Published in final edited form as:

Mol Cell Neurosci. 2012 March ; 49(3): 251–262. doi:10.1016/j.mcn.2011.11.005.

Ovarian Steroids Increase Glutamatergic Related Gene Expression in Serotonin Neurons of Macaques

Cynthia L. Bethea^{1,2,3,4} and Arubala P. Reddy¹

¹Divisions of Reproductive Sciences, Oregon National Primate Research Center, Beaverton, OR 97006

²Divisions of Neuroscience, Oregon National Primate Research Center, Beaverton, OR 97006

³Department of Behavioral Neuroscience, Oregon Health and Science University, Portland, OR 97201

⁴Department of Obstetrics and Gynecology, Oregon Health and Science University, Portland, OR 97201

Abstract

Dendritic spines are the elementary structural units of neuronal plasticity and their proliferation and stabilization involve components of glutamate neurotransmission. In a model of hormone replacement therapy (HT), we sought the effect of estradiol (E) and progesterone (P) on gene expression related to glutamate neurotransmission in a laser captured preparation enriched for serotonin neurons from rhesus macaques. Microarray analysis was conducted (n=2 animals/treatment) and then 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 prior to euthanasia. The midbrain was obtained, sectioned and immunostained for TPH. TPH-positive neurons were laser captured using an Arcturus Laser Dissection Microscope (Pixel II). RNA from laser captured serotonin neurons (n=2 animals/treatment) was hybridized to Rhesus Affymetrix GeneChips for screening purposes. There was a 2-fold or greater change in the expression of 28 probe sets related to glutamate processes in E and E+P treated animals. Quantitative (q) RT-PCR was conducted for 11 genes with a custom Taqman PCR array containing monkey specific primers and analyzed with ANOVA followed by Bonferroni's test. The log of the relative expression values indicated that in general, the responses to E and E+P were similar. Comparison of the relative expression or log relative expression in OvX-controls to combined E and E+P treated groups with t-tests showed a significant increase in AMPA1 (GRIA1), AMPA2 (GRIA2), AMPA4 (GRIA4), NMDA2a (GRIN2A), metabotropic glutamate receptor (GRM1), glutamine synthetase (GLUL), glutamate dehydrogenase (GLUD), glutamate cysteine ligase modifier subunit (GCLM), the glutamate transporter 2 (SLC1A2) and the glutamate transporter 3 (SLC1A3) with steroid treatment. There was no effect of steroid treatment on gene expression of the glutamate cysteine ligase catalytic subunit (GCLC). These data suggest that ovarian steroids target gene expression of ionotropic and metabotropic glutamate receptors in serotonin neurons. These receptors are present on dendritic spines and are necessary for spine maturation. The mRNAs coding for glutamate-related enzymes and transporters are likely derived from astrocytes or glutamate-containing terminals.

© 2011 Elsevier Inc. All rights reserved.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Their induction by ovarian steroids indicates a complex upregulation of multiple components in the glutamate cycle and antioxidation, in addition to spine proliferation.

Keywords

serotonin; macaques; estrogen; progesterone; Affymetrix array; dorsal raphe; glutamate receptors; glutamate enzymes; quantitative PCR

Introduction

The serotonin system modulates a wide range of neural outcomes from emotion to intellect to metabolism and 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 recently reported that ovarian steroids regulate gene and protein expression in laser captured serotonin neurons in a manner that would increase cellular resilience and decrease apoptosis (Bethea and Reddy, 2008; Tokuyama et al., 2008). In addition, ovarian steroid administration decreased DNA fragmentation in serotonin neurons (Bethea et al., 2009; Lima and Bethea, 2009).

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). We postulated that steroid-induced dendritic spine proliferation on serotonin neurons would have a profound effect on serotonergic function and 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. If dendritic spines on serotonin neurons shrink or atrophy due to lack of steroid supported gene expression, then geriatric depression, anxiety, fretfulness, decreased coping skills and increased vulnerability to stress can be predicted outcomes.

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 filopodia extension, spine head enlargement and spine shortening, all of which are necessary for the production of a mature dendritic spine. Hence, if ovarian steroids enhance serotonin neuronal plasticity by increasing spine proliferation on serotonin dendrites, this would facilitate excitatory input to the serotonin neurons and in turn, increase serotonin neural activity.

A critical component of spine maturation and stability are the excitatory ionotropic glutamate receptors, AMPA (α -amino-3-hydroxy-5-methyl-isoxazolepropionate) and NMDA (N-methyl-D-aspartate type). The synthesis, targeting, insertion and clustering of these receptors on spines are highly regulated in time scales ranging from minutes to months. We hypothesized that ovarian steroid administration would increase expression of AMPA and NMDA receptors in laser captured serotonin neurons as a reflection of increased

spine proliferation. In addition, estradiol induces spines and the ionotropic glutamate receptors by enhancing glutamate release independent of transcription (Schwarz et al., 2008). However, upregulation of NMDA receptors also follows NMDA antagonism or blocking of synaptic activity by tetrodotoxin (Bolton et al., 2000). Therefore, we also examined other components of glutamatergic organization since our laser captured serotonin preparation is only enriched for serotonin neurons and likely included glia and presynaptic elements.

Our model involves ovariectomy of adult rhesus macaques for 5-8 months, followed by subcutaneous delivery of estrogen (E) or estrogen plus progesterone (EP) for one month. Therefore, we can only examine one time point, which probably reflects a significant level of maturity and stabilization. We examined the effect of E, with and without supplemental P, on multiple genes related to glutamatergic function in a laser capture preparation enriched for serotonin neurons of rhesus monkeys using the Rhesus Affymetrix cDNA array and quantitative (q) RT-PCR. Eleven 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 and the serum levels achieved by the implants were previously reported (Bethea and Reddy, 2008)

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 RNeasy 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.

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 reported. 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: AMPA1 (GRIA1), AMPA2 (GRIA2), AMPA4 (GRIA4), NMDA2a (GRIN2A), metabotropic glutamate receptor 1 (GRM1), glutamine synthetase (GLUL), glutamate dehydrogenase (GLUD1/2), glutamate cysteine ligase modifier subunit (GCLM), glutamate cysteine ligase catalytic subunit (GCLC), glutamate transporter 2 (SLC1A2) and glutamate transporter 3 (SCL1A3). 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 on the microarrays in the E and E+P groups were examined 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 assay was subjected to ANOVA followed by Bonferroni's posthoc test. If the variance was significantly different between the groups (F test), then a Kruskal-Wallis analysis of variance was conducted followed by Dunn's posthoc test.

The qRT-PCR relative expression values were subjected to a Log transformation and then compared with ANOVA followed by Bonferroni's posthoc pairwise comparison. One gene, GRM1 exhibited near significant difference between groups by ANOVA ($p<0.08$), so a t-test was applied to compare Ovx and E groups. In the ANOVA analysis on the transformed data, the majority of the genes that showed a difference between Ovx and E or E+P treatment, also showed no difference between the treatment groups.

Subsequently, the relative expression values of the E and E+P treated animals were combined and compared to the Ovx-control group with an unpaired 2-tail t-test with Welch's correction for unequal variances. In addition, the log transformed values of the E and E+P treated groups were combined and compared to the Ovx-control group with an unpaired 2-tail t-test.

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 Glutamate Receptors and Enzymes 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. Table 2 contains the average signal intensities for the probe sets corresponding to genes related to glutamate receptors and enzymes in laser captured serotonin neuron preparations from duplicate animals/microarray chips in each group. The first 11 genes were further examined with qRT-PCR. The last 6

genes were interesting, but not pursued. Note that GAD1, GAD2, PAG and VGLUT2 increased, whereas SLC1A4 and GRIK2 (kainate receptor), decreased with steroid treatment. Hence, up and down regulation was observed on the microarray, but only increases were verified with qRT-PCR.

The relative expression values from the Taqman qRT-PCR array for each gene are shown in Table 3. Each animal was assayed in triplicate and a mean for the animal was obtained. Then the overall mean of the animals was obtained and subjected to statistical analysis. With this analysis, 6 of the 11 genes showed an increase in expression in the E and/or E+P treated groups. The expression of two genes, GLUS and GLUD2, was reduced to control levels when P was added to the E regimen. AMPA1, GRM1, GCLC, and the 2 transporters did not exhibit statistical significant changes. However, the variance was significantly different between the groups in this analysis by F test. Therefore, a log transformation was performed on the data.

Glutamate Receptor Expression Changes

The log transformations of the relative expression of the receptor genes are illustrated in Figure 1. There was a significant difference between groups in the expression of the AMPA1 subunit (ANOVA, $p = 0.02$). E treatment increased AMPA1 expression relative to OvX-controls, but was not different from E+P treatment (Bonferroni, $p < 0.05$). There was a significant difference between groups in the expression of the AMPA2 subunit (ANOVA, $p = 0.0025$), the AMPA4 subunit (ANOVA, $p = 0.0001$) and the NMDA2a receptor (ANOVA, $p = 0.002$). Relative to the OvX-control group, E and E+P treatment increased expression of AMPA2 (Bonferroni, $p < 0.05$, both treatments), AMPA4 (Bonferroni, $p < 0.05$, both treatments) and expression of the NMDA2A receptor (Bonferroni, $p < 0.05$, both treatments). There was no difference between the E- and E+P-treated groups. There was a near significant difference between groups in the expression of the metabotropic glutamate receptor or GRM1 (ANOVA, $p = 0.08$). However, there was a significant increase in GRM1 with E only treatment compared to OvX-controls as indicated with an unpaired, 2-tail t-test ($p = 0.006$).

Subsequently, the relative expression values of the E and E+P treated groups were combined and compared to the OvX-control group with a t-test using Welch's correction for unequal variances. The combined relative expression values are illustrated in Figure 2. There was a significant increase in relative expression of AMPA1 ($p = 0.04$), AMPA2 ($p = 0.01$), AMPA4 ($p = 0.0001$), NMDA2a ($p = 0.007$) and in the metabotropic glutamate receptor ($p = 0.028$) with steroid treatment.

In addition, the log transformed expression values of the E and E+P treatment groups were combined and compared to the OvX-control group with an unpaired 2-tail t-test. The results are illustrated in Figure 3. All of the receptor genes exhibited a significant increase with steroid treatment after log transformation. There was a significant increase in expression of AMPA1 ($p = 0.005$), AMPA2 ($p = 0.0003$), AMPA4 ($p = 0.0007$), NMDA2a ($p = 0.0007$) and in the metabotropic glutamate receptor ($p = 0.03$) with steroid treatment.

Glutamate Related Enzyme and Transporter Expression Changes

The log transformations of the relative expression values from OvX-control, E- and E+P treated groups for glutamine synthetase (GLUL), glutamate dehydrogenase (GLUD2), glutamate cysteine ligase modifier subunit (GCLM), glutamate cysteine ligase catalytic subunit (GCLC) and the glutamate transporters 2 and 3 (SLC1A2 and SLC1A3) are illustrated in Figure 4. There was a significant difference between the groups in GLUL (ANOVA, $p = 0.002$), GLUD2 (ANOVA, $p = 0.0014$), and GCLM (ANOVA, $p = 0.004$). E

and E+P significantly increased each of these transcripts relative to the Ovx-control group (Bonferroni, $p < 0.05$, all). In addition, E+P suppressed GLUD2 relative to E treatment alone (Bonferroni, $p < 0.05$). There was a significant difference between the groups in expression of the SLC1A2 (ANOVA, $p = 0.04$) and E treatment increased SLC1A2 expression relative to Ovx-controls (Bonferroni, $p < 0.05$). There was no difference between the groups in expression of the glutamate cysteine ligase catalytic subunit (ANOVA, $p = 0.25$) or the glutamate transporter 3 (ANOVA, $p = 0.11$). It should be noted that there was an outlier in the Ovx-control group of the GCLC results.

Subsequently, the relative expression values were combined and compared to the Ovx-control group with a t-test using Welch's correction for unequal variances. The combined relative expression values are illustrated in Figure 5. Steroid treatment increased gene expression of GLUL ($p=0.002$), GLUD2 ($p=0.023$), GCLM ($p=0.007$) SLC1A2 ($p=0.023$) and SLC1A3 ($p=0.04$). GCLC did not exhibit significant regulation, due to variance ($p=0.2$).

In addition, the log transformed values of the two treatment groups were combined and compared to the Ovx-control group with an unpaired 2-tail t-test. The results are illustrated in Figure 6. As with the relative expression values, each gene, except GCLC, exhibited a significant increase with treatment after log transformation. Steroid treatment increased gene expression of GLUL ($p=0.0012$), GLUD2 ($p=0.014$), GCLM ($p=0.003$) SLC1A2 ($p=0.01$) and SLC1A3 ($p=0.04$). GCLC did not exhibit significant regulation, but the outlier in the control group may have skewed the results ($p=0.08$). The GCLC data need to be confirmed with more animals and may represent a type 2 statistical error.

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. The concentration of P is similar to that observed in the mid-luteal phase. The concentrations of E and P in the serum of the untreated spayed control animals were 11.0 ± 0.1 pg/ml and 0.16 ± 0.13 ng/ml, respectively (significantly different from treated animals by ANOVA, $p < 0.01$).

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 glutamate 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.

Discussion

Glutamate neurotransmission plays a pivotal role in dendritic spine protrusion or retraction, which is a major component of neural remodeling and plasticity in the adult central nervous system (CNS) (Genoux and Montgomery, 2007). Dendritic spines express excitatory glutamate receptors, both ionotropic and metabotropic, and the maintenance of a spine requires continuous glutamate signals from afferent neurons (Butler et al., 1998; 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.

Dendritic spine extension and remodeling is a multi-step process that requires actin reorganization. Filopodia precede spine formation and glutamate promotes filopodia morphogenesis. Filopodia may be transformed into spines, which involves decreased

motility, shortening of the neck and growth of the head (Butler et al., 1998; Ethell and Pasquale, 2005; McKinney et al., 1999). Current evidence suggests that filopodia initially grow without a presynaptic partner. They might however rapidly express functional excitatory receptors, which may be activated by ambient glutamate (Yoshihara et al., 2009).

The dynamic organization of the actin cytoskeleton provides the force for filopodia protrusion and spine morphogenesis. It is regulated by small GTPases of the Rho family, in particular RhoA, Rac1 and CDC42. We previously found that ovarian steroid administration increased gene expression of RhoA, Rac1 and CDC42 and their downstream effectors in laser captured serotonin neurons. In addition, E or E+P increased gene expression of the actin and myosin regulatory proteins cofilin, gelsolin, profilin and ARP2/3 (Bethea and Reddy, 2010).

The transformation of filopodia to a mature spine involves the expression of AMPA and NMDA receptors (McKinney, 2010). Studies suggest a two-step process in which spines initially formed in response to NMDA receptor activation are subsequently stabilized by AMPA receptors (Fischer et al., 2000). Based largely on the combined group data either before or after log transformation, this study indicates that E and/or E+P administration significantly increases expression of the AMPA1 subunit, the AMPA2 subunit, the AMPA4 subunit and the NMDA2a receptor subunit in laser captured serotonin neurons on a rhesus microarray and with qRT-PCR. In addition, ovarian steroids increased the glutamate metabotropic receptor (GRM1). The increase in gene expression for the glutamate receptors suggests that there are more spines on serotonin dendrites after one month of hormone treatment, and maintenance of the spines requires ongoing production of the receptors.

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 the responses of the serotonin neurons to ovarian hormones may be a general phenomena. The ability of estrogen to increase dendritic spines in the hippocampus involves NMDA receptor activity (Woolley et al., 1997) and CREB activation (Murphy and Segal, 1997). Additional work has shown that estrogen and NMDA receptor activation increase dendritic spine number in the developing rat hypothalamus, which is dependent on an increase in presynaptic glutamate release. It has been suggested that the estrogen-enhanced glutamate release occurs via a nongenomic mechanism and activation of PI3 kinase (Schwarz et al., 2008). In addition, AMPA receptors can promote spine formation, transformation, stabilization and maintenance. Moreover, inhibition of synaptic transmission in hippocampal slices causes spine loss and atrophy (Fischer et al., 2000; Matus et al., 2000).

It is important to question whether changes in glutamate receptor gene expression related to dendritic spine proliferation on serotonin neurons are due to a direct action of E or P via steroid receptors or, are due to an indirect action via increased glutamate release, or both. We have shown that serotonin neurons express ER β and PR (Bethea, 1993, 1994; Gundlach et al., 2001), and these receptors are gene transcription factors. Thus, it is reasonable to think that the regulation of gene expression is due to activation of these transcription factors, which leads to the initial extrusion of filopodia and expression of AMPA and NMDA receptors.

Extracellular glutamate then contributes to the transformation of filopodia to mature spines. The additional data in this study indicate a significant increase in the glutamate processing enzymes. Glutamatergic synapses are enveloped in perisynaptic astrocyte processes that play a central role in glutamatergic transmission. Astrocytes are largely responsible for the

clearance of glutamate and glutamate turnover through the 'glutamate cycle' (Anderson and Swanson, 2000; Daikhin and Yudkoff, 2000). It is very likely that our laser captured serotonin neuron preparation, which is enriched about 7-fold for serotonin RNAs, also contains astrocyte-derived RNAs.

We observed a significant increase in glutamine synthetase expression (GLUL; GS) with E and E+P (log) or the combination of the two groups (relative expression and log). Glutamine synthetase is localized to astrocytes and is involved in the metabolism of glutamate to glutamine (Anderson and Swanson, 2000). The resultant glutamine can be exported from the astrocyte, followed by uptake into the glutamatergic neuron and recatalyzed to glutamate by phosphate-activated glutaminase (PAG). The glutamate is packaged into vesicles by VGLUTs (vesicular glutamate transporters) for ultimate release. On the microarray, PAG increased 2-fold with E treatment. Also, VGLUT2 increased 3.6-fold. RNA does migrate to terminals (Mikl et al., 2011) and this data suggests that some RNA in our preparation may have been derived from presynaptic glutamate terminals.

We observed a significant increase in glutamate dehydrogenase (GLUD2; GDH) with E and E+P (log) or both groups combined (relative expression and log). Glutamate dehydrogenase occurs in 2 isoforms. GLUD1 expression is widespread and is considered a housekeeping gene. On the other hand, GLUD2 is specific to neural tissue where it localizes to astrocytes (Zaganas et al., 2009). There is high homology between the two isoforms (Zaganas et al., 2009). The entire sequence of GLUD2 (1677bp) is coding sequence completely contained within the coding sequence of GLUD1 (3501bp) from bp 279 to 1937 (NCBI). On the microarray, the probe sets for GLUD2 would detect GLUD1 and GLUD2. However, the probesets for GLUD1 are outside of the GLUD2 homologous area and therefore, would only detect GLUD1. Since we were applying RNA from brain tissue, the only signal on the Affymetrix array came from the GLUD2 probesets. On the Taqman PCR array, the primers amplified within the coding region of GLUD1 and GLUD2 (identical). However, since GLUD2 is the only isoform present in the brain, it was the isoform detected by the Taqman qRT-PCR array.

Astrocytes can convert glucose via glycolysis and the TCA cycle to α -ketoglutarate, which is converted to glutamate by GLUD2. This enzyme also can act in reverse and metabolize glutamate to α -ketoglutarate as well (Daikhin and Yudkoff, 2000). In the astrocyte, GLUL converts glutamate to glutamine (above) for ultimate transport to a glutamatergic neuron where PAG converts it to glutamate. GLUD2 is also present in presynaptic terminals of glutamate neurons (McKenna et al., 2000), and the potential for laser capture of presynaptic elements on serotonin neurons is present. Hence, the E $\pm$ P-induced increase in GLUD2 may occur in both astrocytes and glutamatergic terminals where it may be contributing to an increase in glutamate production.

We observed an increase in the modifier subunit of glutamate cysteine ligase with E and E +P (log) treatment or both groups combined (relative expression and log), but not in the catalytic subunit. The lack of regulation of the catalytic subunit needs confirmation due to an outlier in the control group and small sample size. The modifier and catalytic subunits (GCLM + GCLC) together, in consecutive action with glutathione synthetase, catalyze the rate-limiting step in glutathione production. Glutathione is a potent antioxidant; it plays a major role in the cellular defense against free radicals, and it is the predominant free radical scavenger in the brain (Had-Aissouni, 2011a). Astrocytes play a central role in the synthesis of glutathione in the brain. They have a high content of glutathione and provide neurons with glutathione precursors. In fact, neuronal glutathione synthesis seems to be strictly under the dependence of glial precursors. Thus, the astrocytes contain a very important mechanism for neuroprotection; i.e., conversion of glutamate to glutathione (Had-Aissouni, 2011b).

Whether of astrocyte or neuronal origin, glutathione probably contributes to the steroid-induced reduction in DNA fragmentation we observed in serotonin neurons (Lima and Bethea, 2009). We propose that ovarian hormones increase glutamine synthetase, glutamate dehydrogenase 2, and glutamate cysteine ligase modifier largely in glia surrounding serotonin neurons, leading to an increase in the activity of the glutamate cycle and in neuroprotection through free radical scavenging by glutathione. The mechanism by which this occurs needs further investigation. Both membrane actions of the steroid hormones, or unique steroid receptors in glia have been suggested (Kipp and Beyer, 2009; Schwarz et al., 2008).

Excitatory amino acid transporters are now in the solute carrier (SLC) gene series and belong to the SLC1A family. Five distinct glutamate transporters have been cloned from mammalian tissues and are differentially expressed in brain cells. A 2-fold or greater steroid-induced increase in the glutamate transporter 2 (SLC1A2 or EAAT1/GLT1) and in the glutamate/aspartate transporter 3 (SLC1A3 or EAAT1/GLAST) was detected on the microarray. Astrocytes express the SLC1A3 subtype, but SLC1A2 can also be found on the presynaptic glutamatergic neuron (Had-Aissouni, 2011b). We observed a significant increase in SLC1A2 and SLC1A3 expression when both treatments were combined (relative expression and log).

Very little is known about the factors that regulate the primary processing and the further fate of the glutamate transporter mRNAs. Extracellular glutamate is the signal for upregulation of the glutamate transporters (Duan et al., 1999; Gegelashvili and Schousboe, 1997); and it must be maintained within the low micromolar range to prevent cytotoxic effects (Schousboe et al., 1997). However, gene and protein expression of these transporters has been disparate in many studies (Gegelashvili and Schousboe, 1997). We propose that the increase in the SLC1A2 and SLC1A3 mRNA may indicate extracellular glutamate was elevated with steroid treatment.

It is curious that the pattern of gene expression detected on the microarray was not always reflected by the pattern of gene expression detected with qRT-PCR. However, the probe sets utilized on the microarray were different from the primer sets used in the qRT-PCR assay. In addition, the variation often observed on microarrays is commonly the reason for confirmation of gene expression changes with qRT-PCR. The results of the qRT-PCR assay are considered more reliable than the signal intensity on the microarray.

In addition, we frequently found that the greatest 'within group variation' in gene expression occurs with E+P treatment. Indeed, often on the microarray and on the Taqman card, this group exhibited the largest variance. The basis for this may be individual neural sensitivity to P since our ratio of E to P was optimal for development of the endometrium (Brenner and Slayden, 1994). Nonetheless, after log transformation and with the exception of GLUD2, there was no difference between the E and E+P treated groups. We have observed this in other serotonin functions, as well as augmentation or suppression with supplemental P. We imagine that the differences in the effect of supplemental P on various genes depend on promoter elements and co-regulators. It would appear that E mediates most of the actions of ovarian steroids on glutamate receptor genes in serotonin neurons and addition of P is neutral. We are eager to further mine the microarray data and look for genes that may be different between E and E+P groups.

All together the data support an increase in AMPA and NMDA receptors on serotonin neurons and an increase in the activity of the glutamate cycle. Extracellular glutamate is tightly regulated; so activated steroid receptors may promote filopodia and receptor expression followed by glutamate-induced maturation. If an increase in spines on serotonin

neurons occurs under a modest increase in glutamate concentrations, then the potential for toxicity is low. The concentration of extracellular glutamate could be examined in more detail with microdialysis of the dorsal raphe. It would also be interesting to examine the effect(s) of glutamate, with and without estrogen, in our cultures of rhesus embryonic stem cell-derived serotonin neurons (Tokuyama et al., 2010). Examination of the protein expression for the genes reported herein is under investigation.

Conclusions

Although the regulation of brain glutamate is much more complex than described here, interpretation of gene expression of the different glutamatergic components is facilitated by understanding their localization. We propose that E or E+P act via steroid receptors in serotonin neurons to (1) induce the RhoGTPases with subsequent effects on actin, (2) induce filopodia extension and (3) induce post-synaptic glutamate receptor expression. The transformation of filopodia to mature spines needs extracellular glutamate, which may increase, but remains tightly regulated. There are also conspicuous changes in expression of enzymes and transporters governing the 'glutamate cycle' and perhaps glutamate production. It is likely that the increases in enzyme gene expression occurred in astrocytes that are in close apposition to serotonin neurons. These astrocytes are the main contributor to glutamate inactivation and recycling. There have been reports of estrogen receptors in astrocytes; however, we hesitate to comment on the mechanism by which the changes in glutamate enzyme or transporter gene expression occur. Finally, the changes observed in gene expression follow one month of steroid treatment, so stabilization of the synapses and maturation of the spines is likely. These actions would enhance serotonin neurotransmission and in turn, improve mood and decrease anxiety.

Acknowledgments

We are deeply grateful to the dedicated staff of the Division of Animal Resources including the staff of the Departments of Surgery and Pathology for their expertise and helpfulness in all aspects of monkey management. The staff of the OHSU Gene Microarray Shared Resource was essential for this study.

Supported by NIH grants: MH62677 to CLB, U54 contraceptive Center Grant HD 18185, RR000163 for the operation of ONPRC

Literature Cited

- Anderson CM, Swanson RA. Astrocyte glutamate transport: review of properties, regulation, and physiological functions. *Glia*. 2000; 32:1–14. [PubMed: 10975906]
- Bethea C, Reddy A. Effect of ovarian hormones on survival genes in laser captured serotonin neurons from macaques. *J Neurochem*. 2008; 105:1129–1143. [PubMed: 18182058]
- Bethea CL. Colocalization of progestin receptors with serotonin in raphe neurons of macaque. *Neuroendocrinology*. 1993; 57:1–6. [PubMed: 8479605]
- Bethea CL. Regulation of progestin receptors in raphe neurons of steroid-treated monkeys. *Neuroendocrinology*. 1994; 60:50–61. [PubMed: 8090282]
- Bethea CL, Reddy AP. Effect of ovarian hormones on genes promoting dendritic spines in laser-captured serotonin neurons from macaques. *Mol Psychiatry*. 2010; 15:1034–1044. [PubMed: 19687787]
- Bethea CL, Reddy AP, Tokuyama Y, Henderson JA, Lima FB. Protective actions of ovarian hormones in the serotonin system of macaques. *Front Neuroendocrinol*. 2009; 30:212–238. [PubMed: 19394356]
- Bolton MM, Blanpied TA, Ehlers MD. Localization and stabilization of ionotropic glutamate receptors at synapses. *Cell Mol Life Sci*. 2000; 57:1517–1525. [PubMed: 11092446]

- Brenner, RM.; Slayden, OD. Cyclic changes in the primate oviduct and endometrium. *The Physiology of Reproduction*. In: Knobil, E.; Neill, JD., editors. Second Edition. Raven Press, Ltd.; New York: 1994. p. 541-569.2 ed
- Butler AK, Uryu K, Chesselet MF. A role for N-methyl-D-aspartate receptors in the regulation of synaptogenesis and expression of the polysialylated form of the neural cell adhesion molecule in the developing striatum. *Dev Neurosci*. 1998; 20:253–262. [PubMed: 9691199]
- Cooke BM, Woolley CS. Gonadal hormone modulation of dendrites in the mammalian CNS. *J Neurobiol*. 2005; 64:34–46. [PubMed: 15884004]
- Daikhin Y, Yudkoff M. Compartmentation of brain glutamate metabolism in neurons and glia. *J Nutr*. 2000; 130:1026S–1031S. [PubMed: 10736375]
- Duan S, Anderson CM, Stein BA, Swanson RA. Glutamate induces rapid upregulation of astrocyte glutamate transport and cell-surface expression of GLAST. *J Neurosci*. 1999; 19:10193–10200. [PubMed: 10575016]
- Ethell IM, Pasquale EB. Molecular mechanisms of dendritic spine development and remodeling. *Prog Neurobiol*. 2005; 75:161–205. [PubMed: 15882774]
- Fischer M, Kaech S, Wagner U, Brinkhaus H, Matus A. Glutamate receptors regulate actin-based plasticity in dendritic spines. *Nat Neurosci*. 2000; 3:887–894. [PubMed: 10966619]
- Gegelashvili G, Schousboe A. High affinity glutamate transporters: regulation of expression and activity. *Mol Pharmacol*. 1997; 52:6–15. [PubMed: 9224806]
- Genoux D, Montgomery JM. Glutamate receptor plasticity at excitatory synapses in the brain. *Clin Exp Pharmacol Physiol*. 2007; 34:1058–1063. [PubMed: 17714094]
- Gundlach C, Lu NZ, Mirkes SJ, Bethea CL. Estrogen receptor beta (ERb) mRNA and protein in serotonin neurons of macaques. *Molecular Brain Research*. 2001; 91:14–22. [PubMed: 11457488]
- Had-Aissouni L. Maintenance of antioxidant defenses of brain cells: plasma membrane glutamate transporters and beyond. *Amino Acids*. 2011a E pub ahead of print.
- Had-Aissouni L. Toward a new role for plasma membrane sodium-dependent glutamate transporters of astrocytes: maintenance of antioxidant defenses beyond extracellular glutamate clearance. *Amino Acids*. 2011b E pub ahead of print.
- Hao J, Janssen WG, Tang Y, Roberts JA, McKay H, Lasley B, Allen PB, Greengard P, Rapp PR, Kordower JH, Hof PR, Morrison JH. Estrogen increases the number of spinophilin-immunoreactive spines in the hippocampus of young and aged female rhesus monkeys. *J Comp Neurol*. 2003; 465:540–550. [PubMed: 12975814]
- Hao J, Rapp PR, Leffler AE, Leffler SR, Janssen WG, Lou W, McKay H, Roberts JA, Wearne SL, Hof PR, Morrison JH. Estrogen alters spine number and morphology in prefrontal cortex of aged female rhesus monkeys. *J Neurosci*. 2006; 26:2571–2578. [PubMed: 16510735]
- Kipp M, Beyer C. Impact of sex steroids on neuroinflammatory processes and experimental multiple sclerosis. *Front Neuroendocrinol*. 2009; 30:188–200. [PubMed: 19393685]
- Lima FB, Bethea CL. Ovarian steroids decrease DNA fragmentation in the serotonin neurons of non-injured rhesus macaques. *Mol Psychiatry*. 2009; 15:657–668. [PubMed: 19823180]
- Manji HK, Quiroz JA, Sporn J, Payne JL, Denicoff K, Gray AN, Zarate CA Jr. Charney DS. Enhancing neuronal plasticity and cellular resilience to develop novel, improved therapeutics for difficult-to-treat depression. *Biol Psychiatry*. 2003; 53:707–742. [PubMed: 12706957]
- Matus A, Brinkhaus H, Wagner U. Actin dynamics in dendritic spines: a form of regulated plasticity at excitatory synapses. *Hippocampus*. 2000; 10:555–560. [PubMed: 11075825]
- McKenna MC, Stevenson JH, Huang X, Hopkins IB. Differential distribution of the enzymes glutamate dehydrogenase and aspartate aminotransferase in cortical synaptic mitochondria contributes to metabolic compartmentation in cortical synaptic terminals. *Neurochem Int*. 2000; 37:229–241. [PubMed: 10812208]
- McKinney RA. Excitatory amino acid involvement in dendritic spine formation, maintenance and remodelling. *J Physiol*. 2010; 588:107–116. [PubMed: 19933758]
- McKinney RA, Capogna M, Durr R, Gähwiler BH, Thompson SM. Miniature synaptic events maintain dendritic spines via AMPA receptor activation. *Nat Neurosci*. 1999; 2:44–49. [PubMed: 10195179]

- Murphy DD, Cole NB, Segal M. Brain-derived neurotrophic factor mediates estradiol-induced dendritic spine formation in hippocampal neurons. *Proc Natl Acad Sci U S A*. 1998; 95:11412–11417. [PubMed: 9736750]
- Murphy DD, Segal M. Morphological plasticity of dendritic spines in central neurons is mediated by activation of cAMP response element binding protein. *Proc Natl Acad Sci U S A*. 1997; 94:1482–1487. [PubMed: 9037079]
- Sasahara K, Shikimi H, Haraguchi S, Sakamoto H, Honda S, Harada N, Tsutsui K. Mode of action and functional significance of estrogen-inducing dendritic growth, spinogenesis, and synaptogenesis in the developing Purkinje cell. *J Neurosci*. 2007; 27:7408–7417. [PubMed: 17626201]
- Schousboe A, Sonnewald U, Civenni G, Gegelashvili G. Role of astrocytes in glutamate homeostasis. Implications for excitotoxicity. *Adv Exp Med Biol*. 1997; 429:195–206. [PubMed: 9413575]
- Schwarz JM, Liang SL, Thompson SM, McCarthy MM. Estradiol induces hypothalamic dendritic spines by enhancing glutamate release: a mechanism for organizational sex differences. *Neuron*. 2008; 58:584–598. [PubMed: 18498739]
- Srivastava DP, Woolfrey KM, Jones KA, Shum CY, Lash LL, Swanson GT, Penzes P. Rapid enhancement of two-step wiring plasticity by estrogen and NMDA receptor activity. *Proc Natl Acad Sci U S A*. 2008; 105:14650–14655. [PubMed: 18801922]
- Tokuyama Y, Ingram SL, Woodward JS, Bethea CL. Functional characterization of rhesus embryonic stem cell-derived serotonin neurons. *Exp Biol Med (Maywood)*. 2010; 235:649–657. [PubMed: 20463306]
- Tokuyama Y, Reddy A, Bethea C. Neuroprotective actions of ovarian hormones without insult in the raphe region of rhesus macaques. *Neuroscience*. 2008; 154:720–731. [PubMed: 18486349]
- Woolley CS, Weiland NG, McEwen BS, Schwartzkroin PA. Estradiol increases the sensitivity of hippocampal CA1 pyramidal cells to NMDA receptor-mediated synaptic input: correlation with dendritic spine density. *J Neurosci*. 1997; 17:1848–1859. [PubMed: 9030643]
- Yoshihara Y, De Roo M, Muller D. Dendritic spine formation and stabilization. *Curr Opin Neurobiol*. 2009; 19:146–153. [PubMed: 19523814]
- Zaganas I, Kanavouras K, Mastorodemos V, Latsoudis H, Spanaki C, Plaitakis A. The human GLUD2 glutamate dehydrogenase: localization and functional aspects. *Neurochem Int*. 2009; 55:52–63. [PubMed: 19428807]

Figure 1.

Scatter plots illustrating changes in glutamate receptor gene expression in laser captured serotonin neurons from OvX-controls, E-treated and E+P treated macaques (n=3 animals/treatment). The gene cDNA/GAPDH cDNA relative expression values were log transformed and subjected to ANOVA with Bonferroni's posthoc analysis.

*different from OvX-control group with Bonferroni's posthoc pairwise comparison (p<0.05);

different from OvX-control group with an unpaired 2-tail t-test (p<0.05).

Figure 2.

Histograms illustrating changes in glutamate receptor gene expression in laser captured serotonin neurons between the Ovx-control group and a combined group containing E- and E+P-treated macaques. The gene cDNA/GAPDH cDNA relative expression values were subjected to an unpaired 2-tailed t-test with Welch's correction for unequal variances.

* different from Ovx-control group with an unpaired 2-tail t-test ($p < 0.05$).

Figure 3.

Scatter plots illustrating changes in glutamate receptor gene expression in laser captured serotonin neurons between the OvX-control group and a combined group containing E- and E+P-treated macaques. The gene cDNA/GAPDH cDNA relative expression values were log transformed and subjected to an unpaired 2-tailed t-test. Circles=ovx-control; Squares = E-treated; Diamonds = E+P treated.

* different from OvX-control group with an unpaired 2-tail t-test (p<0.05).

Figure 4.

Scatter plots illustrating changes in glutamate enzyme and transporter gene expression in laser captured serotonin neurons from OvX-controls, E-treated and E+P treated macaques (n=3 animals/treatment). The gene cDNA/GAPDH cDNA relative expression values were log transformed and subjected to ANOVA with Bonferroni's posthoc analysis.

*different from OvX-control group with Bonferroni's posthoc pairwise comparison ($p < 0.05$);
+ different from E treatment group with Bonferroni's posthoc pairwise comparison ($p < 0.05$).

Figure 5.

Histograms illustrating changes in glutamate enzyme and transporter gene expression in laser captured serotonin neurons between the Ovx-control group and a combined group containing E- and E+P-treated macaques. The gene cDNA/GAPDH cDNA relative expression values were subjected to an unpaired 2-tailed t-test with Welch's correction for unequal variances.

* different from Ovx-control group with an unpaired 2-tail t-test ($p < 0.05$).

Figure 6. Scatter plots illustrating changes in glutamate enzyme and transporter gene expression in laser captured serotonin neurons between the Ovx-control group and a combined group containing E- and E+P-treated macaques. The gene cDNA/GAPDH cDNA relative expression values were log transformed and subjected to an unpaired 2-tailed t-test. Circles=ovx-control; Squares = E-treated; Diamonds = E+P treated. * different from Ovx-control group with an unpaired 2-tail t-test (p<0.05).

Table 1

Available information about ABI custom Taqman qPCR assays.

	Gene			
	Symbol	Assay ID	Context Sequence	NCBI Gene Reference
glutamate receptor, ionotropic, AMPA 1	GRIA1	Rh02829827_m1	CCTTGCAAATCTGGGCTTCATGGAC	XM_001111076.1
glutamate receptor, ionotropic, AMPA 2	GRIA2	Rh02790124_m1	AGGTGATTCCAAGGAAAAGACCAGT	XM_001095129.1,AY723727.1
glutamate receptor, ionotropic, AMPA 4	GRIA4	Rh02829840_m1	CTCAAACAGGTTCTGAATTCAAGGGC	XM_001101031.1
similar to Glutamate [NMDA] receptor subunit epsilon 1 pre	LOC71534!	Rh03986722_m1	ATGATCATGGCTGACAAGGATCCGA	XM_001105525.1
glutamate receptor, metabotropic 1	GRM1	Rh01068380_m1	TCTCACTCATTGGAAGTGATGGATG	XM_001085942.1
glutamate-ammonia ligase (glutamina synthetase)	GLUL	Rh02928658_m1	CCTGCCCAGTGGAATTTCAAATTG	XM_001114827.1
glutamate dehydrogenase	GLUD	Rh03986729_m1	AAGCCCCGAGGCTTCTGCATTGCT	XR_009675.1; NM_012084.3
glutamate-cysteine ligase, modifier subunit	GCLM	Rh00978072_m1	GCTGTATCAGTGCGCACAGGTAAAA	XM_001103381.2; NM_002061
glutamate-cysteine ligase, catalytic subunit	GCLC	Rh02838909_m1	CATCATCAATGGAAGGAAGGCGTG	XM_001103887.1
solute carrier family 1 (glial high affinity glutamate transport)	SLC1A2	Rh02847723_m1	GAGTGCAAGGTAAGTCTGGCAGCCA	XM_001115008.1,AY369853.1
solute carrier family 1 (glial high affinity glutamate transport)	SLC1A3	Rh00904820_m1	CCTGGACTTGATCAGGAACATGTTC	XM_001094417.1

Table 2

Signal intensity for genes of interest on Affymetrix Rhesus macaque arrays (2 animals/arrays per treatment group).

Gene name	Average Signal Intensity (n=2)			
	Gene Symbol		Treatment	
		Placebo	E	E+P
glutamate receptor, ionotropic, AMPA 1	GRIA1	88	53	100
glutamate receptor, ionotropic, AMPA 2	GRIA2	568	4797	1591
glutamate receptor, ionotropic, AMPA 4	GRIA4	369	721	814
N-methyl D-aspartate receptor subtype 2A (NR2A) (NMDAR2A) (hNR2A)	GRIN2A	192	1008	364
glutamate receptor, metabotropic 1	GRM1	238	558	383
glutamate-ammonia ligase (glutamine synthetase)	GLUL	461	3784	1058
glutamate dehydrogenase 2	GLUD2	235	986	402
glutamate-cysteine ligase, modifier subunit	GCLM	57	204	351
glutamate-cysteine ligase, catalytic subunit	GCLC	246	575	455
solute carrier family 1 (glial high affinity glutamate transporter), member 2	SLC1A2	996	6940	4932
solute carrier family 1 (glial high affinity glutamate transporter), member 3	SLC1A3	1804	6166	3846
glutamate decarboxylase 1	GAD1	997	2153	1288
glutamate decarboxylase 2	GAD2	1381	2155	2847
phosphate activated glutaminase	PAG	226	587	281
vesicular glutamate transporter 2	VGLUT2	746	2732	1663
Solute carrier family 1 (glutamate/neutral amino acid transporter), member 4	SLC1A4	271	124	137
glutamate receptor, ionotropic, kainate 2	GRIK2	212	127	78

Table 3Ratio of Gene cDNA/GAPDH cDNA $\pm$ SEM for all groups

	Ovx	E	E+P	p value
Gene				ANOVA or K-W [^]
AMPA1 (GRIA1)	12.5 $\pm$ 2.1	143.9 $\pm$ 76	84.4 $\pm$ 32	0.06 [^]
AMPA2 (GRIA2)	16.4 $\pm$ 1.6	*368.3 $\pm$ 38	*200.4 $\pm$ 66	0.01
AMPA4 (GRIA4)	10.4 $\pm$ 2.2	*189.6 $\pm$ 7.8	*181.3 $\pm$ 33	0.001
NMDA2a (GRIN2A)	12.9 $\pm$ 2.1	52.5 $\pm$ 10	*84.1 $\pm$ 19	0.023
Metabotropic 1 (GRM1)	4.3 $\pm$ 1.4	21.8 $\pm$ 1.5	19.9 $\pm$ 11	0.09 [^]
Glutamine synthetase (GLUL)	2.1 $\pm$ 0.9	*81.0 $\pm$ 15	⁺ 22.2 $\pm$ 2.8	0.002
Glu dehydrogenase (GLUD2)	15.3 $\pm$ 0.3	*116.0 $\pm$ 39	⁺ 35.4 $\pm$ 4.5	0.04
GluCyst ligase mod (GCLM)	8.2 $\pm$ 1.1	*41.5 $\pm$ 6.8	24.3 $\pm$ 5.6	0.01
Glu Cyst ligase cat (GCLC)	5.6 $\pm$ 3.9	14.15 $\pm$ 5.4	12.32 $\pm$ 5.4	0.48
Glu transporter 2 (SLC1A2)	13.2 $\pm$ 2.6	221.2 $\pm$ 112	649.8 $\pm$ 494	0.07 [^]
Glu transporter 3 (SLC1A3)	14.9 $\pm$ 6.6	51.8 $\pm$ 12	156.8 $\pm$ 111	0.12 [^]

* p < 0.05 Bonferroni posthoc test, different from Ovx control group

⁺ p < 0.05 Bonferroni posthoc test, different from E treated group[^] K-W, Kruskal-Wallis ANOVA for unequal variances