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ORIGINAL INVESTIGATION

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Ovarian steroid regulation of monoamine oxidase-A and B mRNAs in the macaque dorsal raphe and hypothalamic nuclei

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Abstract *Rationale:* The serotonin neural system plays a pivotal role in mood, affective regulation and integrative cognition, as well as numerous autonomic functions. We have shown that ovarian steroids alter the expression of several genes in the dorsal raphe of macaques, which may increase serotonin synthesis and decrease serotonin autoinhibition. Another control point in aminergic neurotransmission involves degradation by MAO. This enzyme occurs in two isoforms, A and B, which have different substrate preferences. *Objectives:* We questioned the effect of ovarian steroid hormones on MAO-A and MAO-B mRNA expression in the dorsal raphe nucleus and hypothalamus using in situ hybridization in non-human primates. *Methods:* Rhesus monkeys (*Macaca mulatta*; *n*=5/group) were spayed and either placebo treated (controls), estrogen (E) treated (28 days), progesterone (P) treated (14 days placebo+14 days P), or E+P treated (14 days E+14 days E+P). Perfusion-fixed sections (25 μm) were hybridized with a 233 bp MAO-A, or a 373 bp MAO-B, radiolabeled-antisense monkey specific probes. Autoradiographic films were analyzed by densitometry, which was performed with NIH Image Software. *Results:* MAO-A and -B mRNAs were detected in the dorsal raphe nucleus (DRN) and in the hypothalamic suprachiasmatic nucleus (SCN), preoptic area (POA), paraventricular nucleus (PVN), supraoptic nucleus (SON), lateral hypothalamus (LH) and ventromedial nucleus (VMN). MAO-A mRNA optical density was significantly decreased by E, P, and E+P in the DRN and in the hypothalamic PVN, LH and VMN. Ovarian hormones had no effect on MAO-B mRNA expression in the DRN. However, there was a significant decrease in MAO-B optical density in the hypothalamic POA, LH and VMN with E, P or E+P treatment. Pixel area generally reflected optical density. *Conclusions:* Ovarian steroids decreased MAO-A, but not B, in the raphe nucleus. However, both MAO-A and B were decreased in discrete hypothalamic nuclei by hormone replacement. These data suggest that the transcriptional regulation of MAO by ovarian steroids may play a role in serotonin or catecholamine neurotransmission and hence, mood, affect or cognition in humans.

Keywords Estrogen · Progesterone · Serotonin · Primate · Depression · Mood disorder · Hormone replacement therapy

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Introduction

Monoamine oxidase A and B (MAO-A and MAO-B) are the central enzymes that catalyze oxidative deamination of biogenic amines in the central nervous system and peripheral tissues (Von Korff 1979). Inhibitors of MAO were among the first pharmacotherapies successfully used for the treatment of depression. It is generally thought that serotonin neurotransmission is dysfunctional in depression. Thus, the ability of MAO inhibitors to relieve depression suggests that MAO plays a functionally significant role in serotonin metabolism. Moreover, there is a delay in the onset of therapeutic efficacy which occurs approximately 2 weeks after daily treatment with the MAO-A inhibitor, clorgyline, correlating with desensitization of serotonin autoreceptors (Blier and de Montigny 1985).

The substrate specificity of MAO-A and B varies slightly between species. In human, monkey and rat,

MAO-A selectively degrades serotonin and norepinephrine whereas MAO-B predominantly metabolizes dopamine (Tipton et al. 1982; Youdim and Finberg 1991). Serotonin degradation by MAO-B has also been reported in rat, bovine, and pig brain (Ekstedt and Oreland 1976; Achee and Gabay 1977; Mitra and Guha 1980; Luine and Paden 1982; Tipton et al. 1982). In mice, dopamine is preferentially oxidized by MAO-A (Steyn et al. 2001). Anatomically, MAO-A has been localized largely to catecholaminergic neurons and MAO-B has been largely localized to serotonin neurons. However, MAO-A mRNA has also been detected in the monkey dorsal raphe nucleus, which contains a large population of serotonin neurons (Saura et al. 1982, 1996; Westlund et al. 1985, 1988; Richards et al. 1992; Luque et al. 1996). Among the species, MAO isoform mRNA expression was consistent with protein localization (Richards et al. 1992; Luque et al. 1996).

A body of data suggests that estradiol (E) has the potential to improve mood and cognition (McEwen 1999), although much remains unknown regarding the sites and mechanisms of action of E in the central nervous system (CNS). We have found that E changes the expression of pivotal genes in serotonin neurons in a manner that could increase serotonin synthesis and decrease serotonin autoinhibition (Pecins-Thompson et al. 1996, 1998; Pecins-Thompson and Bethea 1998). In addition, our laboratory has shown that progesterone (P) administered after E priming significantly increased the 5-HT/5-HIAA ratio in CSF of monkeys, due to a significant decrease in 5-HIAA (Bethea et al. 1999). These data led to the hypothesis that overall serotonin neurotransmission may be increased by actions of E on tryptophan hydroxylase (TPH), serotonin reuptake transporter (SERT), and 5HT1A autoreceptor gene expression, and then further facilitated by adding P to the E regimen to act on the expression of MAO-A or B in a fashion that would decrease serotonin degradation. MAO-A and MAO-B are encoded by separate genes (Bach et al. 1988), which also raises the possibility of differential regulation by ovarian steroids.

Previous studies in rats indicated that hypothalamic MAO-A activity decreased with acute E, and the addition of P to E-primed rats restored MAO-A activity (Luine and Rhodes 1983; Ortega-Corona et al. 1994). MAO-B activity in the rat hypothalamus was decreased with E and E+P (Ortega-Corona et al. 1994). However, in the locus coeruleus and cerebellum of the rat, MAO-A activity decreased and MAO-B activity increased with acute and chronic E (Chevillard et al. 1981). The actions of E and P in the serotonin system differ in various respects between rodents and primates. For example, the effect of E on tryptophan hydroxylase mRNA expression and on SERT mRNA expression in the raphe nucleus are different in monkey and rat (Pecins-Thompson et al. 1996, 1998; Alves et al. 1997; McQueen et al. 1997). Therefore, we questioned whether ovarian steroids would decrease MAO-A and MAO-B mRNA in the monkey dorsal raphe and hypothalamic nuclei, areas of the brain that contain significant populations of nuclear receptors for E and P. We also sought evidence that P may amplify the effect of E on the transcription of MAO-A or MAO-B.

Materials and methods

Animals and experimental groups

This study was approved by the Oregon Regional Primate Research Center (ORPRC) Animal Care and Use Committee. Adult female rhesus monkeys (*Macaca mulatta*) were ovariectomized and hysterectomized (spayed) by the surgical personnel of ORPRC between 3 and 6 months before assignment to this project according to accepted veterinary surgical protocol. All animals were born in Oregon, weighed between 4 and 8 kg, and were in good health.

For examination of the regulation of MAO-A and MAO-B mRNA, 20 spayed rhesus macaques were obtained and either treated with placebo (empty Silastic capsules; control group), or treated with E for 28 days (E group), or treated with placebo for 14 days and then treated with P for 14 days (P group), or treated with E for 28 days and then supplemented with P for the final 14 of the 28 days (E+P group). The animals were processed in matched sets containing one animal from each treatment group. Each set was treated with hormones and killed at the same time. A total of five sets of animals were used yielding a final number of five animals in each of the four treatment groups. The administration of E for 28 days supplemented with P for the last 14 days of the 28-day treatment period has been shown to cause differentiation of the uterine endometrium in a manner similar to the normal 28-day menstrual cycle (Brenner and Maslar 1988).

Surgery and treatments

All animals were spayed. The control monkeys were implanted (SC) with empty Silastic capsules. The E-treated monkeys were implanted (SC) with one 4.5-cm E-filled Silastic capsule (i.d. 0.132 in.; o.d. 0.183 in.; Dow Corning, Midland, Mich., USA). The capsule was filled with crystalline estradiol (1,3,5(10)-estratrien-3,17-b-diol; Steraloids, Wilton, N.H., USA). The E+P-treated group received an E-filled capsule, and 14 days later, received one 6-cm capsule filled with crystalline P (4-pregnen-3,20 dione; Steraloids). The P-treated group received an empty Silastic capsule, and 14 days later received a P-filled capsule. All capsules were placed in the periscapular area under ketamine anesthesia (ketamine HCl, 10 mg/kg, SC; Fort Dodge Laboratories, Fort Dodge, Iowa, USA).

Tissue preparation

The monkeys were killed at the end of the treatment period 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, IV), and exsanguinated by severance of the descending aorta. The left ventricle of the heart was cannulated and the head of each animal was perfused with 1 l of saline followed by 7 l of 4% paraformaldehyde in 3.8% borate, pH 9.5 [both solutions made with DEPC treated water (0.1% diethyl pyrocarbonate) to minimize RNase contamination]. The brain was removed and dissected. Tissue blocks were postfixed in 4% paraformaldehyde for 3 h, then transferred to 0.02 M potassium phosphate-buffered saline (KPBS) containing 10%, followed by 20% glycerol and 2% dimethyl sulfoxide (DMSO) at 4°C for 3 days to cryoprotect the tissue. After infiltration, the block was frozen in isopentene cooled to–55°C, and stored at–80°C until sectioning which occurred within 6 months of storage. Sections (25 μm) were cut on a sliding microtome, mounted

on Superfrost Plus slides (Fisher Scientific, Santa Clara, Calif., USA), dehydrated under vacuum overnight and then frozen at–80°C until processing for ISH. Every tenth section (each 250 μm) was stained with hematoxylin for morphological reference and anatomical orientation (Paxinos et al. 2000).

MAO-A and MAO-B cDNA clones

The forward and reverse primers were constructed from sequences of human cDNA (GenBank) which amplified 233 bases and 373 bases of MAO-A and MAO-B DNA, respectively, of the 3' regions within exon 15 of both genes. Although the enzymes are 55% homologous, the 3' region of each is less than 22% homologous. Oligonucleotide primers (24 mers) were synthesized by the ORPRC Molecular and Cellular Biology Core. The primer sequences were as follows:

- MAOA forward: GATCTACACTGACAAATAAGATGG
- MAOA reverse: GCTCTTGCCACGTTCTTGAAGTTC
- MAOB forward: ACCTGTATAGCCTCACTTCCCTAG
- MAOB reverse: TCAGCTGGAGACACACCGCACAAA

RT-PCR was performed in an MJ Research PTC-200 (Watertown, Mass., USA) using genomic DNA. Bands of the correct size were detected on an analytical agarose gel. The PCR products were ligated into pGEM-T (2.9 kb; Promega, Wisc., USA), which contains SP6 and T7 promoters on either side of the multiple cloning site. *Escherichia coli* were transfected, plated, and individual colonies were grown in minipreps and plasmids of the correct size were isolated. The highest yield clones were chosen for amplification using large plasmid preparations.

Riboprobe orientation and synthesis

The orientation of the inserts was determined by restriction enzyme digestion (Biolabs, N.E., USA) and each clone was also sequenced for verification on a 373 Applied Biosystems DNA sequencer in the ORPRC Molecular and Cellular Biology Core. The MAO-A and MAO-B sequences both showed 92% homology with those of the human. The MAO-A clone was linearized with Sal 1 and transcribed for the antisense riboprobe using T7. The same clone was linearized with Nco 1 and transcribed for the MAO-A sense riboprobe using SP6. The MAO-B clone was linearized with Nco 1 and transcribed for the antisense riboprobe using SP6. The same clone was linearized with Sal 1 and transcribed for the MAO-B sense riboprobe using T7.

Due to marked differences in detection, MAO-B sense and antisense riboprobes were synthesized using ^{35}S-labeled UTP, whereas MAO-A riboprobes were synthesized using ^{33}P-labeled UTP (NEN, Mass., USA). Polymerase (Promega, Wisc., USA) was used to transcribe the cRNA from the linearized monkey-specific cDNAs for MAO-A and MAO-B riboprobes. Unincorporated nucleotides were removed with a Nuctrap push column (Stratagene, Calif., USA).

ISH and assay specificity

Brain sections were removed from frozen storage and post-fixed in 4% paraformaldehyde for 15 min, rinsed in TE (0.1 M TRIS, 0.05 M EDTA, in DEPC water, pH 8) for 10 min, permeabilized at 37°C with proteinase K (10 μg/ml) in TE for 30 min then acetylated with 0.2% acetic anhydride in 0.1 M triethanolamine for 10 min, and rinsed in 2× saline sodium citrate (SSC: 0.3 M NaCl, 0.03 M sodium citrate) for 4 min. Following rinses and dehydration in ethanol, sections were hybridized overnight at 55°C for MAO-A and B at probe concentrations of 2×10^5 cpm/μl for MAO-A and 1×10^4 cpm/μl for MAO-B. The following morning, sections were washed with 4× SSC, ribonuclease A (RNase A 22 μg/ml) at 37°C for 30 min, 2× SSC, and 0.1× SSC at 63°C for MAO-A and 65°C for MAO-B. Sections were dehydrated and apposed to β-sensitive film at room temperature for 3–6 days. Sections were subsequently dipped in Kodak NTB-2 emulsion (Eastman Kodak, N.Y., USA), exposed for 8–16 days, then counterstained with thionin for microscopic evaluation. Preliminary trials utilized multiple sections to optimize the probe concentrations, hybridization and wash temperatures and exposure times. Negative controls included the use of ^{35}S-UTP and ^{33}P-UTP labeled sense cRNA on midbrain and hypothalamic tissue and preincubation of similar sections with RNase A prior to hybridization with the ^{35}S-UTP or ^{33}P-UTP labeled antisense cRNA to eliminate all specific signals.

Densitometric analysis of hybridization signal

Sections were anatomically matched between animals using the hematoxylin-stained sections as a guide. Autoradiographic films of sections were developed, illuminated, and images were video-captured using a CCD video camera module (Sony, Japan). The images were digitized with the NIH Image program. A measurement of optical density (OD) was generated from the autoradiographs which required the operator to encircle the anatomical area of interest. The same area size was used on all animals at each anatomical level. The net OD was obtained by subtracting a background measurement of the same size area from the OD measurement of the signal area. In addition, a threshold for the signal intensity was set on a control animal by the operator. The number of positive pixels exceeding the set threshold within the defined area was obtained on all remaining animals. This measurement is called pixel area. Analysis was performed on two to six levels of each hypothalamic nucleus and five to six levels for the dorsal raphe nucleus. The sections spanned ~250–1000 μm in a rostral to caudal direction in both brain regions.

To verify the linearity of the autoradiographic films and to verify that the individual films exhibited similar responsiveness, ^{14}C autoradiographic standards (Amersham, Ill., USA) were used, which yielded an exposure comparable to ^{35}S or ^{33}P. The standards are calibrated to reflect rat brain gray matter impregnated with increasing amounts of ^{14}C. The average optical density measured from experimental regions fell within the linear range of the standards. Also, film responses were consistent between different films. During development of the ISH assay, the films were exposed for various lengths of time to assure that the chosen exposure time was in a linear range and not saturated or overexposed. The MAO-A films were exposed for 6 days and the MAO-B films were exposed for 3 days. After film development, the slides were dipped in NTB-2 emulsion, incubated 16 days for MAO-A and 8 days for MAO-B, and developed for silver grain examination.

Hormone assays

Serum E and P concentrations were measured with radioimmunoassay in the blood sample obtained at necropsy by the ORPRC Endocrine Services Laboratory (Resko et al. 1974, 1975).

Statistics

Each area was represented in more than one section. Thus, the OD or pixel area values in arbitrary units from each section containing a particular area were averaged, generating one value per area for each animal. Then, the average of the animal values was obtained for each area so that the deviation around the mean is related to the variation between individual animals. The treatment averages for the individual regions and the steroid hormone concentrations were compared with a one-way ANOVA followed by Student-Newman-Keul's post-hoc pairwise comparison. Analyses were conducted using the Prism statistic program (GraphPad, San Diego, Calif., USA). A confidence level of $P<0.05$ was considered significant.

A MAOA sequence

```
Mk   GATCTACACT GACAAATAAG ATGGAGCTTG ATCTTACTGA AATTAATTAG GCGATTCGTT GAATTGTTTT TACATTAACA ATGATTGGAA CTAAGCCAAA
H    ---------- ---------- ---------- ----X----- ---------- -T-----T-- ---------- ---T------ ---------- T---------

Mk   GGTAGTTAAA TCCAGTACAC TTAAAAATGG CCCAGTGACA CAGCCTTTAA ATTTGTCAAT ATTTTXCCTX TTGGTGATAT TGAAAATCAA TGAGTAGAGA
H    ---------- ---------- -----C---- ----A----- ---------- -C-------- -----T---C ------G--- ------C--G ----C-----

Mk   GCATAGGAAT AGAACTTCAA GAACGTGGCA AGAGC
H    -----A---C ---------- --C------- G----
```

B MAOB sequence

```
Mk   ACCTGTATAG CCTCACTTCC CTAGTTCTTT GCATTTGTCC TTAGAATACT GTATTGTTAC AGCTGAAAGA CATTAAAGAC CATTTAGTCC TCACXXXCTG
H    ---------- ---------- ---------- -----C---- ----XG---- ---------- ---------- --G------- ---------- ----CTT---

Mk   TTTTAGAGTT GAGCAAACTG AAGCCGACAG GGGTGGAACT TAATTACCTA AGAGCTGCAA TAAGCCACTG GTATCTCGGG GACTAGAACA CAAATCCAAC
H    ---------- ---------- -----C---- A--------- ---------- -----CA--- ---------- ------G--- ---------- -----XA-TT

Mk   GCCTTTCCCA CCTCCCTGGA TGTATTCCCC AATTATCCTC CTTCACTCCC TGTTATTGTC ACTGATGGTG TCCCCTTGTG TGGATTTACT TTGTGCTAAG
H    --T------- ----TT---- ---T------ ---------- ---------- ---C--A--T --C------- ----G----- ---G------ C---------

Mk   TTGTCTTACA CTACTCAAAT GCTACTCAGT ATATAGCCTT AAGTCTTACT GTTTTGTGCG GTGTGTCTCC AGCTGA
H    ---------- --T------- ---------- ---------- --C------- ---------- ---------- ------
```

X-not present in matching sequence

Fig. 1 Nucleotide sequence of amplified monkey (*Mk*) MAO-A (**A**) and MAO-B (**B**) cDNA compared to human (*H*) MAO-A and MAO-B cDNA. *Bold regions* indicate the location of PCR primers. *Bold letters* indicate differences between sequences. Nucleotides that are identical between species appear as *dashes*. Spaces in the sequences that are not matched by a nucleotide appear as *X*. MAO-A and MAO-B were both 92% identical to their human sequences

Results

MAO-A and MAO-B cDNA and controls

The nucleotide sequences of the MAO-A and MAO-B cDNAs obtained from the rhesus monkey genomic library are shown in Fig. 1 with the primers in bold. The MAO-A cDNA contains 233 bp [nucleotides 1639–1872, corresponding to amino acids 524–529 plus the untranslated 3' region, GenBank accession no. M68857.1 (Bach et al. 1988)] The MAO-B cDNA contains 373 bp, [nucleotides 2001–2374, corresponding to the untranslated 3' region, GenBank accession no. M69177.1 (Bach et al. 1988)]. At the nucleotide level, the monkey MAO-A and MAO-B clones are 92% identical to the corresponding regions of human cDNA.

No specific labeling was detected when MAO-A or MAO-B sense cRNA was applied to hypothalamic or raphe sections. RNase A pretreatment also completely eliminated the signal.

Distribution of MAOA and MAOB mRNA

Representative MAO-A and MAO-B autoradiographs from spayed monkey hypothalamus and raphe are shown in Fig. 2 and Fig. 3. A moderately intense MAO-A hybridization signal was detected in the hypothalamic nuclei, including the suprachiasmatic region (SCN), supraoptic nucleus (SON), paraventricular nucleus (PVN), ventromedial nucleus (VMN), and lateral hypothalamus (LH) (Fig. 2A–C). In addition, a somewhat less intense MAO-A hybridization signal was detected in the dorsal raphe nucleus (Fig. 2D–E). The MAO-B hybridization signal was generally more intense and prevalent in the hypothalamus than MAO-A. (This enabled the use of ^{35}S-labeled riboprobes for MAO-B.) MAO-B signal was prominent in the SCN, POA, PVN, SON, LH and VMN (Fig. 3A–C) and it was also intense in the dorsal raphe nucleus (Fig. 3D–E). Figures 2F and 3F show the absence of MAO-A and MAO-B signal, respectively, when sense riboprobes were applied to the dorsal raphe nucleus (negative control).

Steroid regulation of MAO-A and MAO-B mRNA

The OD and positive pixel area (±SEM) for MAO-A mRNA in dorsal raphe nucleus from the spayed, E-treated, P-treated, and E+P-treated macaques is shown in Fig. 4. As reflected both in OD and in pixel area, MAO-A mRNA expression in the dorsal raphe nucleus was significantly decreased by each ovarian steroid treatment, but E and P did not act in an additive manner.

The OD and positive pixel area (±SEM) for MAO-A mRNA in the hypothalamus from the spayed, E-treated, P-treated, and E+P-treated macaques is shown in Fig. 5. E and P treatment, alone and in combination, significantly decreased MAO-A mRNA OD in the hypothalamic PVN, VMN and LH (panel A). The combination of E+P did not appear to be additive compared to the individual treatments. The pixel area analysis for MAO-A mRNA loosely reflected the OD analysis (panel B). MAO-A mRNA pixel area decreased with E and P treatment in the PVN and VMN, but not in the LH.

The OD and positive pixel area (±SEM) for MAO-B mRNA in dorsal raphe nucleus from the spayed, E-treated, P-treated, and E+P-treated macaques is shown Fig. 6. Neither the OD nor the pixel area representing MAO-B

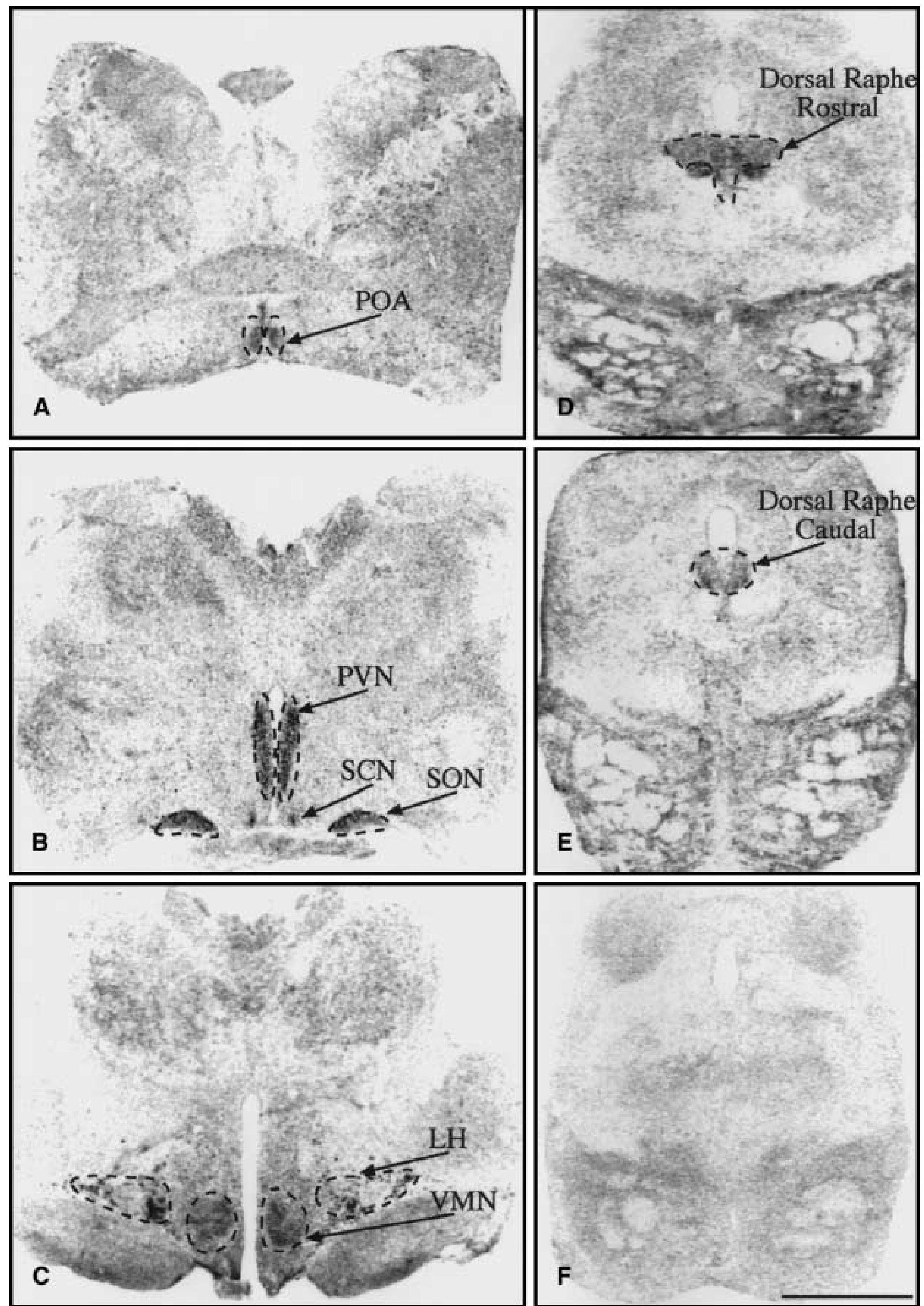

Fig. 2A–F Autoradiographs of representative sections of monkey hypothalamus and raphe illustrating MAO-A mRNA expression. A strong MAO-A signal was localized to the supraoptic nucleus (*SON*), paraventricular nucleus (*PVN*), ventromedial nucleus (*VMN*), and lateral hypothalamus (*LH*), with a less intense signal in the suprachiasmatic nucleus (*SCN*) of the hypothalamus (**A**–**D**). The MAO-A mRNA expression in the dorsal raphe nucleus (*DRN*) was moderate (**E**). The MAO-A sense riboprobe showed no hybridization signal in the DRN (negative control, **F**). Scale bar=5 mm

mRNA expression in the dorsal raphe nucleus was altered by any ovarian steroid treatment.

The OD and positive pixel area (±SEM) for MAO-B mRNA in the hypothalamus from the spayed, E-treated, P-treated, and E+P-treated macaques is shown in Fig. 7. E and P, alone or in combination, decreased the OD signal representing MAO-B mRNA in the POA, LH, and VMN (panel A). The pixel area analysis for MAO-B mRNA (panel B) was in general agreement with the OD analysis. There was a decrease in the pixel area signal representing MAO-B in the hypothalamic POA, LH and VMN in a manner reflecting the OD.

Serum levels of E and P

Serum samples were collected from each animal at necropsy and assayed for E and P by radioimmunoassay.

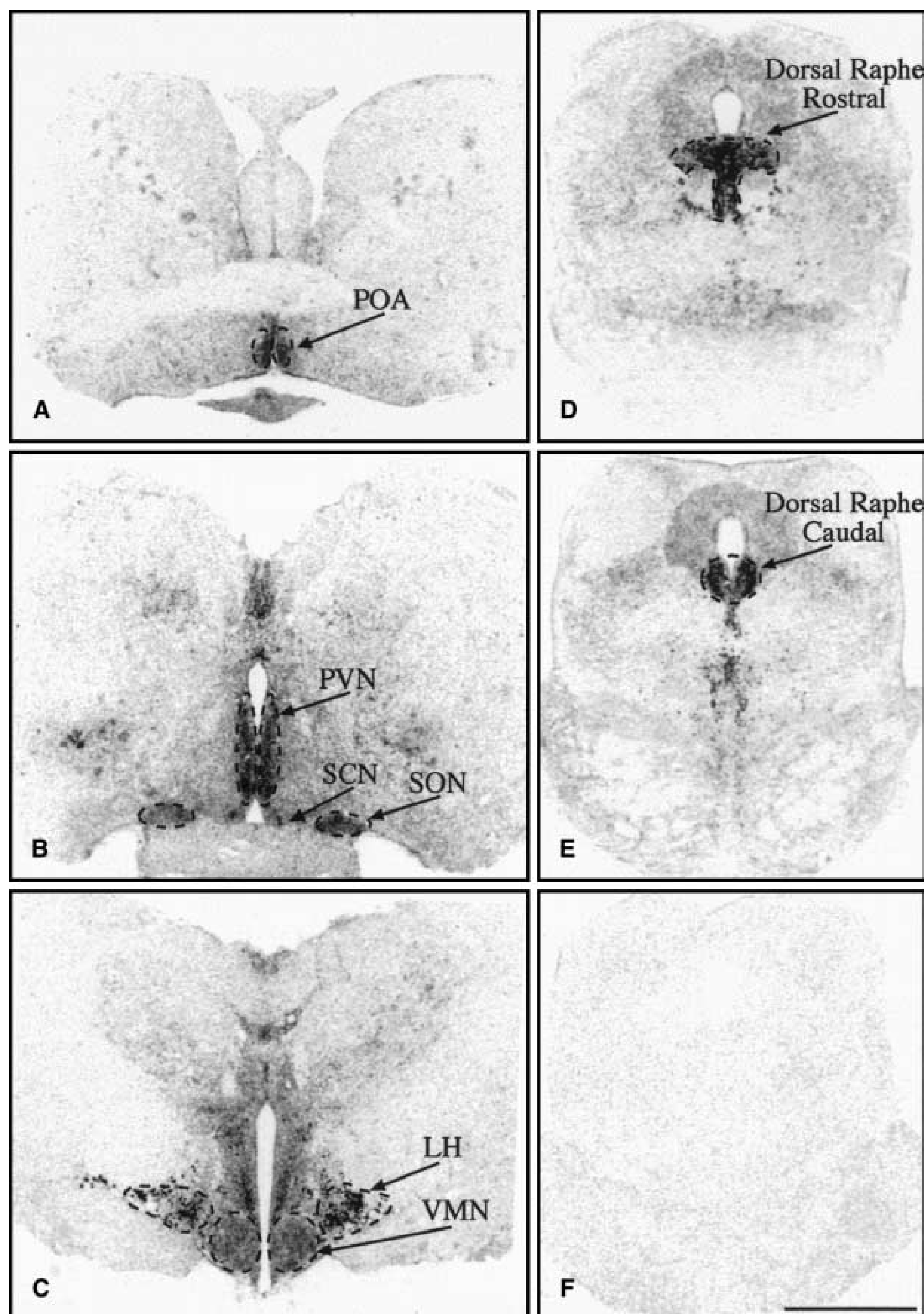

Fig. 3A–F Autoradiographs of representative sections of monkey hypothalamus and raphe illustrating MAO-B mRNA expression. A strong MAO-B signal was localized to the preoptic area (*POA*), supraoptic nucleus (*SON*), paraventricular nucleus (*PVN*), ventromedial nucleus (*VMN*), and lateral hypothalamus (*LH*), with a less intense signal in the suprachiasmatic nucleus (*SCN*) of the hypothalamus (**A**–**D**). The MAO-B mRNA expression in the dorsal raphe nucleus (*DRN*) was the most intense of the observed signals (**E**). The MAO-B sense riboprobe showed no hybridization signal in the DRN (negative control, **F**). Scale bar=5 mm

Each assay exhibited an intra-assay coefficient of variation (CV) of <9%. The sensitivity of the E assay equaled 5 pg/ml and the sensitivity of the P assay equaled 0.1 ng/ml. The mean (±SEM) concentration of E in the serum of the E and E+P-treated groups was 95.2±26.2 pg/ml. The mean (±SEM) concentration of P in the serum of the P and E+P-treated groups was 9.59±1.1 ng/ml. The E level is within the range reported for the mid- to late follicular phase and the P level is within the range reported for the mid-luteal phase of the primate menstrual cycle (Hotchkiss and Knobil 1994). The mean (±SEM.) concentrations of E and P in the serum of the untreated spayed control group were 5.8±0.8 pg/ml and 0.23±0.12 ng/ml, respectively.

Discussion

MAO-A and-B are the main degradative enzymes for catecholamines and serotonin in the CNS and periphery.

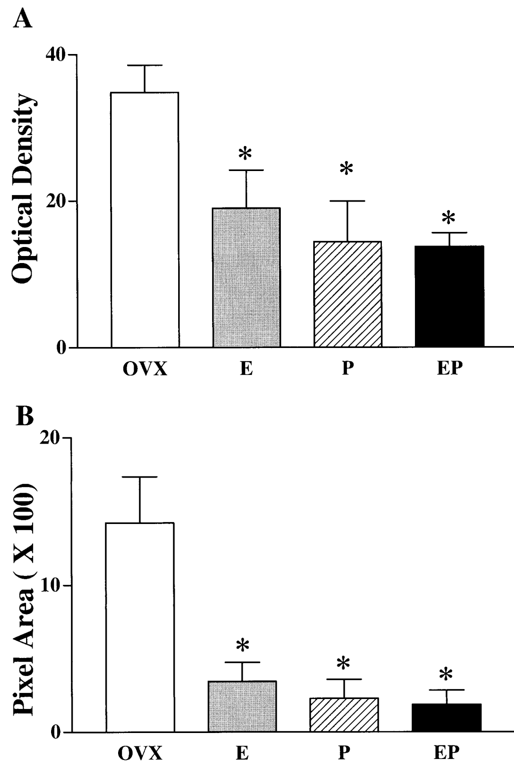

Fig. 4 The mean OD (**A**) and positive pixel area (**B**) (±SEM) for MAO-A mRNA hybridization signal in the dorsal raphe nucleus from spayed, E-treated, P-treated, and E+P-treated animals. The data are presented in arbitrary units. Four to six levels of the dorsal raphe nucleus were analyzed, and the mean was obtained for each animal. There were five animals per treatment group, but statistical outliers (>3 SD away from the mean) were discarded. There was a significant effect of treatment on OD (ANOVA $P<0.0195$, $F=4.5$, $df=3,19$) and pixel area (ANOVA $P<0.0007$, $F=9.8$, $df=3,16$). Post hoc pairwise comparison indicated that groups treated with E, P or E+P were significantly less than the spayed control group ($P<0.05$)

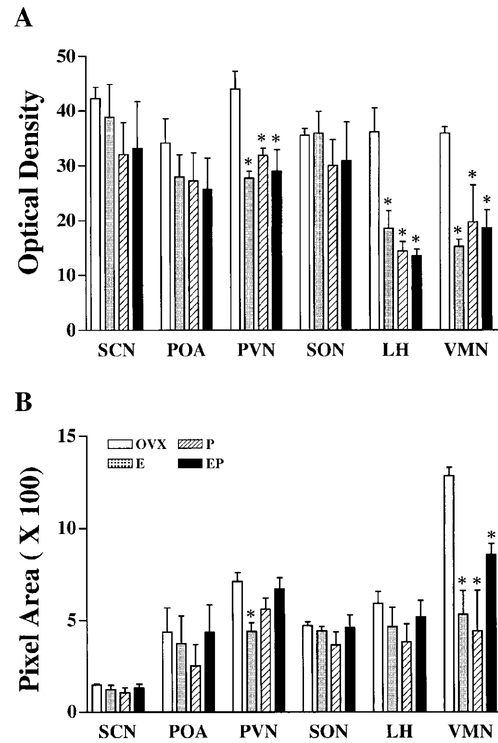

Fig. 5 The mean OD (**A**) and positive pixel area (**B**) (±SEM) for MAO-A mRNA hybridization signal in the hypothalamus from spayed, E-treated, P-treated, and E+P-treated animals. The data are presented in arbitrary units. There were five animals per treatment group, but statistical outliers (>3 SD away from the mean) were discarded. Two sections per animal were analyzed for the suprachiasmatic nucleus (*SCN*), four sections for the preoptic area (*POA*), supraoptic nucleus (*SON*), lateral hypothalamus (*LH*), and six sections were analyzed for the paraventricular nucleus (*PVN*), and ventromedial nucleus (*VMN*). There was a significant effect of treatment on the OD for MAO-A mRNA in the PVN, LH and VMN of the hypothalamus (ANOVAs: PVN $P<0.0053$, $F=6.6$, $df=3,14$; LH $P<0.0004$, $F=12$, $df=3,14$; VMN $P<0.0289$, $F=4.1$, $df=3,13$). Posthoc pairwise comparisons indicated that there was a significant decrease in OD for MAO-A with E, P and E+P treatment in the PVN, LH and VMN ($P<0.05$). There was a significant effect of treatment on the pixel area for MAO-A mRNA in the PVN and VMN (ANOVAs: PVN $P<0.0168$, $F=4.8$, $df=3,14$; VMN $P<0.0011$, $F=10$, $df=3,13$). Post hoc pairwise comparison indicated that there was a significant decrease in pixel area for MAO-A mRNA with E treatment in the PVN and that there was a significant decrease in pixel area for MAO-A mRNA with E, P and E+P treatment in the VMN. No differences were observed in the other hypothalamic regions with ANOVA

The importance of these enzymes to limbic function is underscored by the clinical observation that MAO inhibitors alleviate depression, although their side effect profile has made them less attractive in treatment paradigms since the advent of the selective serotonin reuptake inhibitors (SSRIs). A body of evidence suggests that ovarian steroids, particularly estrogens, also impact mood and cognitive function (Halbreich 1990; Fink et al. 1996; McEwen 1999). We and others have suggested that some of the beneficial psychological effects of E could be transduced by action in serotonin neurons (Halbreich 1990, 1997). However, the location of nuclear receptors for E in the hypothalamus and elsewhere suggests an even wider target. Moreover, a new generation of antidepressants are achieving substantial results by blocking both serotonin and norepinephrine transporters (Rossby et al. 1999; Freeman et al. 2001; Melichar et al. 2001) reviving the notion that catecholamine systems also play a role in affective disorders.

The psychological effects of E in clinical contexts are still not well-characterized, however available data suggest that E has generally favorable effects on mood and cognition. In several studies, E exhibited antidepressant effects (Klaiber et al. 1979; Schmidt et al. 2000; Gregoire et al. 1996), and it improved aspects of cognitive function (Sherwin 2000). Additionally, it has been suggested that antidepressants such as SSRIs may be more efficacious in the presence of E (Schneider et al. 1997), though we note that this report was limited by a

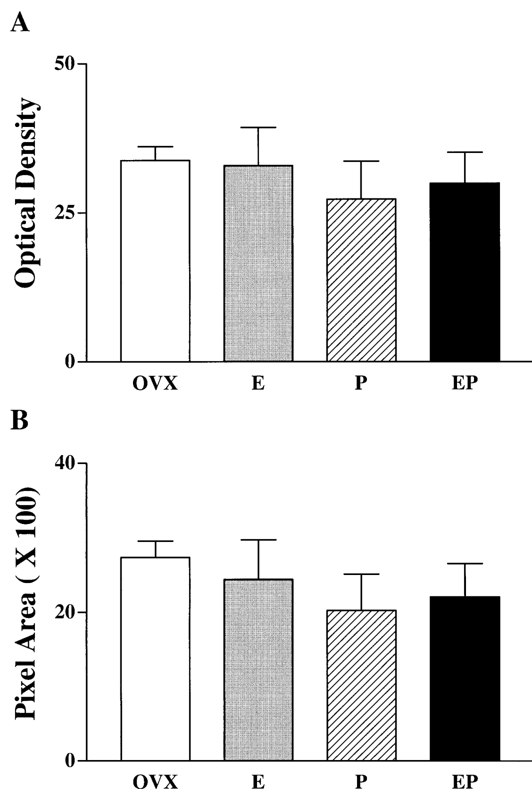

Fig. 6 The mean OD (**A**) and positive pixel area (**B**) (±SEM) for MAO-B mRNA hybridization signal in the dorsal raphe nucleus from spayed, E-treated, P-treated, and E+P-treated animals. The data are presented in arbitrary units. Four to six levels of the dorsal raphe nucleus were analyzed the mean was obtained for each animal. There were five animals per treatment group, but statistical outliers (>3 SD away from the mean) were discarded. There was no effect of treatment on either the OD or the positive pixel area representing the MAO-B hybridization signal in the dorsal raphe nucleus

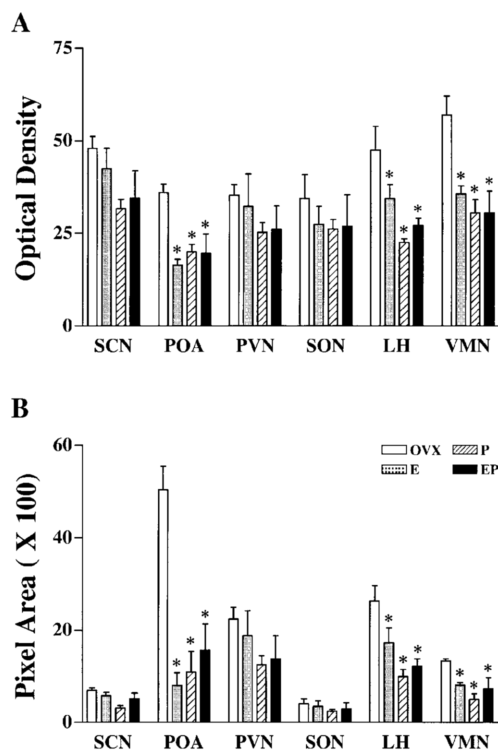

Fig. 7A, B The mean OD (±SEM) for MAO-B mRNA hybridization signal minus background in each hypothalamic nucleus from spayed, E-treated, P-treated, and E+P-treated animals. The data are presented in arbitrary units. There were five animals per treatment group in all regions, except the dorsal raphe (DRN) of the P-treated group, where n=3. Two sections per animal were analyzed for the preoptic area (*POA*) and suprachiasmatic nucleus (*SCN*), three sections for the the paraventricular nucleus (*PVN*), and four to six sections for the supraoptic nucleus (*SON*), lateral hypothalamus (*LH*), ventromedial nucleus (*VMN*). There was a significant effect of treatment on the OD for MAO-B mRNA in the POA, LH and VMN of the hypothalamus (ANOVAs: POA $P<0.0024$, $F=7.7$, $df=3,15$; LH $P<0.0014$, $F=8.7$, $df=3,15$; VMN $P<0.0038$, $F=7.1$, $df=3,14$). Post hoc pairwise comparisons indicated that there was a significant decrease in OD for MAO-B mRNA with E, P and E+P treatment in the POA, LH and VMN ($P<0.05$). There was a significant effect of treatment on the pixel area for MAO-A mRNA in the POA, LH and VMN (ANOVAs: POA $P<0.0001$, $F=17$, $df=3,15$; LH $P<0.0022$, $F=7.8$, $df=3,15$; VMN $P<0.0144$, $F=5$, $df=3,14$). Post hoc pairwise comparison indicated that there was a significant decrease in pixel area for MAO-A mRNA with E, P and E+P treatment in the POA, LH and VMN ($P<0.05$)

number of factors, and that no large, prospective, controlled study addressing this issue has been reported. However, the potency of serotonergic agents in the paradigm of a challenge test does appear to be higher in the presence of E (Halbreich et al. 1995).

At a molecular level, a primary target of estrogenic ligands is gene expression. Estrogens, natural and synthetic, bind with high affinity to nuclear transcription factors called ERα and ERβ (Tsai and O'Malley 1994; Kuiper et al. 1997) which in turn, bind to the promoter region of target genes and/or interact with other transcription factors in a manner so as to either increase or decrease gene expression in a tissue specific fashion (Katzenellenbogen et al. 1996). We recently reported that the macaque serotonin neural system employs ERβ, and not ERα, for transduction of estrogenic action (Gundlah et al. 2000, 2001). In addition, we showed that E treatment of spayed macaques increased progestin receptor (PR) and tryptophan hydroxylase (TPH) gene expression (Bethea 1994; Pecins-Thompson et al. 1996), but decreased expression of the serotonin reuptake transporter (SERT) and $5HT_{1A}$ genes in the dorsal raphe nucleus of macaques (Pecins-Thompson and Bethea 1998; Pecins-Thompson et al. 1998). In concert, these data indicate that E acts via the ERβ receptor/transcription factor in the dorsal nucleus raphe to alter gene expression in serotonin neurons.

In this study, we examined the effect of E, P and E+P on gene expression for MAO-A and MAO-B in the predominantly serotonergic nucleus, the dorsal raphe, and in

various hypothalamic nuclei known to contain dense populations of ERα, ERβ and PR positive neurons (Bethea et al. 1996; Shughrue et al. 1997; Gundlah et al. 2000), and that are heavily innervated by serotonin fibers (Willoughby and Blessing 1987; Rischer et al. 1995). Relying predominantly on the OD signal analysis with support from the pixel area analysis, there was a significant decrease in MAO-A (which prefers serotonin and norepinephrine) in the presence of E, P and E+P in the dorsal raphe nucleus and in several hypothalamic nuclei. MAO-B, which is more promiscuous (Ekstedt and Oreland 1976; Achee and Gabay 1977; Mitra and Guha 1980; Tipton et al. 1982; Luine and Paden 1982) was significantly decreased in several hypothalamic nuclei by E, P or E+P, but it was not regulated in the dorsal raphe. These data indicate that ovarian hormones decrease gene transcription for the major enzymes that degrade aminergic neurotransmitters in the non-human primate CNS. If this change in gene expression is reflected by a change in protein, then it follows that ovarian hormones can increase extracellular concentrations of serotonin, and possibly catecholamines, by decreasing their metabolic oxidation. This action would work in concert with the previously described effects of E and E+P on gene expression leading to serotonin synthesis, reuptake and autoinhibition (Bethea et al. 1999).

On a technical note, we speculate that the OD and pixel area should generally change in a similar fashion. However, if pixel area reflects the number of cells expressing signal over threshold, whereas OD represents the average of the signal intensity in a given area, then discrepancies between the two measurements are conceivable. For example, if all of the cells in a given area manifest a significant decrease in signal yielding a decrease in OD, but the signal remained above threshold then the pixel area may not change. Conversely, we have observed situations where a subpopulation of cells in a given area dropped below threshold producing a decrease in pixel area, but the average OD of the remaining cells was unchanged.

Regarding the anatomical localization of the MAO isoforms, this study indicates that MAO-A and MAO-B mRNA are both expressed in discrete nuclei of the hypothalamus and in the dorsal raphe nucleus of the macaque. We observed a significantly greater expression of MAO-B than MAO-A mRNA in the dorsal raphe. These observations confirm previous reports showing that in the human and/or monkey brainstem, MAO-B mRNA and protein predominate in the indole-containing raphe nucleus, while MAO-A resides predominantly in the catecholaminergic locus coeruleus, although MAO-A mRNA has been observed in the monkey raphe (Westlund et al. 1985, 1988; Richards et al. 1992; Luque et al. 1996). Likewise, there is a 10-fold higher concentration of MAO-B than MAO-A in the monkey striatum whereas in the mouse striatum, MAO-A and B are roughly equal (Irwin et al. 1997). We also observed significant expression of both MAO-A and B mRNAs in the monkey hypothalamus. In rats, MAO-A mRNA is found in the hypothalamus, but not MAO-B mRNA, although MAO-B activity is measured there (Luine and Paden 1982; Luine and Rhodes 1983; Jahng et al. 1997).

At this point, we cannot conclude that the serotonin neurons in the dorsal raphe are the cells expressing MAO-A. Double labeling studies are required to determine the phenotype of the MAO-A or B expressing neurons. However, if serotonin neurons do not express MAO-A, extracellular serotonin still may be balanced by metabolism in adjacent neurons. In addition, MAO-B has been implicated in metabolism of serotonin in vivo (Luine and Paden 1982), although we did not detect a change in MAO-B expression in the raphe with ovarian steroid treatment.

Our data on the regulation of MAO-A and B by E agrees largely with previous reports in rat where 3 weeks of treatment with a high dose of E decreased MAO-A activity in the hypothalamus by 28%, and in the amygdala by 21% (Holschneider et al. 1998). Acute treatment with estradiol benzoate in ovariectomized rats also decreased MAO-A activity in the hypothalamus (Luine and Rhodes 1983; Ortega-Corona et al. 1994).

However, the decrease in MAO-A observed with supplemental P treatment of macaques differs somewhat from previous studies in rodents. Upon addition of P after E priming in rats, an increase in MAO-A activity was observed (Luine and Rhodes 1983; Ortega-Corona et al. 1994). Similarly, P given to E-primed rats decreased VMN serotonin (Farmer et al. 1996), and a nadir in hypothalamic serotonin was seen in estrus rats compared to male control rats (Gundlah et al. 1998), suggesting that P caused an increase in serotonin degradation (or an increase in MAO-A) in rats. Thus, there may be a species difference in the action of P on MAO-A expression in E primed subjects. The decrease in MAO-B with E+P in monkey hypothalamus is similar to the regulation of this isoform reported in rats (Ortega-Corona et al. 1994). Moreover, the lack of regulation of MAO-B in the dorsal raphe is also consistent with a previous study in rats (Holschneider et al. 1998). In summary, these reports indicate that E decreased MAO-A and MAO-B in monkeys and rats. Addition of P to the E regimen decreased MAO-A in monkeys, but increased MAO-A in rats. Addition of P to the E regimen decreased MAO-B in monkeys and rats. Thus, there is a difference between rats and monkeys only in the effect of P on MAO-A.

We often observed an effect of P alone on MAO-A and -B. This is somewhat confounding due to the current dogma that E priming is necessary for the expression of nuclear progestin receptors (PR). If an action of P is transduced by nuclear PR, then the action should be absent without the receptor. However, due to low levels of E from the adrenal or fat tissue, there may have been enough PR present in the spayed macaque brain to mediate the action of P on MAO gene transcription. Recently, we found that 3 months of treatment with the synthetic progestin, cyproterone acetate, decreased the serotonin metabolite, 5-HIAA, in CSF of male macaques and this correlated with a decrease in self-injurious behavior indi-

Table 1 Location of known estrogen receptor isoforms[a] and actions of estrogen on MAO isoforms in discrete areas of the monkey midbrain and hypothalamus. *SCN* superchiasmatic nucleus, *POA* preoptic area, *PVN* paraventricular nucleus, *SON* superoptic nucleus, *LH* lateral hypothalamus, *VMN* ventromedial nucleus

Actions	Receptors present						
	Area Raphe	SCN	POA	PVN	SON	LH	VMN
MAO-A	ERβ			ERβ			
MAO-B			ERα				
A and B						??	ERα and ERβ
None		ERβ			ERβ		

[a] Data from Bethea et a1. (1996) and Gundlah et al. (2000)

cative of increased serotonin (Eaton et al. 1999). Male macaques also have low levels of E and yet there was an apparent P-induced decrease in serotonin degradation. Moreover, we have previously observed significant levels of hypothalamic PR in males as well as, spayed female macaques (Bethea et al. 1992). P metabolites can also have actions via membrane GABA receptors and this mechanism cannot be discounted (Majewska 1992; Bitran et al. 1995; Teschemacher et al. 1995).

Linking the regulation of MAO by E to nuclear ER in discrete areas is not straightforward and suggests that there are gaps in our understanding of the mechanism(s) of action of these hormones in the CNS. As summarized in Table 1, areas with predominantly ERβ, such as the dorsal raphe and PVN, showed regulation of MAO-A, but not B. Areas with predominantly ERα, such as the POA, showed regulation of MAO-B, but not MAO-A. The VMN, which contains both ERα and ERβ, exhibited steroid regulation of both MAO-A and MAO-B. Thus, if these areas are taken as guides, one could simplistically propose that the MAO-A promoter prefers ERβ and the MAO-B promoter prefers ERα. However, there are some notable exceptions to this generalization. ERβ positive neurons have been observed in the SON and the SCN, but there was no regulation of either MAO-A or B in these regions. In addition, the lateral hypothalamic (LH) area is not rich in either ERα or ERβ, and yet it consistently showed a decrease in both MAO-A and MAO-B expression with E treatment. These exceptions do not rule out differential promoter preference for the ER isoforms, but they do suggest that multiple, complex mechanisms of action are probably involved.

It is also difficult to couple the action of P on MAO-A or B tightly to nuclear PR in discrete regions. The areas that exhibit regulation of MAO-A or B generally contain populations of neurons with nuclear PR, although the concentrations vary markedly from nearly none in the lateral hypothalamus (LH), to moderate in the PVN, to very dense in the VMN. Hence, we cannot definitively account for the mechanisms by which E and P regulate MAO-A and -B in hypothalamic versus serotonin neurons. However, it appears that ovarian steroids act on gene expression through their cognate receptors probably as a function of other, cell-specific co-factors that are, as yet, not fully described (Hall and McDonnell 1999; Wijayaratne et al. 1999; Hall et al. 2000; Schaufele et al. 2000). The organization of the MAO-A and MAO-B promoters exhibit significant differences which could provide the basis for differences in their regulation (Zhu et al. 1992). Moreover, in the CNS there can be trans-synaptic input for neuronal regulation.

In conclusion, E and P individually, or in combination, decrease MAO-A expression in the dorsal raphe nucleus and in hypothalamic PVN, LH and VMN. E and P alone, or in combination, have no effect on MAO-B in the dorsal raphe, but they caused a significant reduction in MAO-B in the hypothalamic POA, LH and VMN. If these changes in gene expression are manifested at the protein level, then E and P may reduce degradative oxidation of biogenic amines by decreasing MAO. In turn, this action could elevate extracellular levels of aminergic neurotransmitters, in particular serotonin, and thereby elevate mood or facilitate cognitive functions.

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