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ESTROGEN RECEPTOR BETA REGULATES THE EXPRESSION OF TRYPTOPHAN-HYDROXYLASE 2 mRNA WITHIN SEROTONERGIC NEURONS OF THE RAT DORSAL RAPHE NUCLEI

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Abstract—Dysfunctions of the brain 5-HT system are often associated with affective disorders, such as depression. The raphe nuclei target the limbic system and most forebrain areas and constitute the main source of 5-HT in the brain. All 5-HT neurons express tryptophan hydroxylase-2 (TPH2), the brain specific, rate-limiting enzyme for 5-HT synthesis. Estrogen receptor (ER) beta agonists have been shown to attenuate anxiety- and despair-like behaviors in rodent models. Therefore, we tested the hypothesis that ERbeta may contribute to the regulation of gene expression in 5-HT neurons of the dorsal raphe nuclei (DRN) by examining the effects of systemic and local application of the selective ERbeta agonist diarylpropionitrile (DPN) on *tph2* mRNA expression. Ovariectomized (OVX) female rats were injected s.c. with DPN or vehicle once daily for 8 days. *In situ* hybridization revealed that systemic DPN-treatment elevated basal *tph2* mRNA expression in the caudal and mid-dorsal DRN. Behavioral testing of all animals in the open field (OF) and on the elevated plus maze (EPM) on days 6 and 7 of treatment confirmed the anxiolytic nature of ERbeta activation. Another cohort of female OVX rats was stereotaxically implanted bilaterally with hormone-containing wax pellets flanking the DRN. Pellets contained 17-beta-estradiol (E), DPN, or no hormone. Both DPN and E significantly enhanced *tph2* mRNA expression in the mid-dorsal DRN. DPN also increased *tph2* mRNA in the caudal DRN. DPN- and E-treated rats displayed a more active stress-coping behavior in the forced-swim test (FST). No behavioral differences were found in the OF or on the EPM. These data indicate that ERbeta acts at the level of the rat DRN to modulate *tph2* mRNA expression and thereby influence 5-HT synthesis in DRN subregions. Our results also suggest that local activation of ERbeta neurons in the DRN may be sufficient to decrease despair-like behavior, but not anxiolytic behaviors. **© 2009 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: 5-HT, anxiety, depression, estradiol, raphe, TPH.

Major depressive disorder (MDD) affects about 17% of Americans (Kessler et al., 1994; Williams et al., 2007), and is a complex, heterogeneous disease (Winokur, 1997; Ellard, 2001; Weissman, 2002). A deficiency in 5-HT neurotransmission is a leading hypothesis regarding the development and pathophysiology of this disease (Owens and Nemeroff, 1994; Arango et al., 2002; Perlis et al., 2002; Lesch, 2004). An influence of gonadal hormones on the etiology of depression is indicated based on the incidence, duration, severity and rate of recurrence of depressive disorders, which are over twice as high in women compared to men (Earls, 1987; Angold and Worthman, 1993; Weissman et al., 1993; Kornstein et al., 1995). Moreover, women also tend to respond differently than men to common antidepressant treatments, such as selective 5-HT-reuptake inhibitors (Kornstein, 1997; Gorman, 2006). These observations, together with animal studies reporting sex differences in the regulation of emotion (Steenbergen et al., 1990; Caldarone et al., 2003; Shors and Leuner, 2003; Toufexis, 2007) and interactions between estrogen and the 5-HT system (for review see Amin et al., 2005) suggest an involvement of estrogen receptors (ERs) in the etiology of MDD.

In animal models, estrogen can exert both anxiolytic, and anxiogenic actions depending on the behavioral context (Koss et al., 2004; Hiroi and Neumaier, 2006). This ambiguity may be explained by the existence of two different ER systems, ERalpha and ERbeta. The endogenous ligand estradiol binds to and activates both receptor types with similar affinity (Kuiper et al., 1997). However, ERalpha-selective agonists are anxiogenic, while ERbeta-selective compounds are anxiolytic and anti-depressive (Walf et al., 2004; Lund et al., 2005; Weiser et al., 2009). Moreover, in flinders-sensitive rats, a strain selectively bred for depressive-like behaviors, ERbeta agonists reduce the animals' passive floating and immobility behavior (Overstreet et al., 2006) during the forced swim test (FST), a test established to assess despair-like behavior in rodents (Porsolt et al., 1977). This rat strain also displays abnormal levels of 5-HT(2A) receptor mRNA in the perirhinal cortex, piriform cortex, medial anterodorsal amygdala and in the hippocampus, a phenotype that is reversed by estradiol-treatment (Osterlund et al., 1999).

The brainstem dorsal raphe nuclei (DRN) constitute the primary 5-HT system of the brain. Distinct DRN subdivisions give rise to axons that innervate most forebrain areas, including areas crucial for the regulation of emotion and stress-coping behavior, such as the amygdala and the paraventricular nucleus of the hypothalamus (Imai et al.,

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Abbreviations: AdU, arbitrary density unit; DPN, diarylpropionitrile; DRN, dorsal raphe nuclei; E, 17-beta-estradiol; EPM, elevated plus maze; ERbeta, estrogen receptor beta; FST, forced swim test; MDD, major depressive disorder; OF, open field; OVX, ovariectomy/ovariectomized; TPH2, tryptophan-hydroxylase 2.

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1986; Petrov et al., 1992). Other subregions of the DRN send projections to motivational areas like the prefrontal cortex (Lowry, 2002; Abrams et al., 2004), while axons from the caudal DRN target limbic structures, such as the hippocampus, the entorhinal cortex and the septum (Kohler and Steinbusch, 1982).

Tryptophan-hydroxylase 2 (TPH2), the brain-specific version of TPH (Walther et al., 2003; Zhang et al., 2004), catalyses the rate-limiting step of 5-HT synthesis. Disruption or dysfunction of the *tph2* gene is strongly correlated with affective disorders (Zill et al., 2004; Zhang et al., 2005; Haghighi et al., 2008), and abnormal *tph2* mRNA expression may be responsible for many of those pathologies.

Previous studies have demonstrated that ERbeta is robustly expressed within the DRN of rodents (Shughrue et al., 1997a; Lu et al., 2001; Mitra et al., 2003; Nomura et al., 2005; Vanderhorst et al., 2005), primates (Gundlah et al., 2000, 2001) and guinea pigs (Lu et al., 1999), whereas ERalpha is only expressed to a small extent in the DRN of these species. Given the importance of the 5-HT system in anxiety- and depressive disorders together with the anxiolytic nature of ERbeta and its presence in the raphe complex, we speculate that ERbeta activation may regulate gene expression in 5-HT neurons.

Results from earlier studies suggest that ERbeta activation can regulate transcription and neurotransmission in the brainstem. Alves et al. (2000) reported estradiol-induced progestin receptor expression in the DRN of ERalpha null mice, suggesting a role for ERbeta. Also, phytoestrogens that selectively bind ERbeta have been shown to improve mood and 5-HT neurotransmission in the cynomolgus monkey (Shively et al., 2003). In turn, ERbeta null mice are characterized by increased anxiety in conjunction with lower 5-HT content and decreased *tph* mRNA in the DRN (Imwalle et al., 2005; Nomura et al., 2005). In macaques, estradiol and a combination of the ovarian steroids progesterone and estradiol each caused an elevation of *tph2* mRNA in the DRN (Sanchez et al., 2005). In rats, a recent study showed that estradiol itself increases *tph2* mRNA expression specifically in those DRN subregions that are associated with attenuated anxiety (Hiroi et al., 2006).

These findings support the hypothesis that ERbeta activation in the DRN may be sufficient to alter behavioral parameters and 5-HT-neuronal gene expression in the DRN. Therefore, we examined the effects of chronic systemic versus local, intracerebral delivery of the ERbeta agonist, diarylpropionitrile (DPN), in ovariectomized (OVX) female rats on anxiety- and despair-like behaviors and on *tph2* mRNA expression in the DRN.

EXPERIMENTAL PROCEDURES

Animals

All animal surgeries, behavioral tests and experimental protocols followed NIH guidelines and were approved by the Animal Care and Use Committee (ACUC) at Colorado State University. All experiments were designed to minimize the numbers of animals used and to limit animal pain and suffering, yet maintain sufficient numbers for statistical analysis. Young adult female Sprague–Dawley rats (200–250 g body weight, Charles River Laboratories, Wilmington, MA, USA) were fed a phytoestrogen-free diet (modified AIN-93G with corn oil substituted for soy oil; Dyets, Philadelphia, PA, USA), double housed, and kept under standard laboratory conditions (12-h light/dark cycle, lights on at 06:00 h, 22 °C, 60% humidity, and *ad libitum* access to water and food). The animals were handled daily, and their weight was monitored every other day for the duration of both experiments. Surgical procedures were performed under isoflurane- (for OVX) or ketamine-anesthesia (100 mg/kg of 93% ketamine/5% xylazine/2% acepromazine for stereotaxic surgeries).

Experimental design and surgical procedures

One week after arrival, all rats underwent bilateral OVX to remove circulating gonadal steroids. This procedure also ensured a constant level of ERbeta expression within the brain because receptor levels are regulated by hormones (Suzuki and Handa, 2005). Chronic 8-day systemic (s.c.) or local (intracerebral) treatment with ER ligands began 1 week after OVX. All animals were double housed with a partner of the same treatment group.

I. Experiment 1: systemic DPN treatment. Rats were injected s.c. with the ERbeta agonist, DPN (2 mg/kg; $n=8$) or vehicle (27% hydroxypropyl-beta-cyclodextrin from CTD Inc., High Springs, FL, USA; $n=8$) in phosphate-buffered saline once per day at 06:00 h. DPN was synthesized de novo following an established protocol (Lund et al., 2005). In the morning of day 6, rats were tested for anxiety-like behavior in the open field (OF), and on day 7 on the elevated plus maze (EPM). All animals were killed by decapitation on day 8 between 10:00 and 12:00 h under basal, non-stress conditions. This occurred 4 h after the last DPN injection to avoid potential acute effects of agonist treatment. For all animals, brains were removed from the skull, fresh-frozen in pre-cooled 2-methylbutane (−40 °C) and stored at −80 °C until sectioning.

II. Experiment 2: local treatment with DPN or estradiol. Three groups of rats were stereotaxically implanted bilaterally with wax pellets (2.00 mm long, 0.25 mm in diameter) flanking the DRN at coordinates: 8.0 mm posterior to bregma, ±1.5 mm lateral, 5.5 mm deep, at a 7° angle. Each pellet contained 0.5 μM DPN ($n=10$), 0.5 μM 17-beta-estradiol (Sigma, St. Louis, MO, USA; E, $n=10$) or beeswax only (VWR International, Bristol, CT, USA; vehicle control, $n=10$). The dosage of DPN and E and the procedure for stereotaxic wax pellet implantation were based on previous studies by Lund et al. (2006). A second control group of OVX animals remained unoperated (C, $n=7$). All animals were tested in the OF, EPM and FST on days 5, 6 and 7 of treatment. Animals were returned to their home cage after each test. All animals were killed on day 8 between 10:00 and 12:00 h, and their brains were removed, fresh-frozen and stored at −80 °C until sectioning.

Behavioral testing

To measure anxiety-like behaviors, all rats were tested in the OF and on the EPM on two consecutive days between 10:00 and 12:00 h for 5 min each. In the OF, the following parameters were scored: locomotor activity (total of square line crossings), number of rears at walls, time spent in center squares, time spent in outer squares, time spent grooming, and number of fecal boli. On the EPM, the latency until first open arm entry, the time spent in the open and closed arms, the number of closed and open arm entries (locomotor activity), the time spent grooming, and the number of fecal boli were recorded as described in Lund et al. (2005).

To measure despair-like behavior, rats were tested in the FST (Porsolt et al., 2001) for 5 min on day 7, using 25 °C tap water. In the afternoon of the previous day, all rats were trained for the FST for 3 min each, without recording their behavior. The time spent paddling (normal stress-coping behavior: slow-pace front and hind leg movements), the time spent struggling (active behavior: high-

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pace front leg paddling and strong hind leg strokes), the time spent floating (passive, despair-like behavior: minimal leg movements; stiff, floating body posture) and the number of dives (active behavior) were recorded.

tph2 Riboprobe design

For the production of a riboprobe specific for *tph2* mRNA, total RNA was isolated from microdissected DRN tissue samples (Palkovits et al., 1975), following the protocol established by Chomczynski and Sacchi (1987). One microgram RNA was reverse transcribed into total cDNA with the reverse transcriptase MMLV-RT (Invitrogen, Carlsbad, CA, USA) at 37 °C for 50 min, using 1 μl oligo dT primers, dNTPs (100 mM each), 1st strand buffer (100 mM Tris–Cl, 900 mM KCl, 1 mM MgCl) and 2.5 mM DTT. Subsequently, a 583 bp fragment of *tph2* cDNA was amplified by RT-PCR (forward primer: 5′-GGG GTG TTG TGT TTC GGG-3′, reverse primer: 5′-GTG GTG ATT AGG CAT TCC-3′). PCR conditions were 45 s denaturation at 95 °C, 45 s annealing at 55 °C, 45 s elongation at 72 °C, and a final 7-min elongation step at 72 °C after 35 cycles. The 50 μl PCR reaction volume contained 1.5 mM Mg^{2+}, 0.2 mM dNTPs, 0.2 μM forward and reverse primer, 50 ng template cDNA, and 1.0 U Taq DNA polymerase (Eppendorf, Westbury, NY, USA). After gel-purification (Qiagen, Valencia, CA, USA), the PCR product was subcloned into the linearized 4.0 kb TOPO-vector pCR®II (Invitrogen), and amplified in chemically competent TOP10 bacterial cells (Invitrogen). Successful clones were verified via sequencing (Retrogen, San Diego, CA, USA). Antisense and sense (control) *tph2* cRNAs were transcribed from the plasmid in the presence of [^{35}S]-UTP, following linearization with restriction enzymes, *Blp*I or *Xba*I respectively. As confirmed in two hybridization test runs, the antisense probe specifically detected *tph2* mRNA in the dorsal and median raphe nuclei, but did not hybridize with tryptophan hydroxylase 1 (*tph*1) mRNA in the pineal gland (Patel et al., 2004; Malek et al., 2005). There was no hybridization above background seen when using the sense-directed control probe.

Tissue preparation and *in situ* hybridization

A series of coronal 16-μm brainstem sections between bregma −6.5 to −9.5 mm (Paxinos and Watson, 1998) was cut at −20 °C using a cryostat (Leica, Wetzlar, Germany) and thaw-mounted onto positively charged slides (Superfrost Plus, VWR Scientific, West Chester, PA, USA). All sections were stored at −80 °C until assayed. For *in situ* hybridization, tissue sections were thawed at room temperature, fixed within 10% paraformaldehyde, acetylated with 0.25% acetic anhydride, dehydrated in a graded series of alcohols, and air-dried. Next, sections were incubated with hybridization solution (50% formamide, 0.60 M NaCl, 0.02 M Tris, 0.01 M EDTA, 10% dextran sulfate, 2 M Denhardt's solution, 50 mM dithiothreitol, 0.2% SDS, 100 mg/ml salmon testis DNA, 500 mg/ml total yeast RNA, and 50 mg/ml yeast transfer RNA), containing radiolabeled cRNA at a concentration of 2×10^7 cpm/ml, in humidified chambers at 60 °C overnight. After hybridization, slides were rinsed in 2× SSC. Non-hybridized RNA was digested in a 30 mg/ml RNase A solution for 30 min at 37 °C. A final high stringency wash (0.1× SSC, room temperature) preceded dehydration in graded alcohols. Hybridization was first examined by opposing slides to a ^{35}S-sensitive Biomax MR film (Kodak, Rochester, NY, USA) for 14 h. Subsequently, hybridization was detected using photographic emulsion-coated slide autoradiography (NTB-3; Kodak). After a 2 day-incubation at 4 °C in the dark, all slides were developed (Kodak D-19) and counterstained with Cresyl Violet.

Validation of pellet implantation

We assumed that the compounds used in the present study successfully diffused into all rostrocaudal and medial-to-lateral subregions of the DRN if both pellets were placed within a maximal radius of 0.5 mm from the center of a coronal DRN section. This criterion was defined empirically by Lund et al. (2006) who found the diffusion of [^{3}H]-labeled E to be confined within a 0.5 mm area surrounding the wax pellet. Based on this anatomical criterion, one individual of the vehicle-group had to be excluded from data analysis. Each section containing the DRN (12 sections per animal) was evaluated using bright-field microscopy and the center location of each pellet was estimated and mapped using a rat brain atlas (Paxinos and Watson, 1998).

Image analysis and quantification of mRNA expression

Cytoplasmic detection of *tph2* mRNA in individual 5-HT neurons was verified via photomicrographs. Counterstaining with Cresyl Violet allowed for distinction between silver grain–labeled *tph2* mRNA localized around purple-labeled nuclei. Dark-field images were captured by a Zeiss AxioCam HR camera on an Axioplan 2 microscope controlled by Axiovision, version 3.1, software (Carl Zeiss MicroImaging, Inc., Thornwood, NY, USA). Three rostral, mid and caudal sections per animal were atlas matched (Paxinos and Watson, 1998) and used for analysis via ImageJ software (version 1.31). Matched dark-field images were inverted in order to cause silver grains to appear as dark pixels in the inverted picture. Matrices in approximate shape of the DRN subregions of interest were then utilized to assess *tph2* mRNA expression within the DRN. The density of black pixels was measured and expressed as arbitrary density units [AdU] for each subregion. After subtraction of background activity (determined in an adjacent area devoid of labeling), six (for the lateral DRN) or three values each (for the dorso-rostral, ventro-rostral, dorso-mid, ventro-mid, dorso-caudal, ventro-caudal DRN) were averaged per animal to obtain an individual value for statistical analysis.

Statistics

All data are expressed as the mean±standard error of the mean (SEM). For studies with only two treatment groups, Student's *t*-test was used for pair-wise data comparison. Results of all studies with more than two treatment groups were analyzed by one-way ANOVA (factor treatment) followed by Tukey's post hoc test where appropriate, using SPSS 12.0 for Windows software. Results were considered significantly different when $P<0.05$.

RESULTS

Experiment 1 (systemic treatment)

Weight gain. Vehicle-treated animals gained 25.16±2.79 g, DPN-treated animals 31.17±3.90 g from day 1 to day 8 of treatment. There were no significant group differences in weight gain.

Systemic delivery of DPN decreases anxiety-like behavior. Animals were tested for anxiety-like behaviors in the OF and on the EPM. In the OF (Fig. 1A), DPN-treated animals displayed more rears at the walls than their vehicle-treated counterparts ($P<0.01$). The total number of square line crossings (a measure of overall activity) did not differ between the two groups, suggesting that there was no overall effect on activity. The total time spent in inner or outer squares, the time spent grooming and the number of fecal boli were not significantly different between the two treatment groups.

On the EPM, DPN treatment caused the rats to enter the open arms sooner ($P<0.01$), enter more often ($P<$

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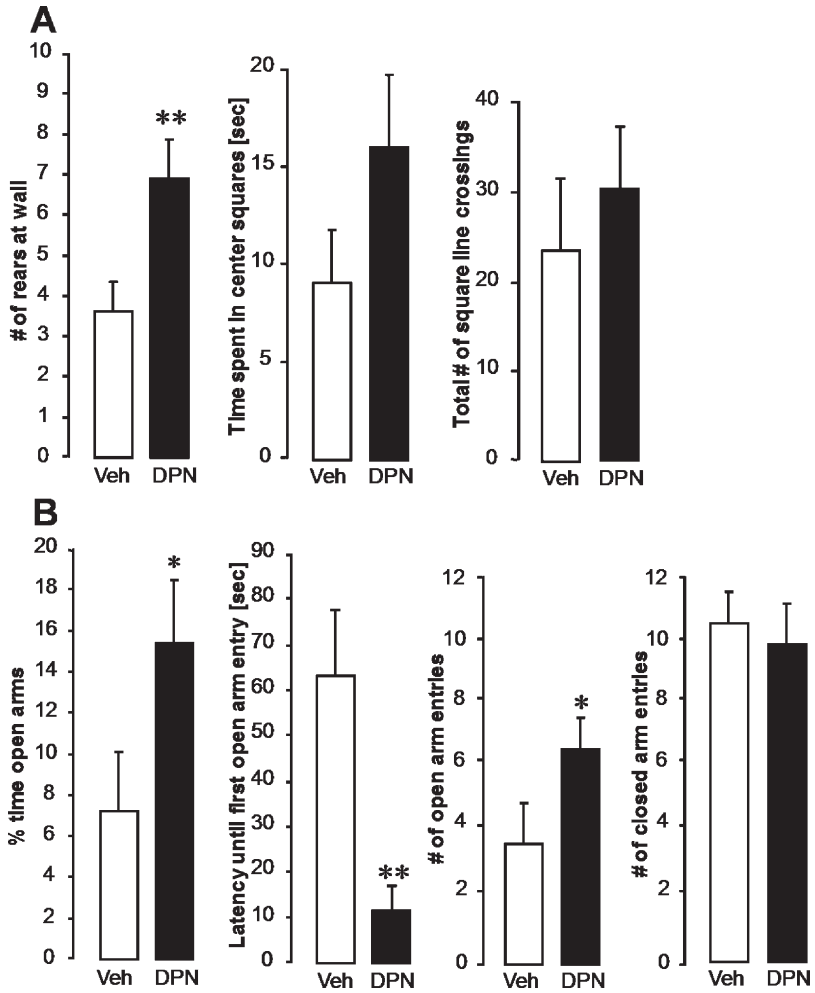

Fig. 1. Effect of systemic delivery of the ERbeta agonist, DPN, on anxiety-like behaviors of female OVX rats. Animals were tested in the OF (A) and on the EPM (B). (A) (Left to right) The number of rears at the walls of the OF, the time the animals spent in the center squares, and the total number of square line crossings. (B) The percent time the animals spent in the open arms of the EPM, the latency until the first open arm entry, the number of open arm entries and the number of closed arm entries (left to right). Each column represents the group mean±SEM of eight individuals per group. Veh=vehicle treated group; DPN=DPN-treated group. * ($P<0.05$) and ** ($P<.01$) indicate significant differences versus vehicle controls (Student's *t*-test).

0.05), and stay on the open arms longer ($P<0.05$) than vehicle controls (Fig. 1B). The number of entries into the closed arms did not differ between the two treatment groups, again indicating that DPN did not increase the rats' overall activity or locomotor behavior. None of the other parameters recorded (time grooming, time in closed arms, fecal boli) revealed significant group differences.

Systemic DPN increases tph2 mRNA expression in the caudal and dorso-mid DRN. To test the hypothesis that systemic ERbeta activation may upregulate *tph2* mRNA expression, *in situ* hybridization was performed, and the density of *tph2* mRNA expression was measured in all subregions of the DRN. Fig. 2 displays representative dark-field photomicrographs of *tph2* mRNA hybridization in the DRN of systemically treated rats. Compared to vehicle-treated animals, daily s.c. administration of DPN significantly enhanced *tph2* mRNA levels in the dorso-mid ($P<0.05$), the dorso-caudal ($P<0.05$) and the ventro-caudal ($P<0.05$) DRN (Fig. 3A). Accordingly, total *tph2* mRNA levels in the entire caudal DRN were almost doubled in the DPN group ($P<0.01$; Fig. 3B), compared to vehicle controls. *tph2* mRNA expression in the rostral DRN was not elevated by DPN. Fig. 3C illustrates in principle the matrix-based, digital analysis of inverted dark-field images that was used for all photomicrographs.

Experiment 2 (central treatment)

Weight gain. The average weight gain during experiment 2 was 28.57±1.87 g for the unoperated control group, 25.00±2.21 g for the vehicle control animals, 25.80±3.30 g for E-treated animals, and 30.50±2.25 g in the DPN group. No significant differences in weight gain were found between any of the treatment groups.

Evaluation of in situ hybridization and wax pellet placement. Fig. 4A shows a representative bright-field photomicrograph of the bilaterally implanted wax pellets

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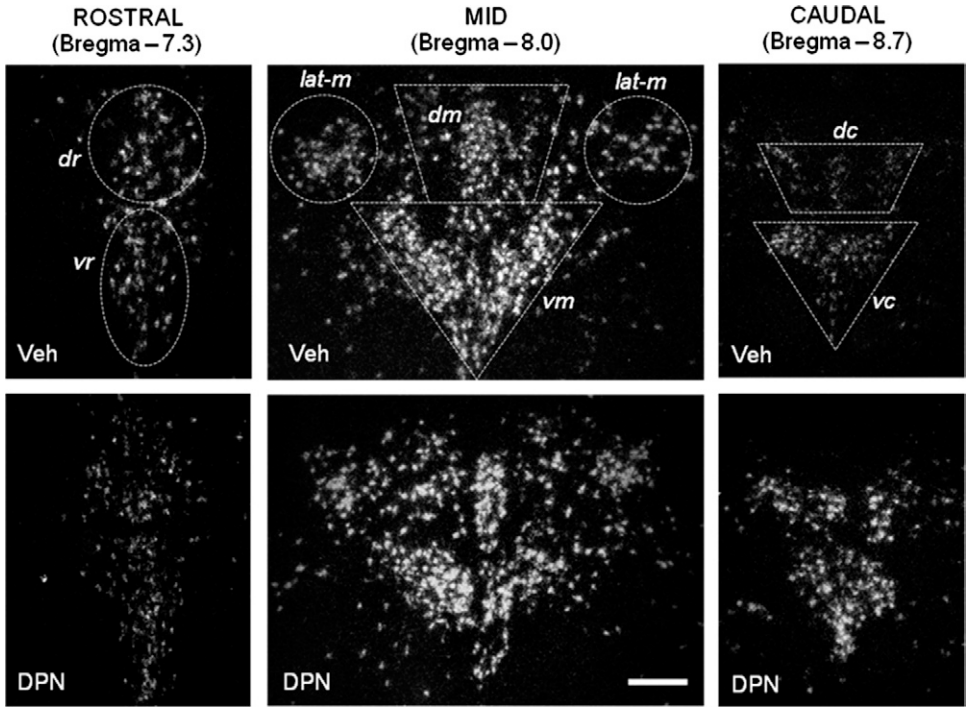

Fig. 2. Representative dark-field photomicrographs from experiment 1. Shown are cells expressing *tph2* mRNA in the rostral (left side, bregma −7.3), mid- (middle, bregma −8.0) and caudal DRN (right side, bregma −8.7) of systemically vehicle- or DPN-treated OVX animals. Vehicle-treated animals (Veh) are shown in the top row of panels, DPN-treated rats (DPN) in the lower row of panels. dr=Dorso-rostral; vr=ventro-rostral; dm=dorso-mid; vm=ventro-mid; lat-m=lateral mid; dc=dorso-caudal; vc=ventro-caudal. Scale bar=40 μm.

placed to flank the DRN. To confirm the cellular silver grain–labeling of neurons containing *tph2* mRNA, brain tissue was counterstained with Cresyl Violet at an intensity sufficient to label the nucleus, but light enough to not alter or interfere with the identification of silver grains (Fig. 4B). A schematic of actual bilateral wax pellet localization in the brainstem of all animals in experiment 2 is depicted in Fig. 4C. If the center of any of the two pellets was found to be more than 0.5 mm away from the estimated center of the DRN, the respective animal was excluded from all data analysis. This was true for one vehicle-treated rat (black dots in Fig. 4C), which reduced the size of this group from 10 to nine animals.

Local DPN or E administration does not alter anxiety-like behaviors, but enhances active stress-coping behavior. All animals were tested for anxiety-related behavior in the OF and on the EPM. Local, DRN-targeted delivery of neither DPN nor E altered any of the parameters measured in the OF and the EPM, compared to unoperated and vehicle controls (Fig. 5A and B). In the FST, rats that were implanted with bilateral, DRN-flanking E- or DPN-pellets displayed a more active stress-coping strategy by spending more time actively struggling in the water than did vehicle controls (Fig. 5C; $F_{3,32}$=4.628; P<0.05). Animals of all treatment groups spent about the same time paddling (neutral stress-coping behavior). Neither E- nor DPN-treatment significantly altered the time spent floating, compared to controls. However, DPN-treated animals spent more time passively floating than their E-treated counterparts (Fig. 5C; $F_{3,32}$=6.040; P<0.05). The number of dives did not differ between any of the groups.

Animals from the unoperated control group did not differ from the regular vehicle control group in any of the behavioral paradigms, indicating that there was no effect of brain surgery on anxiety-like behaviors.

Local exposure to E or DPN strongly elevates tph2 mRNA expression. To investigate whether site-specific activation of ERs through local delivery of DPN or E itself is sufficient to enhance the expression of *tph2* mRNA, *in situ* hybridization was performed. Fig. 6 displays representative dark-field photomicrographs of *tph2* mRNA hybridization in the brainstem of locally DPN-, E-, or vehicle-treated rats. In the dorso-mid DRN, local DPN-administration elevated *tph2* mRNA expression to approximately 2.5-fold of the level seen in controls (Fig 7A; $F_{3,32}$=18.197; both P<.01), resulting in a more than 1.5-fold overall increase of *tph2* mRNA expression for the entire mid-DRN, compared to vehicle and unoperated controls (Fig. 7A and B; $F_{3,32}$= 9.096; both P<0.01). Regarding its effect on *tph2* mRNA in the mid DRN, local DPN treatment also resulted in a stronger response than that of E (Fig. 7A and B; P<.01). In both the dorso- and ventro-caudal DRN, a two- to threefold increase in *tph2* mRNA expression (dorso-caudal: $F_{3,32}$= 13.720; P<.01; ventro-caudal: $F_{3,32}$=14.962; P<0.05) was seen when DPN-treated animals were compared to unoperated and vehicle-treated controls. In summary, local DPN-treatment more than doubled the expression of *tph2* mRNA in the entire caudal DRN, compared to vehicle (Fig.

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A TPH2 mRNA expression [arbitrary density units] – *Experiment 1*

	Vehicle (n=8)	DPN (n=8)
ROSTRAL DRN	**18314 ± 2378**	**16030 ± 2462**
dorso-rostral	8100 ± 1260	6158 ± 1194
ventro-rostral	10214 ± 1382	9872 ±1394
MID DRN	**45192 ± 3815**	**50898 ± 2352**
dorso-mid	7808 ± 721	10710 ± 1105 *
ventro-mid	30810 ± 3515	34065 ± 2546
lateral mid	6574 ± 960	6123 ± 666
CAUDAL DRN	**11340 ± 1219**	**20567 ± 2645 ** **
dorso-caudal	5120 ± 768	9965 ± 1931 *
ventro-caudal	6220 ± 831	10602 ± 1250 *

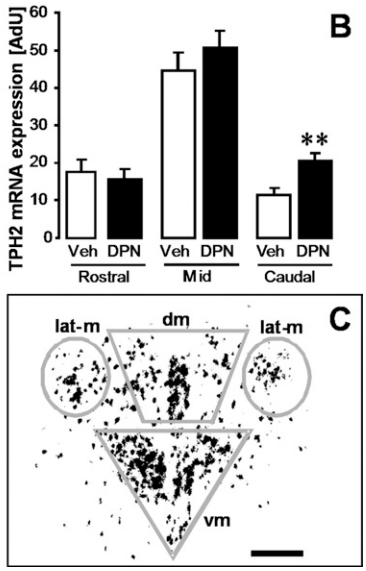

Fig. 3. Effects of systemic DPN-treatment on *tph2* mRNA in OVX females. DPN increases *tph2* mRNA in the dorso-mid and in the dorso- and ventro-caudal DRN. The table in A lists densitometry-determined values for *tph2* mRNA expression in seven subregions throughout the rostro-caudal extent of the DRN (regular letters). For the rostral, mid and caudal DRN, the sum of respective subregional values is displayed in bold letters. Animals were treated s.c. with DPN or vehicle for 8 days. Each value represents the mean±SEM of eight animals per group (numbers in parentheses). * ($P<0.05$) and ** ($P<.01$) indicate significance versus vehicle controls. Data were analyzed by Student's *t*-test for all regions. (B) This diagram is a graphic illustration of *tph2* mRNA expression levels in the entire rostral, mid and caudal DRN, as listed in bold letters in A. All columns represent means±SEM of $n=8$ per group. Veh=vehicle (hydroxypropyl-beta-cyclodextrin) group, DPN=DPN-treated group. ** ($P<.01$) indicates significance versus vehicle controls (Student's *t*-test). (C) Schematic representation of matrix-based densitometry analysis of inverted, normalized dark-field pictures. Silver grains appear as dark dots in the inverted picture. In this example, the densities of silver grains in the dorso-mid (dm), ventro-mid (vm) and lateral mid (lat-m) DRN were measured and subsequently summarized for total *tph2* mRNA expression in the entire mid DRN. Data collection of rostral and caudal subregions of the DRN was performed accordingly. Scale bar=40 μm.

7A and B; $F_{3,32}=6.040$; $P<0.01$) and unoperated controls ($P<0.05$). *tph2* mRNA expression in the rostral DRN was not elevated by DPN.

Treatment with estradiol caused similar, but less intense, effects on *tph2* mRNA expression compared to DPN. Local E-treatment caused *tph2* mRNA levels in the dorso-mid DRN to be about 1.8-fold higher than in either control group (Fig. 7A; $F_{3,32}=10.007$; $P<0.05$), resulting in an overall 1.5-fold increase of *tph2* mRNA in the entire mid DRN (Fig. 7A and B; $F_{3,32}=7.902$; $P<0.05$), compared to both control groups. In the dorso-caudal subregion of the DRN, local delivery of E caused a significant increase in *tph2* mRNA compared to the vehicle control group (Fig. 7A; $F_{3,32}=14.665$; $P<0.05$), in the ventro-caudal part compared to the unoperated control group (Fig. 7A; $F_{3,32}=9.192$; $P<0.05$). Overall expression of *tph2* mRNA in the caudal DRN was almost doubled by local E-treatment compared to vehicle controls (Fig. 7A and B; $F_{3,32}=8.360$; $P<0.05$).

tph2 mRNA levels did not differ for any subregion between the vehicle- and the unoperated control group, ensuring that the implantation of wax pellets adjacent to the DRN did not alter *tph2* gene expression.

DISCUSSION

The results of our studies show that both systemic and local activation of ERbeta in or around the DRN increased the expression of *tph2* mRNA in a subregion-dependent manner. Estrogen treatment caused similar overall effects, but to a lesser extent than DPN. Furthermore, animals treated locally with DPN or E showed decreased despair-like behavior. However, only systemic delivery of DPN decreased anxiety-like behavior, while local administration of DPN failed to have the same effect.

The systemic delivery of DPN to OVX rats reduced several anxiety-related behaviors, confirming the anxiolytic nature of ERbeta (Krezel et al., 2001; Lund et al., 2005; Rocha et al., 2005; Weiser et al., 2009). In contrast, local DPN- or estradiol-treatment of the raphe nuclei failed to decrease anxiety-like behavior, indicating that estradiol's action in regulating anxiety-like behaviors may primarily involve other brain areas, such as the hypothalamic PVN (Herman et al., 2002; Donner et al., 2007; Blume et al., 2008; Neumann, 2008), the lateral septum (Henry et al., 2006), the amygdala (Bosch et al., 2007), or the bed nucleus of the stria terminalis (Davis et al., 1997; Walker et al., 2003). Interestingly, our studies demonstrate that local delivery of DPN or estradiol is sufficient to decrease despair-like behaviors in the FST. It remains unclear why DPN-treated rats spent less time with passive floating behavior than estradiol-treated animals. Yet, in accordance with studies from other research groups, both estradiol and DPN increased the time rats spent actively struggling. While recent studies already revealed an antidepressant function of ERbeta (Walf et al., 2004; Rocha et al., 2005; Hughes et al., 2008), our experiments indicate a

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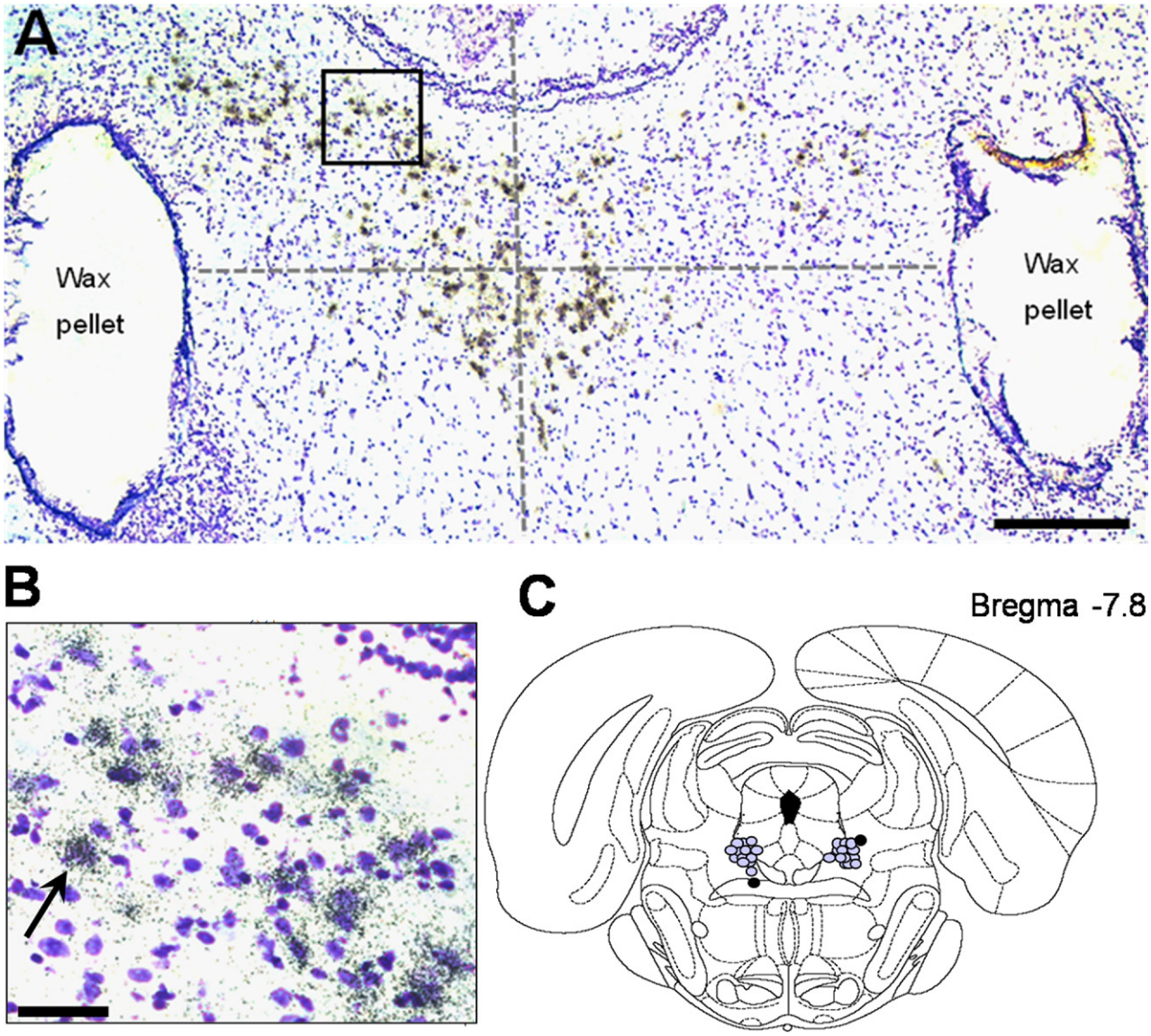

Fig. 4. Localization of hormone containing pellets implanted in experiment 2. Wax pellets containing 0.5 μM DPN or 0.5 μM E were stereotaxically implanted lateral to the DRN. (A) Bright-field image of silver grain–labeled (small black grains) cells at bregma −8.2, hybridized with a riboprobe detecting *tph2* mRNA, and counterstained with Cresyl Violet (purple), is shown. The spread of a compound diffusing from a wax pellet was estimated to be confined within a radius of 0.5 mm (Lund et al., 2006). The predicted center of the DRN is indicated by the crossing point of the two dotted lines. Scale bar=100 μm. (B) Magnification of the outlined area from A. The arrow marks a concentration of silver grain–labeled *tph2* mRNA around a cell nucleus (purple). Scale bar=20 μm. (C) Schematic picture of the rat brainstem at Bregma −7.80 mm (Paxinos and Watson, 1998). Each gray dot represents the center of an implanted wax pellet.

site of action for the observed effect. Thus, high anxiety and despair-like behavior may be closely related to the phenotype of depression (Chaby et al., 1993; Leibbrand et al., 1999; Farabaugh et al., 2005; Godart et al., 2006; Mittal et al., 2006), but the involvement of ERbeta may be through two distinct neuroanatomical regions. Further studies will be required to determine how ERbeta-mediated elevations of *tph2* mRNA expression in the mid-dorsal and caudal DRN are coupled to the attenuation of despair-like behavior.

Not only the route and site of drug administration (systemic versus local), but also the different time course of delivery (once daily s.c. versus constant intracerebral) may have contributed to the discrepancy regarding anxiety-related behaviors between our two experiments. The concentration of DPN in the plasma of male rats that received a 1 mg/kg s.c. injection of DPN has been shown to peak after 1 h and decrease rapidly to undetectable levels within 3 h postinjection (Patisaul et al., 2009). However, this does not mean that the biological activity of DPN decreases within the same time frame. Lund et al. (2005) used an *in vitro* binding approach and differential centrifugation for separation of bound and unbound receptors to estimate that DPN occupies neural ERs with a half-life of about 8 h. These and other studies indicate that DPN, phytoestrogens, and estradiol itself are likely to be sequestered by

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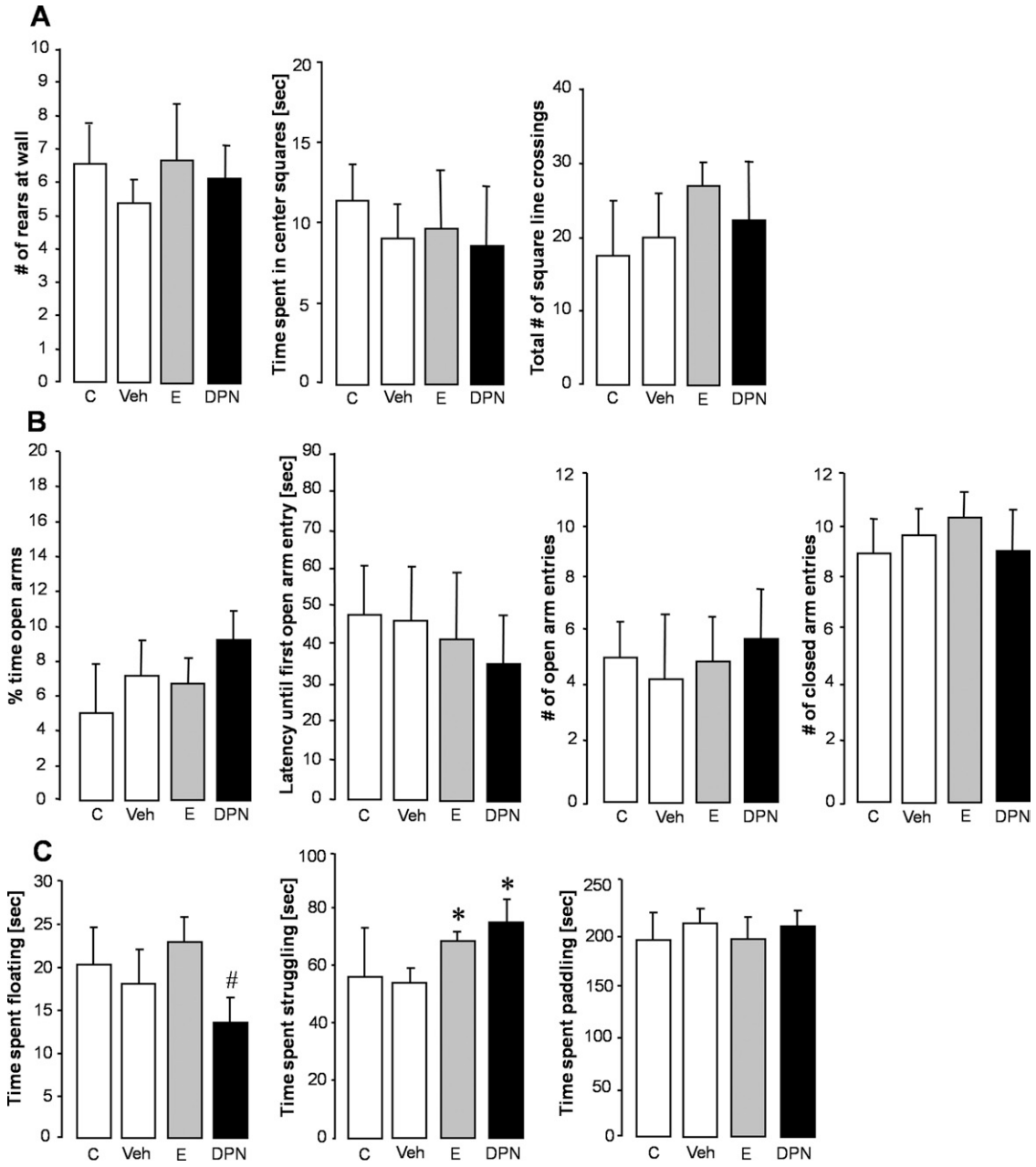

Fig. 5. Effect of local DPN- or E-treatment on anxiety- and depressive-like behavior. Animals were tested in the OF (A) and on the EPM (B) for anxiety-related behavior, and in the FST (C) for depression-related behavior. (A) (Left to right) The number of rears at the walls of the OF, the time the animals spent in the center squares, and the total number of square line crossings. (B) The percent time the animals spent in the open arms of the EPM, the latency until the first open arm entry, the number of open arm entries and the number of closed arm entries. (C) The time spent floating, struggling or paddling when the animals were forced to swim. Each column represents the mean±SEM for seven to 10 animals per group. * ($P<0.05$) indicates significant difference versus vehicle controls, # ($P<0.05$) versus E-treated animals. C=OVX control group without brain surgery ($n=7$); Veh=vehicle control group (blank wax pellets, $n=9$); E=estradiol-treated animals ($n=10$); DPN=DPN-treated animals ($n=10$). ANOVA (factor treatment) was performed, followed by Tukey's post hoc test where appropriate.

ERs in reproductive tissues and in the brain, and are able to exert physiological effects for many hours after incorporation (Blaustein et al., 1979; Dehennin et al., 1982; Morton et al., 1997; Walf et al., 2004; Walf and Frye, 2005). Although animals in our experiment were not sacrificed until 4 h after the last s.c. injection of DPN, it is therefore not unlikely that the behavioral discrepancies observed were due to delayed acute effects of the last DPN injection, compared to a constant chronic exposure to the compound diffusing from locally implanted wax pellets. Chronic wax-mediated, application of DPN for 7 days around the paraventricular nucleus has previously proven sufficient to inhibit the neuronal and corticosterone response of the hypothalamo-pituitary adrenal axis to an acute stressor (Lund et al., 2006). The effects of local DPN-treatment on cellular parameters (*tph2* mRNA) in our study further strengthen

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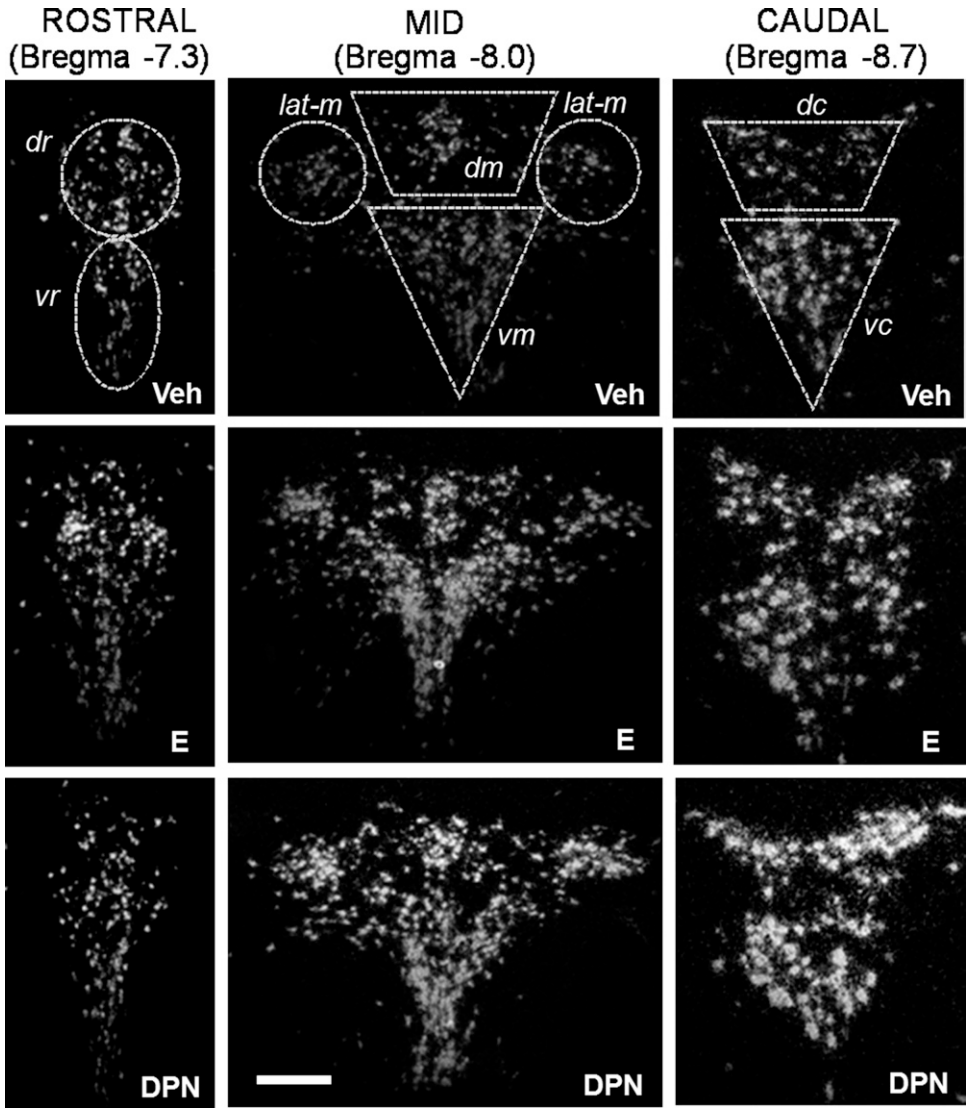

Fig. 6. Representative dark-field photomicrographs from experiment 2. Shown are cells expressing *tph2* mRNA in the rostral (left side, bregma −7.3), mid- (middle, bregma −8.0) and caudal (right side, bregma −8.7) DRN of female OVX rats stereotaxically implanted with vehicle-, E- or DPN-containing pellets. Vehicle-treated animals (Veh) are shown in the top row of panels, E-treated rats (E) in the middle row of panels, and DPN-treated rats (DPN) in the lower row of panels. dr=Dorso-rostral; vr=ventro-rostral; dm=dorso-mid; vm=ventro-mid; lat.-m=lateral mid; dc=dorso-caudal; vc=ventro-caudal. Scale bar=40 μm.

the assumption that DPN is stable and biologically active for a long time when dissolved and administered in beeswax. However, it would be of further interest to identify the biological stability and metabolic fate of DPN following administration.

The observed DPN- or estrogen-induced increase in *tph2* mRNA expression was mainly restricted to the dorso-mid and the caudal DRN. These findings are consistent with observations by Hiroi et al. (2006), who reported elevated *tph2* mRNA expression specifically in the dorso- and ventro-caudal DRN following systemic estrogen treatment in female OVX rats. Furthermore, recent studies in rats and mice indicate that depression-related behavioral paradigms (Keeney and Hogg, 1999; Becker et al., 2007) like social defeat (Gardner et al., 2005) or inescapable stress (Grahn et al., 1999; Amat et al., 2005) selectively activate the dorsal and caudal parts of the DRN. These subregions correspondingly give rise to projections targeting forebrain areas involved in the control of emotional behavior (Lowry et al., 2005, 2008). The mid-dorsal DRN, for instance, sends out collateral projections to emotion- and stress-related brain areas, that could, for instance, simultaneously modulate the hypothalamic PVN and the basolateral or central nucleus of the amygdala (Lowry, 2002; Hale et al., 2008a,b). 5-HT axons from the caudal DRN target limbic structures like the hippocampus, the entorhinal cortex and the septum (Kohler et al., 1982; Kohler and Steinbusch, 1982), indicating that an alteration in *tph2* expression and 5-HT neurotransmission by estrogens may improve memory and learning deficits that are associated with depression (Shors et al., 1998; Burriss et al., 2008; Liu et al., 2008). Within the entire caudal DRN it

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A

TPH2 mRNA expression [arbitrary density units] – *Experiment 2*

	Control (n=7)	Vehicle (n=9)	E (n=10)	DPN (n=10)
ROSTRAL DRN	**17260 ± 2597**	**16757 ± 2420**	**22727 ± 3411**	**13381 ± 2959**
dorso-rostral	7259 ± 2541	7836 ± 1013	12545 ± 3987	5927 ± 1273
ventro-rostral	10001 ± 1853	8921 ± 1650	10182 ± 732	7454 ± 2644
MID DRN	**43428 ± 5898**	**42941 ± 3399**	**61230 ± 2681** $^{*,+}$	**71300 ± 3476** $^{**,++,\#\#}$
dorso-mid	9245 ± 1194	9165 ± 1016	16324 ± 1231 $^{*,+}$	24110 ± 2513 $^{**,++,\#\#}$
ventro-mid	28348 ± 4478	28029 ± 2797	34554 ± 3095	35116 ± 3535
lateral mid	5835 ± 928	5747 ± 1810	10352 ± 1948	12074 ± 3598 $^{+}$
CAUDAL DRN	**15707 ± 1945**	**14623 ± 1751**	**28554 ± 6002** *	**37199 ± 4111** $^{**,+}$
dorso-caudal	9313 ± 1137	6162 ± 1055	16707 ± 4872 *	20138 ± 4831 $^{**,++}$
ventro-caudal	6394 ± 938	8461 ± 577	11847 ± 2342 $^{+}$	17061 ± 3283 $^{*,+}$

B

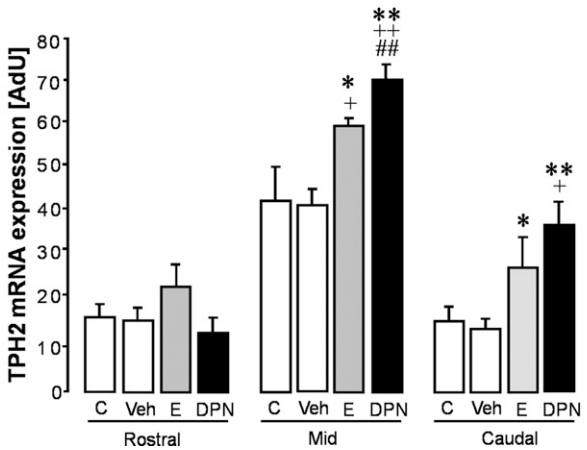

Fig. 7. Effect of local DPN- or E-treatment on *tph2* mRNA levels of OVX females. DPN and E both act locally to enhance *tph2* expression in mid- and caudal subregions of the DRN. The table in A lists densitometry-determined values for *tph2* mRNA expression in seven subregions throughout the rostro-caudal extent of the DRN (regular letters). For the rostral, mid and caudal DRN, the sum of respective subregional values is displayed in bold letters. OVX rats were implanted with DRN-flanking wax pellets containing nothing (vehicle group), E or DPN. An additional control group remained without brain surgery. Numbers in parentheses indicate group size. Each value represents the mean±SEM. (B) This diagram is a graphic illustration of *tph2* mRNA expression levels in the rostral, mid and caudal DRN, as listed in bold letters in A. Each value represents the mean±SEM. * ($P<0.05$) and ** ($P<.01$) indicate significance versus vehicle controls, $^{+}$ ($P<0.05$) and $^{++}$ ($P<.01$) versus unoperated controls, and $^{\#}$ ($P<0.05$) and $^{\#\#}$ ($P<.01$) versus E-treated animals. C=OVX control group without brain surgery ($n=7$); Veh=vehicle control group (blank wax pellets, $n=9$); E=estradiol-treated animals ($n=10$); DPN=DPN-treated animals ($n=10$). ANOVA (factor treatment) was performed, followed by Tukey's post hoc test where appropriate.

is the dorso-caudal subdivision that has been suggested to play a crucial role in changes associated with affective disorders (Commons et al., 2003). In clinical studies of drug-free, depressed suicide victims, the dorso-caudal DRN subregions also exhibited elevated TPH2 protein and *tph2* mRNA expression (Bonkale et al., 2006; Bach-Mizrachi et al., 2008). However, a pathological increase in *tph2* expression may explain this apparent paradox. Pathologically elevated *tph2* mRNA and protein may reflect a compensatory feedback response to low overall 5-HT concentrations in the brain of depressed patients (Mann et al., 1989; Owens and Nemeroff, 1994; Placidi et al., 2001). Also, most of the brains assessed in these clinical studies were derived from male individuals, not females. Consequently, more detailed studies quantifying TPH2 protein and local 5-HT release and turnover within the DRN itself (autoregulation) and in target areas of the DRN in both male and female animal models of anxiety and depression are required to answer this question.

Overall, local estradiol treatment had a similar, but less intense effect on *tph2* mRNA expression and on despair-like behavior than the selective ERbeta agonist. This difference between E and DPN could be explained by the non-selective action of estradiol on both ERalpha and ERbeta. Our previous data and those of others suggest that ERalpha and ERbeta have opposing actions on stress-related behaviors (Liu et al., 2002; Lund et al., 2005; Toufexis et al., 2007; Weiser et al., 2009). While increased ERalpha mRNA and single nucleotide polymorphisms (SNPs) in the gene coding for ERalpha are associated with mental illness, specifically with depression (Perlman et al., 2005; Mill et al., 2008), ERbeta-mediated actions have been found to exert anxiolytic and antidepressant effects in various animal models (Imwalle et al., 2005; Lund et al.,

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2005; Rocha et al., 2005). Since estradiol can bind to both receptor types with equal affinity (Kuiper et al., 1997; Lund et al., 2005), the possibility exists that it could activate two functionally opposing mechanisms, both ultimately balancing *tph2* mRNA expression and 5-HT-dependent behaviors.

At present, the exact patterns for ERbeta expression in DRN 5-HT neurons are controversial. Lu et al. (2001) demonstrated that 5-HT neurons of the DRN of rats contain ERbeta, whereas Sheng et al. (2004) were unable to identify ERbeta in 5-HT neurons. In mice, Nomura et al. (2005) revealed that ERbeta, but not ERalpha is located within 5-HT neurons. ERbeta has also been shown in 5-HT neurons of the guinea pig (Lu et al., 1999) and rhesus monkey (Gundlah et al., 2001). Furthermore, ERbeta2 (Chung et al., 2007), a novel splice variant carrying an 18—amino acid insert between the fifth and the sixth exon in the ligand-binding domain of ERbeta, has been shown in the DRN of female rats. In contrast, ERalpha may only be expressed in non-5-HT-, but possibly GABAergic interneurons (Hart et al., 2001; Su et al., 2001), placing ERalpha in a position to interfere with the negative feedback regulation of 5-HT-neuronal function (Haddjeri et al., 2000; Liu et al., 2000). Differences in the expression of ERalpha versus ERbeta in the midbrain of rats (Shughrue et al., 1997a,b; Lu et al., 2001), mice (Nomura et al., 2005; Vanderhorst et al., 2005), guinea pigs (Lu et al., 1999; Warembourg and Leroy, 2004), and cats (VanderHorst et al., 1998) suggest that species differences may exist in the modulation of the 5-HT system by gonadal steroids. In non-human primates, ERbeta, but not ERalpha, seems to be the predominant ER expressed in raphe 5-HT neurons (Gundlah et al., 2000, 2001; Vanderhorst et al., 2009). While ER expression in the human DRN remains to be fully described, estrogens would be expected to exert mainly anxiolytic and anti-depressive actions in humans if our expression profile of ERbeta in the brain resembles the pattern found in other primates.

The molecular mechanisms by which ERbeta and ERalpha may directly or indirectly modulate *tph2* gene expression are still unknown. Although most ER-induced changes in gene transcription are due to classic effects of the steroid receptors acting as nuclear transcription factors, it is still possible that other mechanisms may be present, particularly given the recent studies showing rapid, membrane-mediated mechanisms of ER action (Cato et al., 2002; Mhyre and Dorsa, 2006; Levin, 2008).

CONCLUSION

In conclusion, our results show that chronic, local activation of ERbeta alters *tph2* mRNA expression in the DRN in a subregion-dependent manner, and, at the same time, facilitates active stress-coping behavior. Interactions between ERbeta and 5-HT neurons of the DRN may be key regulators of anti-depressive behavior, whereas other brain circuits seem to be necessary for ERbeta to exert its anxiolytic actions. Our observations also raise the question whether physiological changes in circulating estradiol can differentially influence behaviors in women across the menstrual cycle. One possibility is that an altered ratio of ERalpha versus ERbeta expression or a disruption of normal ER-regulation of *tph2* expression in the midbrain might contribute to mood disorders like premenstrual syndrome (Rubinow, 1992; Arpels, 1996; Schmidt et al., 1998) or premenstrual dysphoric disorder (Gorman, 2006). These data demonstrate the potential of ERbeta as a pharmaceutical target for treating affective disorders. The future development of an ERbeta agonist for clinical use that facilitates 5-HT function and emotional stability in menopausal women without the risk of breast- or gynecological cancer associated with ERalpha-mediated actions (Chen et al., 2008) would be highly beneficial.

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