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Estrogen decreases 5-HT_{1B} autoreceptor mRNA in selective subregion of rat dorsal raphe nucleus: inverse association between gene expression and anxiety behavior in the open field

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Abstract

We have recently shown that estrogen decreases anxiety and increases expression of tryptophan hydroxylase-2 (TPH2), the rate-limiting enzyme for serotonin synthesis. However, the effects of estrogen on serotonin release and reuptake may also affect the overall availability of serotonin in the forebrain. Estrogen has been previously shown to have no effect on the inhibitory serotonin 1A autoreceptor (5-HT_{1A}) in the rat dorsal raphe nuclei (DRN); however the regulation of the inhibitory serotonin 1B autoreceptor (5-HT_{1B}) in the midbrain raphe by estrogen has not yet been investigated. Therefore, we examined the effects of estrogen on 5-HT_{1B} mRNA in the rat DRN, focusing on specific subregions, and whether 5-HT_{1B} mRNA levels correlated with TPH2 mRNA levels and with anxiety-like behavior. Ovariectomized rats were treated for two weeks with estrogen or placebo, exposed to the open field test, and 5-HT_{1A} and 5-HT_{1B} mRNA was quantified by in situ hybridization histochemistry. Estrogen had no effect on 5HT_{1A} mRNA in any of the DRN subregions examined, confirming a previous report. In contrast, estrogen selectively decreased 5-HT_{1B} mRNA in the mid-ventromedial subregion of the DRN, where 5-HT_{1B} mRNA was associated with higher anxiety-like behavior and inversely correlated with TPH2 mRNA levels. These results suggest that estrogen may reduce 5-HT_{1B} autoreceptor and increase TPH2 synthesis in a coordinated fashion, thereby increasing the capacity for serotonin synthesis and release in distinct forebrain regions that modulate specific components of anxiety behavior.

Keywords

serotonin; tryptophan hydroxylase; 5-HT_{1A}; in situ hybridization; ovarian hormones; ovariectomy

Women suffer from anxiety disorders at twice the rate of men (Palanza, 2001, Pigott, 2003, Steiner et al., 2003). Ovarian hormones, such as estrogen, are thought to play a significant role in regulation of anxiety, perhaps by modulating the serotonin (5-HT) system emanating from the dorsal raphe nucleus (DRN). The DRN, a major source of serotonergic innervation of the forebrain, plays a critical role in stress responsiveness (Maes and Meltzer, 1995, Sandford et al., 2000) and antidepressant drugs commonly used to treat anxiety disorders target the

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serotonergic system. Furthermore, estrogen may have important permissive effects on the actions of serotonergic antidepressants (Schneider et al., 1997, Zweifel and O'Brien, 1997, Zanardi et al., 2007).

Animal studies show that estrogen alters the DRN 5-HT system at multiple control points, including synthesis, release and reuptake (Rubinow et al., 1998). For instance, estrogen has been shown to increase the basal firing rate of serotonergic neurons in female rats (Robichaud and Debonnel, 2005), to increase 5-HT_{2A} and 5-HT transporter mRNA in the DRN and binding sites in various forebrain regions of ovariectomized (OVX) rats (McQueen et al., 1997, Sumner et al., 1999). In addition, we have recently shown that chronic estrogen treatment in OVX rats increases tryptophan hydroxylase-2 (TPH2) mRNA in selective subregions of the dorsal and median raphe (Hiroi et al., 2006), suggesting that estrogen increases the capacity for serotonin biosynthesis.

Autoreceptors are another important means of regulating serotonergic function. For example, somatodendritic 5-HT_{1A} autoreceptors in DRN decrease neuronal firing (Sharp and Hjorth, 1990), thereby limiting 5-HT release from axons and dendrites. On the other hand, 5-HT_{1B} autoreceptors are localized in terminals of the DRN axon projections throughout the forebrain where they regulate synthesis, release, and reuptake of 5-HT in a region-specific manner (Hoyer and Middlemiss, 1989, Hjorth et al., 1995, Barnes and Sharp, 1999, Daws et al., 2000). Thus, 5-HT_{1A} autoreceptors alter DRN neuron firing rates at the cell body, whereas 5-HT_{1B} autoreceptors provide regional refinement of synaptic 5-HT. Estrogen has been shown to have no effect on the 5-HT_{1A} mRNA levels in rat DRN (Birzniece et al., 2001). However, regulation of 5-HT_{1B} autoreceptors by estrogen has not been examined, and our laboratory has shown that 5-HT_{1B} autoreceptors are involved in modulating anxiety and fear behaviors (Clark et al., 2002, Kaiyala et al., 2003, Clark et al., 2004) and SSRIs downregulate 5-HT_{1B} mRNA expression selectively in the DRN (Neumaier et al., 1996a, Anthony et al., 2000). Furthermore, a recent study showed alterations in 5-HT_{1B} function in depression-like states (Svenningsson et al., 2006). These studies suggest that 5-HT_{1B} is likely to play an important role in modulating anxiety and other affective states.

Therefore, in the present study we examined the effect of chronic estrogen treatment on 5-HT_{1B} autoreceptor mRNA in the DRN, focusing on the specific subregions of DRN that project to distinct forebrain regions. Because 5-HT_{1B} receptors in forebrain are expressed in a variety of neurons (Boschert et al., 1994) and both 5-HT_{1B} autoreceptors and heteroreceptors are intermixed throughout the brain, quantitation by binding site analysis cannot reveal regulatory changes in specific neuron types. However, 5-HT_{1B} mRNA, which reflects the changes in the capacity to synthesize 5-HT_{1B} receptors that are then transported to axon terminals, can be localized and quantified in the cell bodies of discrete populations of neurons throughout the brain (Bruinvels et al., 1994). In DRN, where 5-HT_{1B} mRNA leads to synthesis of autoreceptors and not heteroreceptors (Doucet et al., 1995, Neumaier et al., 1996b), several subregions have differential levels of 5-HT_{1B} expression (Clark et al., 2006) and project to distinct forebrain targets that subserve different aspects of behavior (Lowry et al., 2008). Therefore, we quantified the expression of 5-HT_{1B} mRNA throughout the subregions of DRN. The importance of this type of approach has become evident recently as we now appreciate the heterogeneity of gene expression, anatomical connections, and responsiveness to physiological stimuli in the DRN (Lowry et al., 2008). We also measured 5-HT_{1A} mRNA levels for comparison with 5-HT_{1B} mRNA and to confirm previous investigations (Birzniece et al., 2001). Finally, we investigated the association of 5-HT_{1B} mRNA with TPH2 gene expression and with anxiety behaviors, since we have previous evidence showing subregion-specific effects of chronic estrogen treatment on TPH2 and anxiety behaviors (Hiroi et al., 2006).

Experimental procedures

Subjects

A total of 12 female Sprague-Dawley rats were used. Animals were received from the supplier (Charles River) at about 60 days of age, ovariectomized seven days later, and sacrificed 2 weeks later at approximately 80 days of age. Animals were divided into OVX (n=6) or OVX/E (n=6) and were group-housed with 3 animals per cage on a 12-hour light/dark cycle (lights on at 6 a.m.), 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

As described previously (Hiroi and Neumaier, 2006), animals were ovariectomized and implanted with Silastic capsules filled with crystallized 17-beta estradiol (Steraloid, Wilton, NH) or left empty for placebo. Capsules had a total delivery length of 3 mm, which produce serum estradiol levels of 30-40 pg/ml (Bridges, 1984, Hope et al., 1992, Sell et al., 2000, Zhou et al., 2002), approximating the moderate circulating levels of estrogen found during the normal estrous cycle of the adult female rat (Smith et al., 1975, Freeman, 1994).

Vaginal smears and analysis

Vaginal smears were obtained to confirm OVX and hormone replacement as previously described (Hiroi and Neumaier, 2006). The OVX rats had a predominance of leukocytes with nucleated and cornified cells similar to that found in a rat in diestrus stage of the estrous cycle, indicating a successful removal of the ovaries and hormones. On the other hand, OVX/E rats had a predominance of nucleated cells with small number of leukocytes, similar to that found in proestrus. Vaginal smears were obtained at least 2 hours before behavioral testing to allow any stress from this procedure to subside.

Open Field Test

Open field behavior was measured and correlated with TPH2 in a previously published study (Hiroi et al., 2006); 5-HT_{1A} and 5-HT_{1B} mRNA from the same animals were analyzed in the present study. Behavior in the open field was tested 2 weeks after capsule implantation using a start box procedure (Hoplight et al., 2005). Briefly, animals were tested using a 100 cm square black Plexiglas enclosure with 30 cm tall walls 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 rats were allowed access to the arena and latency to exit the start box was recorded. Behavioral data in the OFT were collected for 10 minutes (SMART video tracking software, San Diego Instruments, San Diego, CA). In an OFT arena, center time is considered a reliable index of anxiety, responds to anxiolytic agents (Ramos et al., 1997), and is sensitive to stress-induced anxiety states (Pare, 1994, Izumi et al., 1997, Durand et al., 1999). There are also number of studies examining corner time in the open field (Pohorecky et al., 1989a, Pohorecky et al., 1989b, Bouwknecht et al., 2007, Clark et al., 2007, Anderson and Hughes, 2008) and we have shown, using the same apparatus, that diazepam increases center time and decreases the corner time in the open field (Hoplight et al., 2005). 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 briefly narcotized with CO₂ before decapitation, and the brains were quickly frozen on dry ice and stored at -70°C.

In situ hybridization histochemistry (ISHH)

Tissue sections were processed for ISHH as described previously (Hiroi et al., 2006). Briefly, serial 20- μ m coronal sections of the DRN across the anteroposterior axis were prepared on a cryostat and stored at -70°C until processed for ISHH. Tissue sections were fixed in cold 4% paraformaldehyde for 5 min, rinsed in phosphate-buffered saline, treated with acetic anhydride (0.25% in 0.1 M triethanolamine) for 10 min, dehydrated through a series of graded alcohols, and air dried.

Oligonucleotide probes were designed on the basis of low sequence homology to other known receptor mRNA sequences. For the 5-HT_{1B} probes, three antisense oligonucleotides corresponding to residues 678-717, 866-902, and 1135-1174 of the rat 5-HT_{1B} open reading frame, (NM022225) (Hamblin et al., 1992), were synthesized as previously described (Neumaier et al., 1996a, Neumaier et al., 1996b). For 5-HT_{1A} probes, one antisense oligonucleotide corresponding to nucleotides 1110-1151 was used as previously described (Neumaier et al., 2000). The probes were individually labeled using α [³³P]-ATP (Amersham) and terminal deoxyribonucleotidyl transferase (Promega) and purified on microspin G-25 columns (Amersham). The labeled probes were diluted (2 pmol/ml) in a hybridization buffer containing 50% formamide, 10% dextran sulfate, 0.3 M sodium chloride, 10 mM Tris (pH 8.0), 1 mM EDTA, 1 $\times$ Denhardt's (0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 0.4 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 37°C overnight. Following the hybridization reaction, coverslips were removed and the slides were washed in 1 $\times$ SSC (15 mM NaCl in 1.5 mM sodium citrate) for 1 h at 55°C, and again in 1 $\times$ SSC for 1 h at room temperature. The slides were dehydrated through a series of graded alcohol rinses containing 300 mM ammonium acetate, and air dried. Slides were exposed to phosphor storage screens for 48 hrs, and autoradiographic signal was detected using a Cyclone storage phosphor scanner (Packard Instruments, Meridian, CT) at 600 dpi resolution; images were stored on optical disks.

Data analysis

Densitometry and data analysis: In situ hybridization signal was quantified using a computer-based densitometry system (MCID, InterFocus Imaging Ltd (Linton, UK). The most intense hybridization signal was measured from each subregion of each brain region and expressed relative to ¹⁴C-plastic standards coexposed on each phosphor screen, which yields a linear relationship between tissue radioactivity and measured signal intensity; tissue background, measured within the same section, was subtracted from each DRN measurement. The rater was blinded to the treatment group of the sections analyzed. Three rostral-caudal levels were analyzed and hybridization signal within anatomically defined subregions (Abrams et al., 2004, Clark et al., 2006) were quantified separately: rostral DRN (AP: -7.4mm) included two subregions, rostral dorsomedial and ventromedial DRN (rDM and rVM, respectively); mid DRN (AP: -8.0) included mid-ventromedial DRN (mVM); caudal DRN (AP: -8.5mm) included caudal dorsomedial and ventromedial DRN (cDM, cVM). The two halves of the mDL and cDM subregions were averaged and used as one value for each region. TPH2 mRNA from the same brains was quantified previously (Hiroi et al., 2006); these values were used for correlation analysis.

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, NC), with $p < 0.05$ considered significant.

Results

We previously reported that chronic estrogen treatment in OVX rats increased TPH2 mRNA in mVM, cDM and cVM DRN and that this increase in TPH2 in the caudal DRN was associated with decreased anxiety-like behavior, as measured by increased time spent in the center of the open field (Hiroi et al., 2006). On the other hand, we also found that higher TPH2 in rDM DRN was associated with increased anxiety like behavior, as measured by more time spent in the corner of the open field (Hiroi et al., 2006).

The present study is an extension of this study, examining the effects of estrogen on 5-HT_{1A} and 5-HT_{1B} mRNA levels in rostral, mid, and caudal levels of the DRN (see Figure 1) in the same animals used in the above study. Expression values of mRNA in each of these subregions were then pooled for all animals, regardless of treatment, to determine if there were any significant associations with TPH2 mRNA and with anxiety-like behavior measured in the open field test.

Chronic estrogen treatment in OVX rats had no effect on 5-HT_{1A} mRNA in the DRN

There were no significant differences between OVX and OVX/E groups in 5-HT_{1A} mRNA in any of the DRN subregions examined in this study (Table 1).

Chronic estrogen treatment in OVX rats selectively decreased 5-HT_{1B} mRNA in mid-ventromedial subregion of the DRN

Chronic estrogen treatment in OVX rats had no effect on 5-HT_{1B} mRNA in the DRN when all subregions were pooled ($t(79) = 1.242$; $p > 0.22$; Figure 2A). Analysis of the subregions, however, revealed that estrogen selectively decreased 5-HT_{1B} mRNA in mVM DRN ($t(10) = 5.548$; $p < 0.0002$; Figure 2C) but had no effect on any other subregions of the DRN (Figure 2B, 2D).

5-HT_{1B} mRNA levels were inversely associated with TPH2 mRNA levels in the mid-ventromedial and rostral dorsolateral subregions of the DRN

5-HT_{1B} mRNA levels had a significant negative correlation with the TPH2 mRNA levels in the mVM ($r = -0.679$; $p < 0.02$) and rDM ($r = -0.643$; $p < 0.05$) subregions of the DRN (Figure 3). No other regions showed significant correlation between 5-HT_{1B} and TPH2 mRNA (data not shown).

Anxiety-like behavior had a negative association with 5-HT_{1B} mRNA in the mid-ventromedial DRN and a positive association with TPH2 mRNA in the rostral DRN

Percent time spent in the corners of the open field (an indication of increased anxiety) had a significant negative correlation with 5-HT_{1B} levels only in the mVM subregion of the DRN ($r = -0.585$; $p < 0.05$; Figure 4). No other regions showed significant correlation between 5-HT_{1B} and time spent in corners ($p > 0.05$). Total distance traveled (a measure of overall locomotion) or time spent in the center of the open field (an indication of decreased anxiety) had no significant correlation with 5-HT_{1B} levels in any DRN subregions examined (data not shown).

Discussion

In the present study, we examined the effects of estrogen on 5-HT_{1A} and 5-HT_{1B} mRNA in subregions of rat DRN that have distinct projection targets and functional implications (Lowry et al., 2008). We found that chronic estrogen treatment in OVX rats had no effect on 5-HT_{1A} mRNA in any of the subregions of DRN examined, corroborating a previous report using comparable estrogen treatment protocol (Birzniece et al., 2001). That study examined only the

mid DRN; our study extends these results to include the entire rostral to caudal axis; this is relevant since we previously found that TPH2 in caudal DRN was especially strongly regulated by estrogen (Hiroi et al., 2006) and 5-HT_{1A} receptors in caudal DRN have been reported to play important roles in rat models of stress disorders such as learned helplessness (Maier et al., 1995, Maswood et al., 1998, Greenwood et al., 2003, Day et al., 2004). These results suggest that the effects of chronic estrogen treatment on the serotonin system do not involve changes in 5-HT_{1A} autoreceptors expression, at least in the DRN.

We did, however, find that the same estrogen treatment selectively decreases 5-HT_{1B} mRNA expression in the mVM DRN, suggesting a diminished autoinhibitory action in those neurons, which preferentially project to forebrain regions such as ventral tegmental area, caudate putamen, and various cortical regions. Activation of these forebrain regions are thought to regulate many aspects of motor functions (Lowry et al., 2008). In addition, we found an inverse association between 5-HT_{1B} and TPH2 mRNA in both the mVM and rDM DRN when all animals were pooled together; this association suggests that animals with lower 5-HT_{1B} mRNA, regardless of hormone treatment, had the tendency to have higher TPH2 mRNA levels in these regions. While this experiment did not determine whether 5-HT_{1B} receptors regulate TPH2 expression or if they are coordinately regulated by other factors (including E), 5-HT_{1B} autoreceptors have previously been shown to inhibit serotonin synthesis (Hjorth et al., 1995). The combined effect of having low 5-HT_{1B} autoreceptor and high TPH2 enzymatic activity would be to increase availability of 5-HT via disinhibition of 5-HT release (Sharp et al., 1989, Auerbach et al., 1991, Hjorth and Tao, 1991, Martin et al., 1992, Barnes and Sharp, 1999, Sari, 2004), reduced 5-HT uptake by SERT (Daws et al., 2000), and increased 5-HT biosynthesis, respectively, in the terminal regions receiving input from these DRN subregions. The midrostral DRN preferentially project to forebrain regions such as caudate putamen, substantia nigra, ventral tegmental area and diverse cortical targets, and are thought to be important for motor function and behavioral arousal (Lowry et al., 2008).

Although the effects of serotonin release in these terminal sites on anxiety behavior are unknown, we speculate that enhanced serotonin release from mVM DRN would increase behavioral arousal and may result in increased anxiety-like behavior in certain situations, such as a high stress/threatening environment. Thus, it may be possible that estrogen may increase anxiety via enhanced serotonin release from these forebrain regions during threatening situations. This may seem contradictory to our earlier finding that estrogen is anxiolytic; however, estrogen, as well as serotonin, has been shown to be both anxiolytic and anxiogenic depending on context. The open field test performed under red light in our present study is considered a relatively low stress-environment and thus estrogen reduced anxiety. However, numerous studies, including our previous study on anxiety and fear (Hiroi and Neumaier, 2006), have demonstrated that estrogen can be anxiolytic in low stress and anxiogenic in high stress environment (for review, see (Morgan et al., 2004)). Taken together, our results indicate that estrogen may increase serotonin neurotransmission in these forebrain regions, which in turn may influence anxiety by modulating motor functions and behavioral arousal. However, the present study does not address the effects of estrogen on 5-HT_{1B} protein or serotonin release in these forebrain regions and further studies are warranted to confirm this idea.

It is interesting to note that although estrogen had no effect on 5-HT_{1B} or TPH2 mRNA in the rDM, there was an inverse association between 5-HT_{1B} and TPH2 in this region. This estrogen-independent association between 5-HT_{1B} and TPH2 may suggest either a pre-existing association between these two genes or that factors other than estrogen may influence serotonin activity in the rDM DRN. Conversely, the lack of correlation between 5-HT_{1B} and TPH2 in the cDM and cVM, regions where we previously found estrogen-induced increase in TPH2, indicates that these two genes are not regulated in a completely coordinated manner. Perhaps additional factors besides estrogen (like stress related peptides and neurotransmitters) also

regulate gene expression in various subdivisions of the DRN that were not investigated in this experiment.

The present study was able to directly examine the 5-HT_{1B} autoreceptor population on serotonin neurons originating from the DRN, rather than the heteroreceptors located in nonserotonergic neurons throughout the forebrain. This approach was critical for distinguishing the two populations, because they are intermixed throughout the forebrain. However, 5-HT_{1B} heteroreceptors located in various forebrain regions, including amygdala and hippocampus, may also play important roles in regulating anxiety behavior. Estrogen receptors are found in these regions, making it possible that estrogen regulates both 5-HT_{1B} auto- and heteroreceptors. The intricate interaction between these distinct populations of 5-HT_{1B} may be important for regulating different aspects of anxiety behaviors by altering both serotonergic and nonserotonergic neurotransmission. Further studies investigating the effects of estrogen on 5-HT heteroreceptors throughout the forebrain are warranted.

In order to assess the role of the decreased 5-HT_{1B} mRNA on anxiety, we correlated the level of 5-HT_{1B} mRNA in each subregion of the DRN with anxiety-like behavior in the open field. We found a negative correlation between 5-HT_{1B} mRNA and anxiety; animals with decreased 5-HT_{1B} mRNA in the mVM DRN tended to spend more time in the corner of the open field, an indicator of increased anxiety-like behavior (Pohorecky et al., 1989a, Pohorecky et al., 1989b, Hoplight et al., 2005, Xu et al., 2006, Bouwknecht et al., 2007, Clark et al., 2007, Anderson and Hughes, 2008). However, there was not a significant correlation between 5-HT_{1B} mRNA and center time as one would expect. There was a non-significant inverse correlation between corner and center time indicating a possible reciprocal relationship between these two compartments as expected (data not shown). Previously we found that center and corner time were both significantly changed by diazepam in opposite directions and this procedure is sensitive to changes in serotonergic function (Hoplight et al., 2005), but the presence of a start box on one wall changes the behavior compared to a symmetric arena, and may tend to interfere with reciprocal relationship between corner and center time. We have chronic estrogen replacement to consistently produce anxiolytic effects in this apparatus, increasing center time while decreasing corner time, although one or the other factor has not been statistically significant every time (perhaps due to the presence of a start box). Thus, we interpret the inverse correlation between 5-HT_{1B} mRNA and corner time cautiously; further studies are necessary to confirm our interpretations.

Nevertheless, this finding is in agreement with a previous report that 5-HT_{1B} mRNA levels in mid-rostral DRN in the male rats were negatively correlated with anxiety behavior in the elevated plus maze (Kaiyala et al., 2003). A second study, however, found that unstressed rats from two models of differential stress reactivity (congenital learned helplessness and maternal separation) showed high 5-HT_{1B} mRNA in the mVM DRN in the low vulnerability groups only in male but not in female rats (Neumaier et al., 2002). Therefore, it is likely that 5-HT_{1B} autoreceptors play a complex role in modulating anxiety that is dependent on multiple factors, including stress, gender, and hormonal status.

At first glance, this finding is contradictory to previous reports showing chronic estrogen treatment in OVX rats decreased anxiety, as measured by increased time spent in the center of the open field (Hiroi and Neumaier, 2006). Recently, we also found that estrogen increases TPH2 mRNA in the caudal DRN and that this increase was associated with increased time spent in the center of the open field, while TPH2 mRNA in the rostral DRN was associated with increased time spent in the corner of the open field (Hiroi et al., 2006). Therefore, anxiolytic effects of estrogen may be primarily mediated through increased capacity of 5-HT synthesis in the caudal DRN, while increase in 5-HT availability in the mid or rostral DRN may mediate anxiogenic behavior. Given the complex function of the serotonergic system in

regulating stress responses, it is not surprising that increased serotonergic activity has been linked to both increases and decreases in anxiety, depending on the context (Johnston and File, 1986, Gorman et al., 1987, Boyer and Feighner, 1992, Masand and Gupta, 1999, Clark et al., 2002, Burghardt et al., 2004, Clark et al., 2004, Gordon and Hen, 2004). These studies demonstrate the complex regulation of the serotonin system in the DRN by estrogen and reiterate the importance of elucidating the intricate relationship between serotonergic activity in the DRN, particularly in select subregions, and anxiety behaviors.

The complex effect of estrogen on DRN function and the bimodal effect of serotonergic activity in distinct subregions on anxiety may be expected as estrogen, like 5-HT, has been shown to be anxiolytic or anxiogenic, partly depending on the relative actions of estrogen on two estrogen receptor types, ER α and ER β (Lund et al., 2005, Walf and Frye, 2005). These studies demonstrated that ER α and ER β have opposing effects on anxiety, in which ER α agonist, propyl-pyrazole-triol (PPT), is anxiogenic, whereas ER β agonist, diarylpropionitrile (DPN), is anxiolytic. The unique distributions of ER α and ER β throughout the rat brain, including the subregions of the DRN (Shughrue et al., 1997, Alves et al., 1998, Lu et al., 2001), suggest a possible direct mechanism underlying the hormonal regulation of serotonergic neurons and may explain the bimodal effects on anxiety.

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 (Molliver, 1987, Price et al., 1998, Kirby et al., 2000, Hammack et al., 2002, Lowry, 2002, Roche et al., 2003, Abrams et al., 2004). Estrogen selectively decreased 5-HT_{1B} mRNA in the mVM DRN and this decrease was associated with increased TPH2 mRNA, suggesting that estrogen increases the availability of 5-HT in specific regions of the forebrain via a combined effect of disinhibition of 5-HT release from and increased biosynthesis of 5-HT in selective terminal regions of the forebrain. In addition, decreased 5-HT_{1B} mRNA levels in the mVM DRN was associated with anxiogenic behavior in the open field, indicating a complex relationship between serotonergic activity and anxiety behavior. Although correlative data has clear limitations, these results support an interesting hypothesis --that estrogen regulates the DRN serotonergic system differentially in discrete subregions. Because these DRN subregions have segregated projections to forebrain regions that regulate different components of affective states, estrogen may alter the intricate balance of serotonergic activity in different brain areas, thereby moderating the effects of serotonin on emotional behavior in a context-dependent manner. Further investigation using direct manipulations of gene expression in selected DRN subregions are needed.

Although the present study reports decreased 5-HT_{1B} mRNA after chronic estrogen treatment in ovariectomized animals, it is also important to note that there are possible changes in 5-HT_{1B} expression with acute estrogen treatment and in cycling females. For example, elevated estrogenic states during estrus cycle may enhance serotonergic function in rodents (Gundlah et al., 1998, Rubinow et al., 1998, Maswood et al., 1999) and during the menstrual cycle in women (Halbreich et al., 1995). Further, a number of studies found differences in anxiety-related behaviors across the estrus cycle (Fernandez-Guasti and Picazo, 1992, Frye et al., 2000, Morgan et al., 2004, Schneider and Popik, 2007). Therefore, further studies examining effects of the estrus cycle on 5-HT_{1B} expression may be important in understanding how estrogen modulates the serotonergic system and anxiety.

The paradigm used in the present study, nevertheless, closely resembles menopausal women undergoing hormone replacement therapy and may have clinical significance, since anxiety disorders are associated with relatively low serum estrogen levels in several conditions, including menopause, and are often relieved by estrogen treatment (Best et al., 1992, Sichel et al., 1995, Arpels, 1996, Gregoire et al., 1996). Antidepressants targeting the serotonin system,

collectively known as selective serotonin reuptake inhibitors (SSRIs), also successfully treat some symptoms of menopause, yet there is evidence that a lower response rate is augmented by estrogen treatment. These studies suggest a possible interaction of estrogen and serotonin in regulating anxiety and thus elucidating the exact mechanisms of the effects of estrogen on anxiety via the DRN serotonergic system may lead to further advancement in treating affective disorders.

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List of abbreviations

5-HT	Serotonin
5-HT_{1A}	Serotonin receptor type 1A
5-HT_{1B}	Serotonin receptor type 1B
DRN	Dorsal raphe nucleus
mRNA	Messenger ribonucleic acid

TPH2	Tryptophan hydroxylase-2
OVX	Ovariectomized
OFT	Open field test

Figure 1. Subregional anatomy of the raphe nuclei. Schematic diagrams (Paxinos and Watson, 1986) (left panel) and representative photomicrographs (two right panels) of the subregions analyzed for 5-HT_{1A} and 5-HT_{1B} in situ hybridization signal in rostral, mid, and caudal levels of the dorsal raphe. DRN = dorsal raphe nucleus, DM = dorsomedial, VM = ventromedial, DL = dorsolateral.

Figure 2.

Estrogen decreases 5-HT_{1B} mRNA in the mid-VM DRN (C), but has no effect in any other subregions (B, D) or when subregions were combined (A). * $p < 0.05$, t-test. DRN = dorsal raphe nucleus, DM = dorsomedial, VM = ventromedial, DL = dorsolateral. Each bar represents the mean $\pm$ SEM 5-HT_{1B} optical density in subregions of the DRN from each treatment group.

Figure 3. Correlation between 5-HT_{1B} mRNA and TPH2 mRNA levels in subregions of the DRN. 5-HT_{1B} mRNA and TPH2 mRNA levels in the mVM (left panel) and rDM (right panel) negatively correlated with TPH2 mRNA. Alpha = 0.05. Open circle = OVX, closed circle = OVX/E, DRN = dorsal raphe nucleus, mVM = mid ventromedial, rDM = rostral dorsomedial.

Figure 4.

Correlation between 5-HT_{1B} mRNA level in subregions of the DRN and specific behavior in the open field test. 5-HT_{1B} mRNA levels in the mVM DRN negatively correlated with time in the CORNERS of the open field (higher anxiety). Alpha = 0.05. Open circle = OVX, closed circle = OVX/E, DRN = dorsal raphe nucleus, mVM = mid ventromedial.

Estrogen has no effect on 5-HT_{1A} mRNA in the DRN when subregions of the DRN were combined or when subregions were analyzed separately. DRN = dorsal raphe nucleus, r = rostral, m = mid, c = caudal, DM = dorsomedial, VM = ventromedial, DL = dorsolateral, Comb Subreg = Combined Subregions. Each number represents the mean $\pm$ SEM 5-HT_{1A} optical density in the DRN from each treatment group.

	Comb Subreg	rDM	rVM	mDM	mDL	mVM	cDL	cVM
OVX	0.27 $\pm$ 0.02	0.23 $\pm$ 0.05	0.48 $\pm$ 0.06	0.17 $\pm$ 0.02	0.16 $\pm$ 0.04	0.52 $\pm$ 0.02	0.14 $\pm$ 0.01	0.22 $\pm$ 0.03
OVX/E	0.26 $\pm$ 0.02	0.24 $\pm$ 0.03	0.41 $\pm$ 0.03	0.12 $\pm$ 0.01	0.13 $\pm$ 0.01	0.56 $\pm$ 0.03	0.14 $\pm$ 0.01	0.24 $\pm$ 0.04