact mood in a significant subset of women (Epperson et al. 1999; Heikkinen et al. 2006; Joffe and Cohen 1998; Kugaya et al. 2003; Schmidt et al. 2004; Sherwin 1991; Soares et al. 2003).

It has been suggested that antidepressants and other pharmacotherapies may act by promoting neuronal plasticity (Manji et al. 2003). The underlying structural element of neuronal plasticity in the adult nervous system is the dendritic spine (Ethell and Pasquale 2005). In addition, dendritic spines are the morphological basis for excitatory neurotransmission (Butler et al. 1998; McKinney et al. 1999). A significant body of literature has demonstrated that estrogen (E) increases dendritic spines in the hippocampus and cortex (Cooke and Woolley 2005; Hao et al. 2003; Hao et al. 2006; Murphy et al. 1998), but the dorsal raphe nucleus has not been examined. A recent role for ER β in spine proliferation has emerged (Liu et al. 2008; Srivastava et al. 2010) and serotonin neurons exclusively express ER β in macaques (Gundlah et al. 2000; Gundlah et al. 2001). We hypothesized that ovarian steroids induce dendritic spine proliferation on serotonin neurons, which could have a profound effect on serotonergic neurotransmission.

The issue of steroid supported dendritic spine proliferation on serotonin neurons may be important for menopausal women grappling with issues surrounding hormone therapy (HT). Women experience ovarian failure and loss of ovarian steroid production around 50 years of age. Thus, with extended life spans, a woman may live 35-40 years without ovarian steroids. We speculate that dendritic spines on serotonin neurons shrink or atrophy due to lack of steroid supported gene expression, which may decrease serotonergic support of higher neural functions.

Dendrite protrusion, maturation and stabilization involve a complex repertoire of cytoskeletal reorganization, expression of glutamate receptors and synapse assembly. We recently reported that ovarian steroids increase gene expression in laser captured serotonin neurons for the effector GTPase proteins called cdc42, Rac 1 and Rho A in monkeys (Bethea and Reddy 2010). These small molecules activate cascades that lead to actin reorganization and production of a mature dendritic spine. In addition, we have found that ovarian steroids increase expression of AMPA and NMDA subunits in laser captured serotonin neurons and increase expression of pivotal components of the glutamate cycle within glia and glutamate neurons (Bethea and Reddy 2011). The glutamate receptors activate calcium calmodulin-dependent kinases, which also lead to activation of the RhoGTPases through intermediaries (Saneyoshi et al. 2010). In addition, upstream of the RhoGTPase are integral membrane proteins, which play a role in cell adhesion and cell-cell or cell-matrix communication, and which can directly activate RhoGTPases (Ethell and Pasquale 2005). Cell adhesion molecules are also involved in synapse assembly.

Hormone therapy has been under assault for treatment of menopausal women since release of the results of the Women's Health Initiative, which administered conjugated equine estrogens and medroxyprogesterone acetate to women approximately 10 years after onset of menopause. Estrogenic compounds can be used in women after complete hysterectomy and estrogenic plus progestogenic compounds should be used in women with a reproductive tract. Women are usually prescribed HT during perimenopause and comply with treatment for years. However, our cost effective model involves ovariectomy of adult rhesus macaques for a relatively short time, i.e. 5-8 months, followed by subcutaneous delivery of bioidentical estradiol (E) or estradiol plus progesterone (EP) for one month. Therefore, we only examine one time point. However, it probably reflects a significant level of maturity and stabilization, which were shown to occur by 24 hours after protrusion in the hippocampus (De Roo et al. 2008). Using the Rhesus Affymetrix cDNA array and quantitative (q) RT-PCR, we examined the effect of E, with and without supplemental P, on multiple genes related to synapse assembly in a laser capture preparation enriched for serotonin neurons from rhesus monkeys. Nine pivotal gene changes predicted by the microarray were examined by qRT-PCR.

Materials and Methods

The Oregon National Primate Research Center (ONPRC) Institutional Animal Care and Use Committee approved this study.

Animals and treatments

Nine adult female rhesus monkeys (*Macaca mulatta*) were oophorectomized (Ovx) by the surgical personnel of ONPRC between 5 and 8 months before assignment to this project according to accepted veterinary surgical protocol. All animals were born in China, were aged between 7-14 years by dental exam, weighed between 5 and 8 kg, and were in good health.

Animals were either treated with placebo (**Ovx-control group; n=3**), or treated with estradiol (E) for 28 days (**E group; n=3**), or treated with E for 28 days and then supplemented with progesterone (P) for the final 14 of the 28 days (**E+P group; n=3**). The placebo treatment of the spay-control monkeys consisted of implantation with empty Silastic capsules (s.c.). The E-treated monkeys were implanted with two 4.5-cm E-filled Silastic capsules (i.d. 0.132 in.; o.d. 0.183 in.; Dow Corning, Midland, MI). The capsule was filled with crystalline estradiol (1,3,5(10)-estratrien-3,17-b-diol; Steraloids, Wilton, NH). The E+P- treated group received E-filled capsules, and 14 days later, received one 6-cm capsule filled with crystalline progesterone (4-pregnen-3,20 dione; Steraloids). All capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10mg/kg, s.c.; Fort Dodge Laboratories, Fort Dodge, IA).

The monkeys were euthanized at the end of the treatment periods according to procedures recommended by the Panel on Euthanasia of the American Veterinary Association. Each animal was sedated with ketamine, given an overdose of pentobarbital (25 mg/kg, i.v.), and exsanguinated by severance of the descending aorta.

Steroid Hormone Assays

Assays for E and P were performed utilizing a Roche Diagnostics 2010 Elecsys assay instrument. Prior to these analyses, measurements of estradiol and progesterone on this platform were compared to traditional RIA's as previously reported (Bethea et al. 2005). The E+P treatment regimen has been shown to cause differentiation of the uterine endometrium in a manner similar to the normal 28-day menstrual cycle (Brenner and Slayden 1994).

Tissue preparation and Laser Capture Dissection (n=9; 3 animals/treatment group)

The left ventricle of the heart was cannulated and the head of each animal was perfused with 3 liters of 1X cold RNA-later buffer (Ambion Inc., Austin, TX) plus 20% sucrose. The brain was removed from the cranium, dissected into blocks and frozen at -80°C . The pontine midbrain block was placed in a cryostat (Microm HM500OM) and brought to -20°C . Thin sections ($7\text{ }\mu\text{m}$) through the dorsal raphe nucleus were thaw mounted onto plain glass slides and frozen at -80°C . The next morning, the sections were processed in a rapid, RNase free immunohistochemical assay for tryptophan hydroxylase (TPH). The sections were immersed in cold acetone for 1 minute, cold ethanol for 30 seconds, cold PBS for 3 minutes, and then covered with normal rabbit serum (NRS, 1/500) containing 1% RNase inhibitor for 10 minutes. The NRS was blotted and the sections were covered with sheep anti-TPH (1/300; Chemicon, Temecula, CA) containing 1% RNase inhibitor for 30 minutes, then immersion washed in PBS for 3 minutes, and covered with biotinylated rabbit anti-sheep serum (1/120; Vector Laboratories, Burlingame, CA) containing 1% RNase inhibitor for 20 minutes. The sections were then immersed in PBS for 3 minutes, covered with Vector ABC reagent for 30 minutes, immersed in cold 0.2 M Tris (pH 8.2), immersed in cold diaminobenzidine (DAB) containing H_2O_2 (30% solution diluted 1/5000) and dehydrated in 100% ethanol for 2 minutes. Finally, the slides were immersed in xylene for 2 minutes and then dried under vacuum for 1 hr prior to laser capture. Serotonin neurons appeared darkly stained and were captured with an Arcturus Laser Dissection Microscope (PixCell II). After capture to the microcap film (Capsure macro-211), the films were removed from the caps and immersed in lysis buffer. Up to 10 films with 1000-3000 pulses each were collected into one microtube containing 200 μl of lysis buffer. Approximately 150,000 laser pulses were executed for a pool (12-15 microtubes were pooled). Individuals interested in obtaining other areas of the brain for study are encouraged to contact the authors.

RNA extraction from laser captured neurons

Two pools were prepared from two placebo, two E-treated and two E+P treated animals. One of the pools was used for hybridization to the microarray and one pool was set aside for qRT-PCR. An additional pool was prepared from another placebo, E-treated and E+P treated animal for qRT-PCR. Thus, 2 animals per group were used for hybridization and 3 animals per group were used to confirm gene changes with qRT-PCR.

The tubes containing the lysis buffer and films were vortexed to dislodge the captured material from the films. Each tube was adjusted to 350 μl of lysis buffer and then 350 μl of 70% ethanol was added and mixed well. The samples were extracted with the RNAeasy microRNA kit from Qiagen according to the directions. The final eluates were pooled and evaporated in a Speedvac. The RNA was suspended in 12 μl of TE (0.01MTris+0.005M EDTA). The quantity of RNA in the resuspended sample was determined with the Ribogreen Quantitation Kit (Molecular Probes, Eugene, OR) or with a Nanodrop Spectrophotometer (ND 1000 V3.3, Wilmington, DE). The integrity of the RNA was examined with the Agilent Bioanalyzer using the pico-chip according to the directions of the manufacturer. The laser capture preparation was previously shown to be enriched approximately 7-fold for serotonin neuron-related mRNA (Bethea and Reddy 2008).

Affymetrix hybridization

To screen a large number of genes the Rhesus Affymetrix GeneChip was utilized. Labeled target cRNA was prepared from 6 pools of laser captured serotonin neurons (n=2 animals/treatment group). The cRNA from the laser capture pools was hybridized to Rhesus Affymetrix GeneChip arrays. The Affymetrix GeneChip® Rhesus Macaque Genome Array interrogates over 47,000 M. mulatta transcripts. The array contains 52,024 rhesus probe sets and was designed using public data sources including data from the University of Nebraska

(R. Norgren), the Baylor School of Medicine's rhesus macaque whole-genome shotgun assembly (October 1, 2004), and GenBank® STSs, ESTs, and mRNAs up to March 30, 2005. Additionally, probe sets were designed to interrogate rhesus transcripts orthologous to the 3' end of human transcripts (GeneChip® Human Genome U133 Plus 2.0 Array and RefSeq sequences up to March 2005), sixteen viral sequences from human and other primate species, three different rhesus r(ribosomal) RNAs, and thirteen rhesus mitochondrial genes along with control and reporter sequences. Microarray assays were performed in the Affymetrix Microarray Core of the OHSU Gene Microarray Shared Resource.

Data Analysis with GeneSifter Software

The data was processed with Affymetrix GCOS interface software and compressed into CHP files and uploaded to GeneSifter (VisX Labs, Seattle, WA). The software calculated the mean signal intensity and the standard error of the mean for each treatment group. Probe sets that were undetectable in all treatment groups were eliminated. The remaining probe sets were filtered and those probes sets exhibiting a 2-fold or greater change between the treatment groups were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis and GeneSifter nomenclature analysis. The use of a 2-fold filter was chosen based upon a survey of other microarray studies. Probe sets with robust signal intensities that showed a 2-fold or greater change with E or E+P and that were consistent across multiple representations on the chip were noted. No statistical tests were performed on this data as it was only used to indicate potential glutamate related genes.

Taqman qRT-PCR array

We designed a custom Taqman qRT-PCR array (Applied Biosystems, Foster City, CA) containing pivotal genes related to glutamate function. This platform utilizes microfluidic distribution of samples into wells containing custom primers, which eliminated pipetting and sample-to-sample variation. The primers utilize a 5' fluorescent reporter, FAM (Fluorescein amidite; Molecular Probes, Eugene, OR) and a 3' quencher, TAMRA (tetramethylrhodamine), which improves sensitivity. The cards also contain the passive reference dye, ROX, which enables standardization. In addition, we obtained an extra quantity of each primer set. The genes examined were: Ephrin receptor A4 (EPHA4), Ephrin receptor B4 (EPHB4), Integrin A8 (ITGA8), Integrin B8 (ITGB8), cadherin 11 (CDH11), neuroligin 3 (NLGN3), neurexin 3 (NRXN3), neural cell adhesion molecule1 (NCAM1), syndecan 2 (SCD2) and calcium calmodulin-dependent kinase 2B (CAMK2B). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), GUSB and PIAA were measured for reference. GAPDH was used for normalization of the data. TPH2, which is known to increase with E±P treatment of Ovx macaques, was included on the Taqman array card as an additional gene regulation control.

Preamplification of laser captured samples followed by Taqman qPCR

Reverse transcription and complementary DNA (cDNA) synthesis was performed using Oligo-dT 15 and Random hexamer primers (Invitrogen Life Technologies, Carlsbad, CA) and Superscript III reverse transcriptase (200 U/μg of RNA, Invitrogen Life Technologies) at 42°C for 1 hr. cDNA was treated with RNase H to disintegrate dsDNA. Total RNA (1.4 to 1.8 μg) from each laser capture pool and from a pool of rhesus tissues was transcribed and stored as cDNA at a concentration of 250 ng/μl. Each laser captured sample and an aliquot of the rhesus pool cDNA was preamplified with a master mix containing Platinum Taq polymerase and all primer sets of interest (200μM each; separately provided by ABI). The preamplification PCR reaction was run for 14 cycles. The PCR product from the multiplex reaction on the rhesus standard pool was diluted to generate a standard curve for each of the primer sets. The PCR product from the laser-captured samples was diluted 1/20. Then, the preamplified standards and samples were loaded onto the custom Taqman cards

for qPCR in 100 μ l of reaction mix. There was a log linear increase in fluorescence detected as the concentration of amplified double-stranded product cDNA increased during the reaction. The fluorescence was detected as cycle threshold (Ct) with an ABI 7900 thermal cycler (Applied Biosystems Inc.) during 40 cycles. The slope of the curve was used to calculate the relative picograms of each transcript in the RNA extracted from the laser-captured pools. Then, the ratio of each transcript to GAPDH was calculated for each sample.

Primer selection

For our genes of interest, the Applied Biosystems Rhesus Monkey primer inventory was examined. For the genes not present in the library, Applied Biosystems designed and constructed new primers, which were then added to the Rhesus Monkey inventory. The exact primer sequences are proprietary, but the gene names, symbol, AB assay ID, context sequence, and NCBI gene reference information are shown in Table 1.

Statistical analysis

The average signal intensities above threshold on the microarrays were examined in the E and E+P groups for 2-fold or greater increases or decreases from the Ovx-control group (n=2 animals or chips/group). No further statistical test was performed on the signal intensities. The average relative expression of the 3 groups (n=3 animals/group) from the qRT-PCR assays were compared with ANOVA followed by Bonferroni's posthoc pairwise comparison. In addition, the data were subjected to a log transformation and again compared with ANOVA followed by Bonferroni's posthoc pairwise comparison. Variance between animals is not unusual for this type of preparation, and more animals would reduce the chance of making a type 2 error. Therefore, negative results need further confirmation. Comparisons were considered significantly different when there was greater than a 95% chance that the groups were different ($p < 0.05$). Prism 5.0 from Graph Pad (San Diego, CA) was used for all comparisons.

Results

Expression Changes Related to Adhesion in Laser Captured Serotonin Neurons

The entire data set of the 6 Affymetrix microarray chips has been submitted to the Gene Expression Omnibus public database (www.ncbi.nlm.nih.gov/geo/info/linking.html) and assigned the GEO accession number GSE16169. After exclusion of probe sets that were below threshold (n=32,761), the remaining probe sets (19263) were filtered for 2-fold or greater differences between the groups leaving 10,493 probes for categorization. KEGG analysis of the Cell Adhesion Molecule (CAM) category indicated that 63 probe sets were altered 2-fold or greater by treatment. Genesifter Function analysis of the Synapse Assembly category indicated 31 probe sets were altered 2-fold or greater by treatment. Genesifter Function analysis of the Adhesion category indicated 258 probe sets were altered 2-fold or greater by treatment. The probe sets in the CAM and Synapse Assembly categories were represented in the Adhesion data set. This study is focused upon the *membrane proteins* involved in neuron-neuron adhesion and synaptic assembly. The adhesion data set also contained numerous extracellular matrix (ECM) related genes, cell mediators of immunity (CD molecules), pleckstins, cytoplasm molecules and cytoskeletal molecules, which are involved in other aspects of spine creation and synapse assembly, but that were not considered further in this study.

Table 2 contains the average signal intensities for the probe sets corresponding to pivotal genes related to membrane adhesion and synapse assembly in laser captured serotonin neuron preparations from duplicate animals/microarray chips in each group. A number of these genes were represented by multiple probe sets. Illustrated are 21 genes that were

increased 2-fold or more by E±P and 9 genes that decreased 2-fold or more, all of which have been strongly implicated in neural cell adhesion and synapse assembly. Nine representative genes were selected that were both up- and downregulated for further examination with qRT-PCR.

Adhesion genes that were very common, or in related synapse support categories, or that exhibited modest signal intensity were not listed in the table, but merit mention. Other common adhesion genes coding for catenin delta2 (CTNND2), disintegrin (ADAM9), protocadherins (PCDH; 7, 10, 18, GA4, GC3), cell adhesion molecule 3 (ISGF4B), paxillin (PXN) and contactin associated protein like 2 (CNTNAP2) increased between 2- and 9-fold with E±P treatment. Shank2, Shank3 and Homer1 are intracellular scaffold coding genes that increased between 2- to 3-fold with E±P treatment. Genes coding for microtubule attachment sites included microtubule associated proteins (MAP1A, MAP1B, MAP1LC3A, MAP1LC3B), kinectin1 (KTN1; kinesin receptor), dystonin (DST) and vinculin (VCL) that increased 2-fold or greater with E±P treatment. Neural genes coding for limbic system associated membrane protein (LSAMP), neural growth regulator (NEGR1), neurofibromin 1 (NF1), transforming growth factor-beta2 (TGFB2) and cellubrevin (VAMP3) increased 2-fold or more with E±P treatment, but the signal intensity was modest. Genes coding for ECM proteins such as laminins (LAMA) α 1, 2, 4, testican 2 (SPOCK2), brevican (BCAN), lectin (LGALS1), fibronectins (FN1; FNDC3A) and spondin 1 (SPON1) exhibited 2- to 10-fold increases in E±P treated animals.

Validation of Expression Changes in Laser Captured Serotonin Neurons

Figure 1 illustrates the relative expression of 5 genes that code for cell adhesion and/or synapse assembly proteins. Ephrin receptor A4 subunit expression was significantly different between the treatment groups (ANOVA, $p < 0.0001$). E treatment significantly increased EphA4 expression (Bonferroni's, $p < 0.05$) relative to the Ovx-placebo treated group. Supplemental P treatment significantly suppressed EphA4 expression relative to the E-placebo treated group (Bonferroni's, $p < 0.05$). However, there was no difference in Ephrin receptor B4 subunit expression between the groups (ANOVA, $p = 0.38$).

Integrin A8 subunit (ITGA8) expression was significantly different between the groups (ANOVA, $p = 0.05$). E treatment significantly increased Integrin A8 expression relative to the Ovx-placebo treated group (Bonferroni's, $p < 0.05$). The E+P treated group was not different from the Ovx-placebo group, indicating that supplemental P treatment suppressed the E-induced increase in ITGA8. However, there was no difference in Integrin B8 subunit (ITGB8) expression between the groups (ANOVA, $p = 0.41$).

Cadherin 11 (CDH11) expression was significantly different between the groups (ANOVA, $p = 0.007$). E treatment significantly increased CDH11 expression relative to the Ovx-placebo treated group (Bonferroni's, $p < 0.05$). The E+P treated group was not different from the Ovx-placebo group, indicating that supplemental P treatment suppressed the E-induced increase in CDH11.

Figure 2 illustrates the relative expression of an additional 4 genes that code for cell adhesion and/or synapse assembly proteins. Neuroligin 3 (NLGN3) expression was significantly different between the groups (ANOVA, $p = 0.02$). The E-treated group was not significantly different from the Ovx-placebo group. However, E+P treatment significantly suppressed NLGN3 expression from the E treated group (Bonferroni's, $p < 0.05$). Neurexin 3 (NRXN3) expression was significantly different between the groups (ANOVA, $p = 0.014$). E treatment significantly increased NRXN3 expression relative to the Ovx-placebo treated group (Bonferroni's, $p < 0.05$). The E+P treated group was not different from the Ovx-placebo group, indicating that supplemental P treatment suppressed the E-

induced increase in NRXN3. Syndecan 2 (SCD2) expression was significantly different between the groups (ANOVA, $p = 0.016$). E treatment significantly increased SCD2 expression relative to the Ovx-placebo treated group (Bonferroni's, $p < 0.05$). The E+P treated group was not different from the Ovx-placebo group, indicating that supplemental P treatment suppressed the E-induced increase in SCD2. Neural cell adhesion molecule 1 (NCAM1) expression was significantly different between the groups (ANOVA, $p < 0.0001$). E treatment significantly increased NCAM1 expression relative to the Ovx-placebo treated group (Bonferroni's, $p < 0.05$). The E+P treated group was not different from the Ovx-placebo group, indicating that supplemental P treatment suppressed the E-induced increase in NCAM1.

Following log transformation of the data and re-analysis with ANOVA and Bonferroni's posthoc test, the results were the same as those reported above on the original data.

Steroid Hormone Verification

The concentration of E and P in a serum sample obtained from each animal at necropsy was obtained to verify the efficacy of the Silastic implants. The concentration of E in the serum of the E and E+P-treated animals was 136.5 ± 15 pg/ml and the concentration of P in the serum of the E+P-treated animals was 8.17 ± 1.15 ng/ml. The concentration of E is similar to that observed in the mid-follicular or mid-luteal phase and the concentration of P is similar to that observed in the mid-luteal phase (Hotchkiss and Knobil 1994). The concentrations of E and P in the serum of the placebo-control animals were 11.0 ± 0.1 pg/ml and 0.16 ± 0.13 ng/ml, respectively (significantly different from treated animals by ANOVA, $p < 0.01$).

TPH2 Internal control

TPH2 was determined in the pools of laser captured serotonin neurons on the same Taqman card and at the same time as the adhesion genes. The TPH2/GAPDH ratio increased 20-fold and 40-fold in the E and E+P-treated groups, respectively, over the placebo-control group indicating that serotonin neurons responded to the treatments. In turn, the increases in TPH2 support the validity of the regulation of other genes.

Discussion

Dendritic spine extrusion or retraction is a major component of neural remodeling and plasticity in the adult central nervous system (CNS) (Genoux and Montgomery 2007). Dendritic spine development is a multi-step process that requires actin reorganization. Filopodia precede spine formation and then filopodia may be transformed into spines. This process involves expression of glutamate receptors, decreased motility, shortening of the neck, growth of the head and elaboration of a postsynaptic density (Butler et al. 1998; Ethell and Pasquale 2005; Fischer et al. 2000; McKinney 2010; McKinney et al. 1999).

Based upon a significant body of evidence (Cooke and Woolley 2005), we hypothesized that ovarian steroid administration to Ovx monkeys would increase dendritic spines on serotonin neurons. We recently found that E±P administration significantly increases expression of the Rho GTPases and their downstream effectors of actin reorganization, AMPA2 subunit, the AMPA4 subunit and the NMDA2a receptor subunit in laser captured serotonin neurons (Bethea and Reddy 2010; Bethea and Reddy 2011).

The ability of E to induce spine proliferation is well documented in the hippocampus (Hao et al. 2003; Woolley et al. 1997), hypothalamus (Schwarz et al. 2008), cerebellum (Sasahara et al. 2007) and cortex (Hao et al. 2006; Srivastava et al. 2008), so spine proliferation on serotonin neurons in response to ovarian hormones may be a general phenomena. For a spine to be functionally excitatory it has to develop a synapse with the afferent

glutamatergic axon. It is well accepted that trans-synaptic interaction of adhesion molecules is critical for synaptogenesis. The interaction between adhesion molecules stabilizes the initial axon–dendrite contact thereby allowing simultaneous bi-directional communication between the pre-synaptic and post-synaptic specialization. This study examined the effect of E±P on the expression of a small number of cell surface molecules thought to be involved in synapse assembly in an enriched preparation of laser captured serotonin neurons from macaques.

We detected a significant increase in Eph4A in the laser-captured preparation of serotonin neurons from E-treated monkeys. There are 14 Eph receptor genes that include A and B subtypes. Eph receptors are tyrosine kinase receptors (Klein 2004). EphA4 is especially important for regulating dendritic spine morphology in the adult brain. The interaction of neural EphA4 with ephrin-A3, located on peri-synaptic astrocytes is believed to contribute to spine maintenance and stabilization (Murai and Pasquale 2011). EphA4 signaling causes spine retraction by *reducing* β -*integrin* function. No ephrines were detected with the 2-fold filter in our analysis.

The subtype of EphB receptors and ephrin-B family members detected at synapses depends on the developmental stage of the neuron and the kind of synapse being studied. During synapse formation in cortical and hippocampal neurons, presynaptic ephrin-Bs trigger activation of postsynaptic EphB receptors to cluster NMDA receptors and promote dendritic spine maturation (Murai and Pasquale 2011). EphB4 was detected in our preparation and exhibited a 2-fold decrease with E treatment on the microarray. However, there was not a significant difference with qRT-PCR. Due to the small number of animals, this needs to be confirmed. We speculate that with one month of steroid treatment, the spines largely require maintenance, which is a function of EphA4 (Klein 2004). Hence, after one month of E treatment, EphA4 expression may be favored over EphB4.

We found a significant increase in integrin A8 (ITGA8) in our preparation from E-treated monkeys. The integrins are a structurally complex family of adhesion molecules that transduce signals bidirectionally across the plasma membrane by undergoing rearrangement; and they link the external environment to internal cytoskeletal components. The integrin ligands on the cell surface include some members of the immunoglobulin superfamily (IgSF) and the cadherin family. Various components of ECM are also integrin ligands (Ethell and Pasquale 2005). Integrin is composed of α and β subunits. There are 18 different α subunits and 8 different β subunits in vertebrates that can form at least 24 α/β heterodimers (Springer and Wang 2004). The $\alpha 8$ -integrins are required for long-term potentiation at hippocampal CA1 synapses (Chan et al. 2010). If a similar role is subsumed in serotonin neurons, then the increase in ITGA8 expression with E treatment underscores an important role in synapse formation.

Integrin B8 (ITGB8) has been detected in perivascular astrocytes of humans (Su et al. 2010) and analysis of the promoter region suggests that ITGB8 expression is under the control of transcription factors in stress-activated and pro-inflammatory pathways (Markovics et al. 2010). In this study, the trend toward decreased ITGB8 expression in treated animals is consistent with the increase in EphA4 (see above) and could indicate that steroid administration ameliorates proinflammatory activation of ITGB8 in astrocytes. Nonetheless, due to the small number of animals, this observation needs to be confirmed. Moreover, the mechanism of E action in astrocytes is not adequately understood.

In our preparation of serotonin neurons, cadherin 11 (CDH11) exhibited a 4-fold increase on the microarray and a significant increase in the qRT-PCR assay in the E-treated monkeys. An increase in numerous protocadherins was also detected on the microarray. In addition,

the microarray detected an increase in catenin $\alpha 1$, the cytoskeleton linker. Cadherins (CDHs) and protocadherins are integral membrane proteins that mediate calcium-dependent cell-cell adhesion. They complex with the β -catenins, which are cytosolic proteins that anchor cadherins to the actin cytoskeleton (Suzuki and Takeichi 2008). Cadherins and catenins are found on both sides of the synapse; they use binding partners in both compartments to mediate intracellular signaling pathways required for construction and ongoing stabilization of the synapse (Arikkath and Reichardt 2008). Recent RNAi experiments confirmed that CDH11 has a pivotal role in glutamatergic synapse development (Paradis et al. 2007). Further studies have suggested that in the hippocampus, N-cadherin plays a critical role in embryonic synaptogenesis, whereas CDH11 is more important for stabilization of synapses in adulthood (Bartelt-Kirbach et al. 2010). This notion is consistent with our speculation that the serotonin synapses in our preparation were likely mature.

Neurexins are a family of proteins that function in the vertebrate nervous system as cell adhesion molecules and receptors. The neurexins form a complex with the neuroligins. Neurexins and neuroligins play a central role in organization of excitatory glutamatergic and inhibitory GABAergic synapses. They function as cell adhesion molecules, bridging the synaptic cleft. In general, neuroligins trigger presynaptic differentiation and neurexins trigger postsynaptic differentiation (Craig and Kang 2007). Our data indicate that E treatment induced a significant increase in Neurexin 3 (NRXN3) suggesting an increased number of postsynaptic densities. Moreover, its partner Neuroligin 3 (NLGN3), which was presumably located on the presynaptic bouton, exhibited a weak signal on the microarray; and it exhibited a decrease with E+P treatment on both the microarray and in the qRT-PCR assay. Thus, presynaptic components were present in our preparation.

Syndecan 2 (SCD2) expression was significantly increased in our serotonin neuron preparation in E-treated monkeys. Syndecans are cell surface heparin sulfate proteoglycans localized to sites of cell-cell and cell-matrix contacts. SCD2 plays a pivotal role in the maturation of dendritic spines and in the molecular mechanisms underlying postsynaptic modifications. It is highly concentrated on the spines of mature hippocampal neurons and it clusters with the morphological maturation of spines (Ethell and Yamaguchi 1999).

NCAM1, a well-known mediator of neural cell attachment, is polysialylated in its active form. Gene expression of NCAM1 increased in our preparation nearly 3-fold on the microarray and was significantly increased in the qRT-PCR assay in E-treated monkeys. NCAM is expressed in the synapses of adult brain structures where it is involved in activity-induced synaptic plasticity (Gascon et al. 2007). NCAM1 has been localized to the postsynaptic density; whereas L1 cell adhesion molecule and tenascin (Table 2) do not label synaptic membranes (Persohn et al. 1989; Schuster et al. 2001) and were weakly expressed and/or decreased by hormone treatment on the microarray.

There were a number of other interesting genes related to adhesion or synapse assembly that increased with E-treatment on the microarray (Table 2) and given the consistency between the 9 confirmed genes and the microarray expression, it is likely that this regulation could be confirmed. Of note, CAMK2B, SNAP25, TrkB, FEZ1 and Reelin exhibited 2- to 20-fold increases with E treatment, and their specific roles in synapse assembly are supported by the literature in this field.

The adhesion genes that increased with E treatment usually exhibited a decrease in expression with addition of P to the E regimen. We previously observed this pattern of expression with RhoA and Rac1, but not with cdc42, Rock1, WAVE, gelsolin or profilin, the latter of which were as high or higher with P supplementation than with E alone (Bethea and Reddy 2008). We recently observed that expression of the glutamate receptor genes in

serotonin neurons was equal with E and E+P (Bethea and Reddy, in press). So within serotonin neurons, we have observed 3 types of P action; P may be inhibitory, neutral or additive with E on the expression of various genes within serotonin neurons and we do not know how this occurs. Preliminary studies indicate that E and E+P equally stimulate immunogold staining for psd95 in the dorsal raphe (n=2 animals/group; 3rd set of animals in preparation). Thus, we have reason to believe that the suppressive effect of P on gene expression of the adhesion proteins reported herein does not impact maintenance of spines on serotonin neurons, but how that follows is not clear.

ER β is constitutively expressed in serotonin neurons of macaques (Gundlah et al. 2000; Gundlah et al. 2001) and E treatment induces the expression of nuclear PR (progesterone receptors) in the monkey raphe (Bethea 1994). PR occurs in two isoforms, PR-A and PR-B (Tung et al. 1993). Although PR-A greatly exceeds PR-B in peripheral tissues, the isoforms are nearly equal in the monkey brain (Bethea and Widmann 1998), and differences in the action of the isoforms has been reported for tyrosine hydroxylase gene expression (Jensik and Arbogast 2011). Promoter analysis of each gene would be informative as the presence or absence of PRE's could play a role. Different actions of ER through ERE or Ap1 sites, or by blocking NF κ B-induced transcription, may also be involved (McKay and Cidlowski 1998; Paech et al. 1997). In addition, gene expression does not always reflect protein expression, stability or turnover. Finally, differential actions of membrane receptors cannot be ruled out. Clearly, we are a long way from fully understanding the different actions of P on gene expression when it is added to an E treatment regimen.

Conclusions

Gene expression and involvement of the plethora of adhesion molecules in spine targeting and synapse assembly is much more complex than described here. Nonetheless, we have genomic evidence that the molecular action of E $\pm$ P in serotonin neurons might lead to the elaboration of dendritic spines on serotonin neurons. We propose that E and/or E+P act via steroid receptors in serotonin neurons to (1) induce the RhoGTPases with subsequent effects on actin (Bethea and Reddy 2010); (2) induce post-synaptic glutamate receptor expression (Bethea and Reddy 2011) and, (3) induce specific adhesion proteins that are involved in synapse assembly and spine stabilization. Together these data support our preliminary observation that there is a significant increase in dendritic spines in the dorsal raphe with immunogold staining for psd95. Golgi staining and stereological analysis of spines in the dorsal raphe is underway. Since the differences observed in gene expression between groups follow one month of steroid treatment in this study, the spines are likely mature (De Roo et al. 2008). Altogether, these actions would enhance serotonin neurotransmission to forebrain areas.

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Figure 1.

Histograms illustrating changes in expression of selected genes related to synapse assembly obtained with a Taqman custom qRT-PCR array on RNA/cDNA extracted from laser captured serotonin neurons (n=3 animals/treatment).

*p<0.05 different from OvX control with Bonferroni's posthoc pairwise comparison after ANOVA.

p<0.05 different from E treated group with Bonferroni's posthoc pairwise comparison after ANOVA.

Figure 2.

Histograms illustrating changes in expression of selected genes related to synapse assembly obtained with a Taqman custom qRT-PCR array on RNA/cDNA extracted from laser captured serotonin neurons (n=3 animals/treatment).

*p<0.05 different from OvX control with Bonferroni's posthoc pairwise comparison after ANOVA.

p<0.05 different from E treated group with Bonferroni's posthoc pairwise comparison after ANOVA.

Table 1

Available information about ABI proprietary primers used for Taqman qRT-PCR.

Gene Name	Gene Symbol	Assay ID	Context Sequence	Category	NCBI Gene Reference
EPH receptor A4	EPHA4	Rh02848603_m1	AAGTTATAGGAGTTGGTGAAATTTGG	Receptor	XM_001106493.1; XM_001106876.1
EPH receptor B4	EPHB4	Rh01119118_g1	TGCACCCCTGCACCAACCCCTCCCTCG	Cell adhesion molecule	XM_002803225.1
integrin, alpha 8	ITGA8	Rh00943516_m1	GCTGTTTACAGAGCAAGACCGGTTG	Cell adhesion molecule	XM_001089317.1
integrin, beta 8	ITGB8	Rh02841381_m1	GCAGCTGTCTGTGAAAGTCATATTG	Receptor	XM_001103130.1; XM_001103047.1
cadherin 11, type 2 preproprotein	CDH11	Rh02839805_m1	CAGCCCAATAAGGTATTCCATCGAT	Cell adhesion molecule	XM_001104523.1; XM_001104597.1
neuregulin 3	NLGN3	Rh03986723_m1	CAGATGAAAAAAGATGGTTTCTCTGAG	Signaling molecule	XM_001111843.1
neurexin 3	NRXN3	Rh01028194_m1	CTTCATGGGCTGCCTTAAAGAGGTT	Receptor	XM_001097688.1
neural cell adhesion molecule 1	NCAM1	Rh00354742_m1	TCCTGAAAAAAGATGTCCGATTTCAT	Cell adhesion molecule	XM_001083486.1; XM_001083597.1
syndecan 2	SCD2	Rh02790313_s1	GCTAAACAGTATTTTAAAGAAAGTTGCT	Cell adhesion molecule	XM_002805408.1
glyceraldehyde-3-phos dehydrogenase	GAPDH	Rh02621745_g1	CATCTTCCAGGAGCGAGATCCCTCC	Oxidoreductase	XM_001105471.1
tryptophan hydroxylase 2	TPH2	Rh02788839_m1	CTACTCGGCAACTTAAACACTAAATA	serotonin synthesis	NM_001039946.1; AY827483.1

Table 2

Signal Intensity of genes related to adhesion and synapse assembly in laser captured preparations enriched in serotonin neurons.

Gene name	Gene Symbol	Ovx	E	E + P
Increased				
Synaptosomal –associated protein	SNAP25	6144	13757	7746
Eph Receptor A4	EPHA4	143	550	296
Integrin Receptor alpha 6	ITGA6	238	495	425
Integrin Receptor alpha 8	ITGA8	20	122	77
Neurexin 1	NRXN1	537	2699	1740
Neurexin 3	NRXN3	653	2020	874
Neural cell adhesion molecule	NCAM1	609	1732	819
Syndecan 2	SDC2	207	417	343
Roundabout, axon guidance receptor, homolog 1	ROBO	282	663	519
Cadherin 11	CDH11	17	409	183
Protocadherin 9	PCDH9	1615	5896	3008
Catenin alpha 1(Cadherin-associated protein)	CTNNA1	125	816	479
Neurotrophic tyrosine kinase (Trk B)	NTRK2	2025	9778	3295
Cell adhesion molecule 1 (IGSF4)	CADM1	1203	2559	1258
Contactin 1	CNTN1	861	2323	1476
Fasciculation and elongation protein zeta1	FEZ1	1080	5522	2538
Neurotrimin	HNT	451	1309	814
Laminin receptor 1	LAMR1	157	1989	763
Reelin	RELN	3051	6361	2987
Apolipoprotein E	APOE	300	5308	1231
Calcium calmodulin Kinase 2B	CAMK2B	152	2352	902
Decreased				
Eph Receptor B4	EPHB4	392	139	353
Integrin Receptor beta 3	ITGB3	951	155	826
Integrin Receptor beta 8	ITGB8	296	88	260
L1 cell adhesion molecule	L1CAM	1082	890	454
Neuroigin 3	NLGN3	376	311	153
Neuronal cell adhesion molecule	NRCAM	143	74	70
Neurfascin	NFASC	158	93	61
Tenascin N	TNN	261	75	84
transmembrane protein 8	TMEM8	489	195	370