Estrogen Selectively Increases Tryptophan Hydroxylase-2 mRNA Expression in Distinct Subregions of Rat Midbrain Raphe Nucleus: Association between Gene Expression and Anxiety Behavior in the Open Field

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Background: *Ovarian steroids modulate anxiety behavior, perhaps by regulating the serotonergic neurons in the midbrain raphe nucleus. The regulation of the brain-specific isoform of rat tryptophan hydroxylase (TPH2) by ovarian hormones has not yet been investigated. Therefore, we examined the effects of estrogen and progesterone on TPH2 mRNA in the rat dorsal and median raphe nuclei (DRN and MRN, respectively) and whether TPH2 mRNA levels correlated with anxiety behavior.*

Methods: *Ovariectomized rats were treated for two weeks with placebo, estrogen or estrogen plus progesterone, exposed to the open field test, and TPH2 mRNA was quantified by in situ hybridization histochemistry.*

Results: *Estrogen increased TPH2 mRNA in the mid-ventromedial and caudal subregions of the DRN and the caudal MRN. Combined estrogen and progesterone treatment did not change TPH2 mRNA relative to ovariectomized controls. TPH2 mRNA in caudal DRN was associated with lower anxiety-like behavior, whereas TPH2 mRNA in rostral dorsomedial DRN was associated with increased anxiety-like behavior.*

Conclusions: *These results suggest that estrogen may increase the capacity for serotonin synthesis in discrete subgroups of raphe neurons, and reinforce previous observations that different subregions of DRN contribute to distinct components of anxiety behavior.*

Key Words: Ovarian hormones, progesterone, serotonin, TPH2, dorsal raphe, median raphe

Serotonin (5-HT) neurons emanating from the midbrain raphe nuclei (dorsal and median raphe nucleus; DRN and MRN, respectively), a major source of serotonergic innervation to the forebrain, play a critical role in stress responsiveness (Maes and Meltzer 1995; Sandford et al 2000). Mood and anxiety disorders often respond favorably to antidepressants that alter serotonergic function, such as selective serotonin reuptake inhibitors (SSRIs). There are also several lines of evidence showing that anxiety and affective disorders disproportionately affect women compared to men (Palanza 2001; Pigott 2003; Steiner et al 2003) and that the brain serotonin system is sexually dimorphic and is influenced by ovarian hormones (Rubinow et al 1998). Animal studies demonstrate increased synthesis, turnover, and overall levels of brain 5-HT in female rats compared to male rats (Carlsson et al 1985; Haleem et al 1990; Rosecrans 1970; Vaccari et al 1977). This increase may be due to estrogen, because estrogen has shown to increase the basal firing rate of serotonergic neurons in female rats (Robichaud and Debonnel 2005), to increase 5-HT2A and 5-HT transporter (SERT) mRNA in the DRN and binding sites in various forebrain regions of ovariectomized rats (McQueen et al 1997; Sumner et al 1999). Furthermore, estrogen (E) and progesterone (P) have complex effects on anxiety-like behavior in rats (Hiroi and Neumaier 2006; Koss et al 2004; Lund et al 1999; Morgan et al 2004).

Tryptophan hydroxylase (TPH), the rate-limiting enzyme for 5-HT synthesis, is one of the key control points in regulation of the serotonergic system and may be a target for ovarian steroids during modulation of anxiety. TPH mRNA expression in the DRN has been shown to increase with E treatment in macaques (Pecins-Thompson et al 1996) and mice (Gundlah et al 2005), but not in rats (Alves et al, unpublished data). TPH protein levels have also been shown to increase with E and E plus P treatment in DRN of macaques (Bethea et al 2000) and guinea pigs (Lu et al 1999). However, a second, brain-specific, isoform of TPH has recently been described (TPH2; Walther and Bader 2003) and older studies of TPH mRNA (now called TPH1) may not have accurately reflected the expression of TPH in the raphe nuclei. Thus, recent efforts have been made to examine the expression of TPH2 following hormone treatment. TPH2 mRNA in the DRN has been shown to increase with E and E plus P treatment in macaques (Sanchez et al 2005) but not in mice (Clark et al 2005). Regulation of TPH2 in the midbrain raphe by gonadal steroids in rats has not been reported. The interaction of ovarian hormones with TPH2 expression is particularly relevant; recent studies identified changes in TPH-immunoreactive neurons in the DRN in depressed suicide victims (Boldrini et al 2005), TPH1 polymorphisms in depression and anxiety (Nash et al 2005; Sun et al 2004; You et al 2005), and TPH2 in the genetic susceptibility to major depression (Zhang et al 2005; Zill et al 2004), implicating TPH as an important factor in stress-related dysfunctions. Furthermore, it is now recognized that DRN consists of multiple subregions that have differential responses to stress exposure and distinct output targets in forebrain (Abrams et al 2004; Hammack et al 2002; Kirby et al 2000; Lowry 2002; Molliver 1987; Price et al 1998; Roche et al 2003). Therefore, we investigated the effects of E and P on TPH2 mRNA expression in the subregions of the DRN and MRN in ovariectomized rats treated with placebo, E, or E plus P for two weeks. In addition, to examine its relevance

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to anxiety behaviors, we correlated TPH2 mRNA levels with behavior in the open field test in these same rats.

Methods and Materials

Subjects

A total of 26 female Sprague-Dawley rats between the ages of 60 and 90 days were used. In the first group, animals were divided into ovariectomized (OVX) ($n = 6$) or OVX/E ($n = 6$). In the second, animals were divided into OVX ($n = 7$) or OVX/EP ($n = 7$). They were group-housed with 3 animals per cage on a 12-hour light/dark cycle (lights on at 6 am), and all behavioral measures were performed during the light period. Rats were acclimated to the colony rooms for at least 1 week before any experimental manipulation.

Ovariectomy and Hormone Capsule Implantation

Animals were anesthesized with 3% isoflurane, given a single injection of buprenorphine (.05 mg/kg subcutaneous [SC]), and the abdominal skin was shaved and wiped with an alcohol pad. Using sterile equipment, a small incision was made on the side of the abdomen below the ribs and the ovary was located using blunt dissection. The fallopian tube was clamped and tied well below the ovary and then cut above the clamp. The muscle was sutured, and the skin was closed with 2 or 3 wound clips. This procedure was repeated on the opposite side. Then an incision was made in the skin below the neck and a tunnel was made under the skin for capsule implantation. The skin was closed with wound clips. Hormone capsules were made a day prior to the surgery day using Silastic tubing (Dow Corning, Midland, Michigan) filled with crystallized 17-beta estradiol or progesterone (Steraloid, Wilton, New Hampshire), or left empty for placebo. The ends were plugged with small pieces of wooden dowel and sealed with silicone glue. The length of the Silastic tube determined the rate of hormone release (Smith 1978). Estrogen capsules had a total delivery length of 3 mm while progesterone capsules were 36 mm. According to previous studies using a similar method of hormone administration (Bridges 1984; Hope et al 1992; Sell et al 2000; Zhou et al 2002), capsules of these lengths produce serum estradiol levels of 30–40 pg/ml and progesterone levels of 10–20 ng/ml, approximating the moderate circulating levels of E and P found during the normal estrous cycle of the adult female rat (Freeman 1994; Smith et al 1975). Radioimmunoassay confirmed that mean ± SEM plasma estradiol level in estrogen-treated OVX rats was significantly higher than OVX rats treated with blank capsules (32.0 ± 2.8 pg/ml vs. 7.4 ± .9 pg/ml, respectively; $t(12) = 8.34$, $p < .0001$) and that mean ± SEM plasma progesterone level of progesterone-treated OVX rats was significantly higher than OVX rats with blank capsules (13.4 ± 2.3 pg/ml vs. 1.9 ± .5 pg/ml, respectively; $t(12) = 4.853$, $p < .0003$) (Hiroi and Neumaier 2006).

Vaginal Smears and Analysis

Vaginal smears were obtained to confirm OVX and hormone replacement. A wet cotton swab was inserted approximately 5 mm into the vagina. These swabs were smeared on glass slides and the resulting vaginal cell samples were stained shortly after the smear. Cells were fixed for 5 min in 100% methanol, dried for 10 min, and stained for 15 min in 5% Giemsa stain (Sigma, St. Louis, Missouri). Stained cells were analyzed via light microscopy. The OVX rats had a predominance of leukocytes with nucleated (N) and cornified (C) cells similar to that found in a rat in diestrus stage of the estrous cycle, indicating a successful removal of the ovaries and hormones; in general, OVX/E and OVX/EP rats had a predominance of N or C cells with small number of leukocytes, similar to that found in proestrus or estrus, respectively. Vaginal smears were obtained at least 2 hours before behavioral testing to allow any stress from this procedure to subside.

Open Field Test

Behavior in the open field was tested 2 weeks after capsule implantation using a start box procedure (Hoplight et al 2005). Animals were tested using a 100 cm square black Plexiglas enclosure with 30 cm tall walls set on a nonreflective black plastic base. This open field was located in a small, quiet room fitted with a video camera 2 m above the apparatus. Animals were tested under a low-illumination red light to simulate darkness and minimize stress levels. After a pre-test acclimation of at least 30 min in the testing rooms, animals were placed in a closed 9 × 12 cm start box located on one of the walls of the open field. After a 2-min acclimation period, a remotely operated sliding door was opened, allowing the rat access to the arena. Latency to exit the start box was recorded, and behavioral data in the open field test (OFT) were collected for 10 min (SMART video tracking software, San Diego Instruments, San Diego, California). Rats were then removed from the OFT arena and returned to their home cages. In an OFT arena, rats prefer the periphery to the center of the apparatus. Center entering assesses approach-avoidance toward novel stimuli, which is considered a reliable index of anxiety, responds to anxiolytic agents (Ramos et al 1997), and is sensitive to stress-induced anxiety states (Durand et al 1999; Izumi et al 1997; Pare 1994). We quantified the following parameters: percent time spent in the center and corners of the OFT arena (anxiety-like behavior) and total distance traveled (overall locomotor activity). Animals were narcotized with CO_2 before decapitation, and the brains were quickly frozen on dry ice and stored at −70°C.

In Situ Hybridization Histochemistry

Using a Leica Jung CM3000 cryostat, serial 20-μm coronal sections of the DRN across the anteroposterior axis were prepared and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, Pennsylvania). The sections were stored at −70°C until processed for in situ hybridization histochemistry (ISHH). In brief, tissue sections were briefly thawed at room temperature and fixed in cold 4% paraformaldehyde for 5 min. After rinsing in phosphate-buffered saline, sections were treated with acetic anhydride (.25% in .1 M triethanolamine) for 10 min, dehydrated through a series of graded alcohols, and air dried.

TPH2 riboprobes were prepared from a partial cDNA clone of TPH2 kindly provided by Dr. Michael Bader (Max-Delbrück Center, Berlin-Buch, Germany). The SacI fragment corresponding to bases 780–1060 of the cDNA was cloned into pGEM-3Zf(+) such that the antisense probe was generated using T7 RNA polymerase. Riboprobes were transcribed using α[^{33}P]-UTP (Amersham, Piscataway, New Jersey) constituting 25% of total UTP and T7 RNA polymerase. Labeled probes were purified from unlabeled nucleotides using LiCl precipitation and were diluted (1.0 pmol/ml) in a hybridization buffer containing 50% formamide, 10% dextran sulfate, .3 M sodium chloride, 10 mM Tris (pH 8.0), 1 mM EDTA, 1× Denhardt's (.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), .4 mg/ml yeast tRNA, and 200 mM dithiothreitol. Fifty microliters of the hybridization mixture was applied to each slide, and the sections were covered with HybriSlips (Sigma). The slides were incubated in moist

covered trays at 50°C overnight. Following the hybridization reaction, coverslips were removed and the slides were incubated in RNAse buffer (500 mM NaCl, 10 mM Tris, 1mM EDTA, pH 8.0) containing 20 μg/mL RNAse A for 30 min at 37°C, then again for 30 min at room temperature in 1× saline sodium citrate (SSC; 15 mM NaCl in 1.5 mM sodium citrate). Slides were then washed twice in .1× SSC for 30 min at 60°C, and once again in .1× SSC at room temperature for 30 minutes. The slides were dehydrated through a series of graded alcohol rinses containing 300 mM ammonium acetate, and air dried. Previous studies in our laboratory (Clark et al, submitted) have shown these conditions to be saturating for TPH2 mRNA in DRN tissue, and sense controls produced no detectable signal above background in DRN, MRN or elsewhere, indicating that antisense signal observed was specific to TPH2. Autoradiographic signal was detected using a Cyclone storage phosphor scanner (Packard Instruments, Meridian, Connecticut) at 600 dpi resolution, and images were stored on CD-ROM disks; exposure times were 24–26 hours.

Data Analysis

Densitometry and Data Analysis. In situ hybridization signal was quantified using a computer-based densitometry system (microcomputer imaging device, Imaging Research, St. Catherine's, Ontario, Canada). Hybridization signal (measured in arbitrary digital light units) from one section was measured from each subregion of each brain and was expressed relative to ^{14}C-plastic standards coexposed on each phosphor screen, which yielded a linear relationship between tissue radioactivity and measured signal intensity; tissue background within the same section of each measurement was subtracted. The rater was blinded to the treatment of the sections analyzed. There were ten subregions analyzed separately: rostral DRN included two subregions, rostral dorsomedial and ventromedial DRN (rDM and rVM, respectively); mid DRN included mid-dorsolateral, -dorsomedial, and -ventromedial DRN (mDL, mDM, mVM); caudal DRN included caudal dorsomedial and ventromedial DRN (cDM, cVM); and MRN included rostral, mid and caudal MRN (rMRN, mMRN, and cMRN). Each of these subregions were also analyzed separately. The two halves of the mDL and cDM subregions were averaged and used as one value for each region. Representative shapes used in quantification for each subregion are shown in Figure 1.

Between-group differences were analyzed using Student's t test for gene expression in each subregion, and Fisher's r to z test was used to analyze correlations between gene expression levels and behavior. All analyses were performed using Statview software (SAS, Cary, North Carolina), with $p \leq .05$ considered significant.

Results

The hormonal effects on TPH2 mRNA levels were assessed in rostral, mid, and caudal levels of the DRN and MRN as a function of hormonal treatment (see Figure 1). TPH2 mRNA expression values in each of these subregions were then pooled for all animals, regardless of treatment, to determine if there were any significant associations with anxiety-like behavior measured in the open field test.

Chronic E Treatment in OVX Rats Increased TPH2 mRNA in Distinct Subregions of the Midbrain Raphe; Combined E and P Treatment in OVX Rats Had No Effect

Chronic E treatment in OVX rats significantly increased TPH2 mRNA in the DRN ($t(97) = 4.497$; $p < .0001$; Figure 2). Analysis

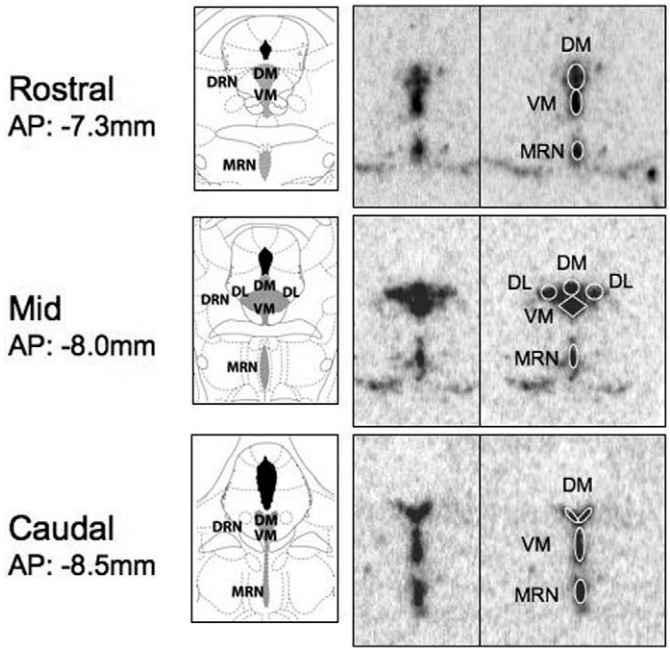

Figure 1. Subregional anatomy of the raphe nuclei. Schematic diagrams (Paxinos and Watson 1986) (left panel) and representative photomicrographs (middle panel) of the subregions analyzed for TPH2 in situ hybridization signal in rostral, mid, and caudal levels of the midbrain raphe. Ovals and diamonds are shown in the right panel to illustrate the shapes used for the densitometry analysis. DRN, dorsal raphe nucleus; MRN, median raphe nucleus; DM, dorsomedial; VM, ventromedial; DL, dorsolateral; TPH2, brain-specific isoform of rat tryptophan hydroxylase.

of the subregions revealed that E selectively increased TPH2 mRNA in mid ($t(20) = 3.528$; $p < .0009$) and caudal ($t(31)$; $p < .002$) DRN but had no effect on rostral DRN ($t(20) = .850$; $p = .41$). Further analysis showed that these increases were restricted to mVM (56% increase; $t(9) = 3.560$; $p < .007$), cDM (78% increase; $t(9) = 2.328$; $p < .05$), and cVM (85% increase; $t(9)$; $p < .05$) subregions of the DRN (Figure 3A). TPH2 mRNA levels in OVX/EP rats did not differ from those of OVX rats in any region examined (Figure 2B, 3B).

Chronic E treatment in OVX rats also increased TPH2 mRNA in the MRN ($t(30) = 2.254$; $p < .04$; Figure 2A) and this increase was restricted to caudal MRN ($t(9) = 2.844$; $p < .02$). However, there was no effect in rostral ($t(8) = 1.541$; $p = .16$), mid ($t(9) = 1.609$; $p = .14$) MRN. Combined E and P treatment did not change TPH2 mRNA relative to OVX controls ($t(30) = -.096$; $p = .92$; Figure 2B, 3B).

Anxiety-Like Behavior Had a Negative Association with TPH2 mRNA in the Caudal DRN and a Positive Association with TPH2 mRNA in the Rostral DRN

In the first group of animals (OVX and OVX/E), percent time spent in the center of the open field (an indication of decreased anxiety) positively correlated with TPH2 mRNA levels in cDM ($r = .698$; $p < .01$) and cVM ($r = .627$; $p < .04$) subregions of the DRN, regardless of treatment (Figure 4). This behavioral measure had no significant correlation with TPH2 levels in any other subregions of DRN or MRN. In addition, percent time spent in the corners of the open field (an indication of increased anxiety) had a significant positive correlation with TPH2 levels only in the rDM subregion of the DRN ($r = .733$; $p < .007$; Figure 3). No other regions showed significant correlation between TPH2 and

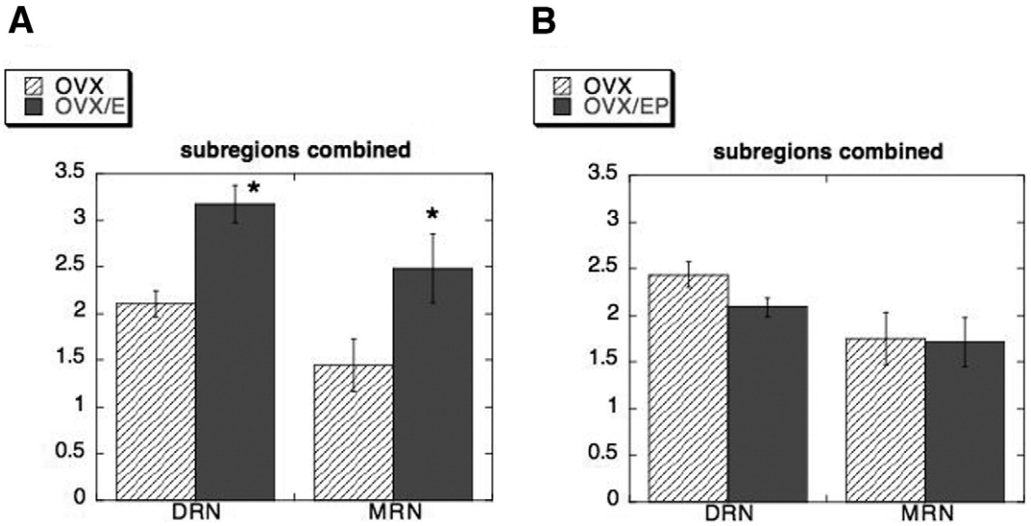

Figure 2. Estrogen increases TPH2 mRNA in the raphe nuclei. **(A)** E treatment significantly increased TPH2 mRNA in the DRN and MRN. **(B)** Combined E and P treatment had no effect on TPH2 mRNA in either the DRN or the MRN. Each bar represents the mean ± SEM TPH2 optical density in subregions of the midbrain raphe from each treatment group. *$p < .05$, *t*-test. DRN, dorsal raphe nucleus; MRN, median raphe nucleus; TPH2, brain-specific isoform of rat tryptophan hydroxylase; OVX, ovariectomized rats; E, estrogen-treated rats; P, progesterone-treated rats.

time spent in corners ($p > .05$). Total distance traveled (a measure of overall locomotion) had no significant correlation with TPH2 levels in any DRN or MRN subregions examined ($p > .05$). Table 1 summarizes these results. TPH2 levels in the raphe were not significantly correlated with anxiety behavior in the open field in the second group of animals (OVX and OVX/EP, data not shown). This lack of correlation is most likely due to the narrow range of TPH2 expression and anxiety behavior, because OVX and OVX/EP animals did not differ in these measures.

Discussion

In the present study, we examined the effects of E and P on TPH2 mRNA in the rat DRN and MRN with a detailed analysis of raphe subregions, which have not been rigorously examined in the past. We found that chronic E treatment in OVX rats increased TPH2 mRNA expression in distinct subregions of the DRN, namely mVM and caudal DRN (both cDM and cVM). The same E treatment had no significant effect on other areas examined, although there was a trend for E to increase TPH2 mRNA in mid and caudal MRN. Although we chose a two-week regimen so as to investigate the effects of physiological concentrations of E and P at a steady state, other dosing and duration parameters might have different effects on these regions. In contrast, TPH2 mRNA in E and P treated animals was never different from OVX rats in any of the regions examined, indicating that P reversed the effects of E. This result is generally consistent with the concept that P is an antiestrogen (Arpels 1996; Diaz-Veliz et al 1994; Herzog 1999); however, between-species differences may exist, since P has been shown to augment E-induced TPH2 mRNA expression in DRN of macaques (Sanchez et al 2005). In any case, chronic hormone treatments showed highly selective subregional control of TPH2 mRNA expression in the midbrain raphe of rats. Assuming that the mRNA levels measured in these subregions reflect the production of mature TPH2 protein, E may be enhancing 5-HT synthetic capacity in specific clusters of serotonergic neurons.

The second main finding of this study was that TPH2 mRNA levels in some subregions of the DRN correlated with anxiety-like behavior in the open field test. TPH2 mRNA levels in the caudal DRN were positively correlated with increased exploration of the center of the open field; in another words, animals that had higher TPH2 expression in the caudal DRN also tended to show decreased anxiety, suggesting that increased TPH2 expression in this region by E may be anxiolytic. Recently, we found that chronic E treatment in OVX rats decreased anxiety, as measured by increased exploration of the center of the open field, and P reversed this effect (Hiroi and Neumaier 2006). Perhaps the anxiolytic effect of E may be mediated by an increase in TPH2 mRNA in the caudal DRN while P can block this anxiolytic effect by opposing TPH2 mRNA induction.

However, the relationship between TPH2 and behavior is not simple. Despite the lack of E modulation of TPH2 mRNA in the rostral DRN, increased TPH2 in the rostral dorsomedial DRN was associated with increased time spent in the corners of the open field. Increased corner time is thought to represent anxiety-like behavior since the corners appear to be perceived as "safer" than the center of the open field (Prut and Belzung 2003). Moreover, TPH2 mRNA in mid DRN was not associated with any change in behavior in the open field, despite the enhancement of TPH2 mRNA levels in this region by E. Since the associations between TPH2 mRNA and behavior reported here are correlational and used only one type of behavioral test, further investigation is required with controlled manipulations using more than one assay of anxiety-like behavior. In any case, together these results illustrate that there is an E effect on both TPH2 and anxiety behavior (Hiroi and Neumaier 2006) as well as an E-independent effect of TPH2 on behavior, depending on the subregion of DRN. These results are consistent with the hypothesis that serotonergic activity in subregions of DRN that project to different areas of forebrain may mediate complex effects of E on anxiety-like behavior.

This complex regulation is not surprising given the structural and functional intricacy demonstrated by the DRN. Electrophysiological and microdialysis data from animal models indicate that 5-HT cell firing and 5-HT release can be increased in some regions while decreased in others simultaneously (Adell et al 1997; Kirby et al 1995, 1997; Price et al 1998; Thomas et al 2003). Evidence from animal and clinical studies also suggests that

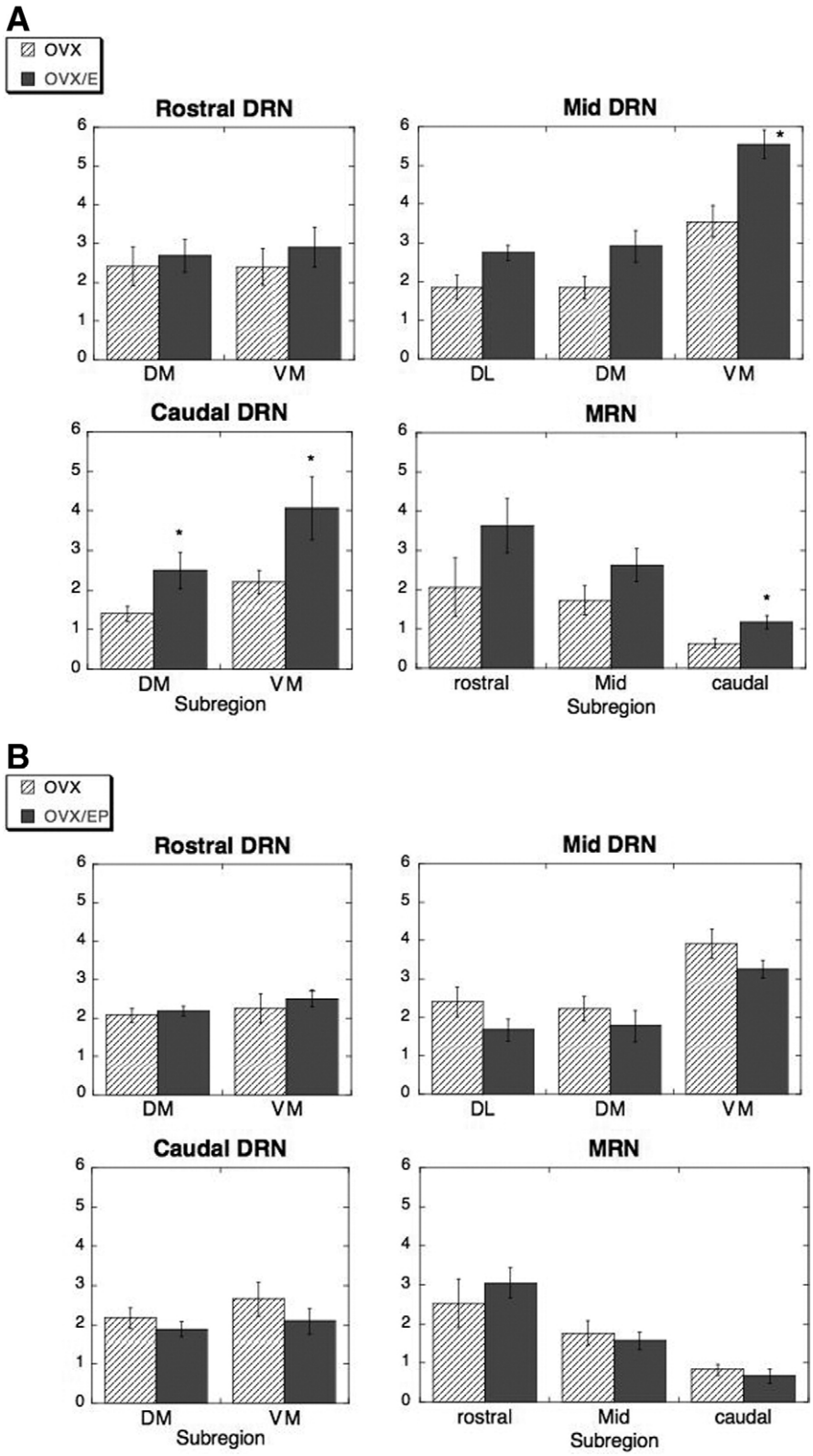

Figure 3. Estrogen selectively increases TPH2 mRNA in specific subregions of the raphe nuclei. **(A)** E treatment significantly increased TPH2 mRNA in the mid-VM, caudal DM and VM subregions of the DRN and the caudal MRN. **(B)** Combined E and P treatment had no effect on TPH2 mRNA in the raphe nuclei, suggesting that E-induced increase in TPH2 mRNA in some regions of the raphe are reversed by P. Each bar represents the mean ± SEM TPH2 optical density in subregions of the midbrain raphe from each treatment group. *$p < .05$, *t*-test. DRN, dorsal raphe nucleus; MRN, median raphe nucleus; DM, dorsomedial; VM, ventromedial; DL, dorsolateral; TPH2, brain-specific isoform of rat tryptophan hydroxylase; OVX, ovariectomized rats; E, estrogen-treated rats; P, progesterone-treated rats.

serotonin (Bagdy et al 2001; Boyer and Feighner 1992; Burghardt et al 2004; Griebel 1995; Handley et al 1993; Lowry 2002), as well as estrogen (Lund et al 2005; Morgan et al 2004), can be either anxiolytic or anxiogenic. Because serotonergic neurons in the subregions of DRN and MRN have distinct inputs from and project to diverse forebrain regions involved in stress response (Lowry 2002; Peyron et al 1998; Vertes 1991; Vertes et al 1999), differential regulation of 5-HT synthesis and subsequent increase in serotonergic input to distinct forebrain stress circuits can have characteristic behavioral outcomes. For instance, E-induced ac-

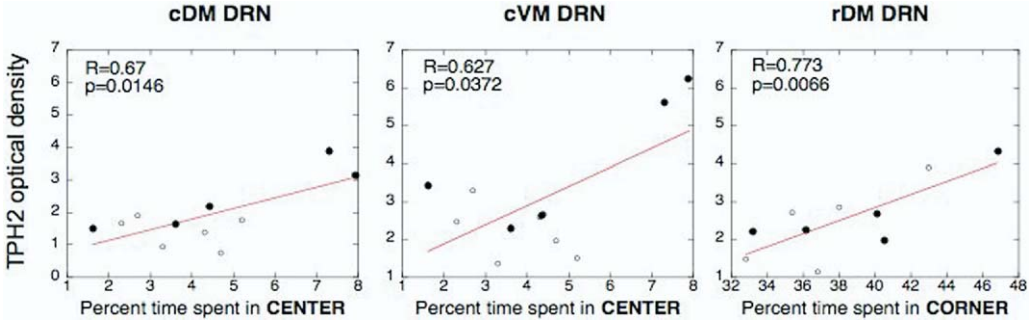

Figure 4. Correlation between TPH2 mRNA level in subregions of the DRN and specific behavior in the open field test. Scatter plot showing the correlation between behavior in the open field and the TPH2 level in subregions of the DRN. TPH2 mRNA levels in the cDM (left panel) and cVM (middle panel) positively correlated with percent time spent in the CENTER of the open field (lower anxiety); whereas in the rDM (right panel), TPH2 mRNA level positively correlated with time in the CORNERS of the open field (higher anxiety). Alpha = .05. Open circle = OVX, closed circle = OVX/E. DRN, dorsal raphe nucleus; cDM, caudal dorsomedial; cVM, caudal ventromedial; rDM, rostral dorsomedial; TPH2, brain-specific isoform of rat tryptophan hydroxylase; OVX, ovariectomized rats; E, estrogen-treated rats; P, progesterone-treated rats.

tivation of TPH2 in mVM DRN, which preferentially projects to forebrain areas, such as caudate putamen, may be important for motor-related movements involved in behavioral arousal during stress (Lowry 2002). On the other hand, activation in caudal DRN, whose efferents preferentially project to limbic regions such as hippocampus, locus coeruleus and lateral septum (Vertes 1991), may be important for emotional coping responses leading to decreased anxiety (Lowry 2002). In addition to distinct circuitry, cells in subdivisions of the DRN have unique morphology and express different arrays of cotransmitters, neuropeptides, and receptors (Lowry 2002). Thus, depending on the nature of the stimuli and which circuits are activated, serotonergic neurons in the raphe nucleus are equipped to produce appropriate responses to a variety of situations.

Table 1. Correlation (*r* Values) between TPH2 mRNA Levels in Each Subregion and Specific Behavior in the Open Field Test

	Correlation *r* : TPH2 and OFT		
Subregions	% Center	% Corner	Total dist
Rostral			
DM	−.040	.733[b]	−.011
VM	.314	.510	.196
MRN	.410	.258	.367
Mid			
DL	.545	.323	−.124
DM	.319	.401	−.350
VM	.400	.285	−.062
MRN	−.302	.449	−.544
Caudal			
DM	.698[c]	−.276	.351
VM	.627[a]	−.249	.319
MRN	.374	.537	.120

TPH2 mRNA level in the cDM and cVM DRN positively correlated with percent time spent in the center of the open field (lower anxiety); whereas in the rDM DRN, TPH2 mRNA level positively correlated with time in the corners of the open field (higher anxiety). Behavior in the open field test was not significantly correlated with TPH2 mRNA in any other subregions. TPH2, tryptophan hydroxylase-2; DM, dorsomedial DRN; VM, ventromedial DRN; DL, dorsolateral DRN; % Center, percent time spent in center of open field; % Corner, percent time spent in corners of open field; Total dist, total distance traveled in the open field; OFT, open field test.

[a] $p < .037$.
[b] $p < .007$.
[c] $p < .0001$ (Fisher's *r* to *z* test); alpha = .05.

The mechanisms by which ovarian steroids may modulate these distinct groups of serotonergic raphe neurons are unknown. It is possible that E and P affect TPH2 mRNA expression both directly (via regionally distinct expression of steroid receptors) and indirectly (via afferents to DRN from steroid-sensitive brain regions). The two types of E receptors (ERα and ERβ) have unique distributions throughout the rat brain (Shughrue et al 1997). Within the DRN, ERα and P receptors are localized in the nonserotonergic neurons preferentially in the rostral DRN (Alves et al 1998), whereas ERβ, known to oppose ERα effects on gene transcription (Lindberg et al 2003; Liu et al 2002; Matthews and Gustafsson 2003), are localized in the 5-HT neurons of the DRN (Lu et al 2001). Although subregional levels of expression have not been explicitly reported, from the data available, ERβ is clearly expressed in mid and caudal levels of the DRN (Lu et al 2001; Shughrue et al 1997). The differential localization of ERα and ERβ in subregions of the DRN suggests a possible direct mechanism underlying the hormonal regulation of serotonergic neurons and anxiety.

The present findings are consistent with clinical studies suggesting E interacts with the serotonergic system to influence anxiety. Women are far more susceptible to anxiety disorders than men and fluctuation of ovarian hormones has been associated with changes in mood; in fact, anxiety disorders are associated with relatively low serum E levels in several conditions including premenstrual syndrome, premenstrual dysphoric disorder, postpartum depression, and menopause and are often relieved by E treatment (Arpels 1996; Best et al 1992; Gregoire et al 1996; Sichel et al 1995). Notably, recent studies suggest that postmenopausal women have decreased serotonergic activity, which is reversed by E treatment (Halbreich et al 1995). It is well-established that deficiency in 5-HT neurotransmission is implicated in the etiology of anxiety disorders (Sun et al 2004; You et al 2005), and these conditions often respond favorably to serotonin-enhancing agents (e.g., selective serotonin reuptake inhibitors, SSRIs). The efficacy of these E and SSRI treatment is dependent on the context in that these treatments produce changes in mood in susceptible women with prior history of these disorders, but has no effect in normal women (Rubinow 2005; Rubinow et al 1998; Schmidt et al 1998). Therefore, instead of absolute levels of hormone, it is rather the altered sensitivity to these hormones in these susceptible women that may result in mood disturbances. It is possible that serotonergic dysfunction may represent this vulnerability and E-induced increase in sero-

tonin-related gene function, such as that shown in the present study, may be a therapeutic target that can restore serotonergic functions in these women.

In conclusion, the present study underscores recent assertions that the differences in activity of 5-HT neurons in different compartments of the DRN may be key to understanding the complex regulation of anxiety by the serotonergic system (Abrams et al 2004; Hammack et al 2002; Kirby et al 2000; Lowry 2002; Molliver 1987; Price et al 1998; Roche et al 2003) and demonstrates the ability of ovarian hormones to modify this system with remarkable specificity. E increased TPH2 mRNA in the caudal DRN, which may play an important role in the anxiolytic effect of E by increasing the synthetic capacity for serotonin in this region and thereby enhancing serotonergic input to specific forebrain projection areas. Although E also increased TPH2 mRNA level in the mVM DRN and caudal MRN, these serotonergic neurons project to other forebrain circuits that may be involved in different behavioral functions that were not examined in this study. While E did not seem to regulate TPH2 in rostral DRN, our behavioral data suggest a possible dissociation of the effects of TPH2 between rostral versus caudal DRN on anxiety behavior. These bimodal effects of TPH2 in rostral versus caudal DRN on anxiety may have implications in elucidating the complex relationship between serotonin activity and anxiety and affective disorders.

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