Modulation of responses to stress by estradiol benzoate and selective estrogen receptor agonists

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Abstract

Previously, pretreatment with estradiol benzoate (EB) was found to modulate the response of hypothalamic–pituitary–adrenal (HPA) axis and gene expression in several catecholaminergic neuronal locations in ovariectomized (OVX) rats exposed to single immobilization stress (IMO). Here, we investigated the role of estrogen receptor (ER) subtypes, using selective agonists for ER α (propyl pyrazole triol, PPT) or ER β (WAY-200070) in two major central noradrenergic systems and the HPA axis after exposure to single and repeated IMO. OVX female rats received 21 daily injections of either EB (25 μ g/kg), PPT (10 mg/kg), WAY-200070 (10 mg/kg), or vehicle. Injections of EB and PPT, but not WAY-200070, elicited reduced body weight and increased uterine weight, showing their selectivity. Both EB and PPT increased corticosterone levels about two- to threefold, but prevented any further rise with either single or repeated

IMO, indicating an ER α (ESR1)-, but not ER β (ESR2)-, mediated mechanism. In the locus coeruleus (LC), the rise in dopamine- β -hydroxylase (*Dhli*) mRNA with both stress paradigms was abrogated in EB- or PPT-injected animals. However, WAY-200070 blocked the response of *DBH* mRNA to single IMO but not to repeated IMO. In the nucleus of the solitary tract (NTS), the rise in tyrosine hydroxylase and *DBH* mRNAs with both IMOs was absent, or greatly attenuated, in EB- or PPT-treated rats. In most cases, WAY-200070 inhibited the response to single IMO but not to repeated IMO. The results demonstrate that pretreatment with estradiol, or ER-selective agonists, modulates the stress-triggered induction of gene expression of norepinephrine biosynthetic enzymes in LC and NTS, with ER selectivity depending on duration of the stress. *Journal of Endocrinology* (2010) **205**, 253–262

Introduction

It is now widely appreciated that there are distinct gender-related differences in stress-responses and in susceptibility to stress-associated disorders (Baum & Grunberg 1991, Figueiredo *et al.* 2002, Kajantie & Phillips 2006). Women are more susceptible than men to stress-related neuropsychiatric diseases. It is crucially important to determine the cause for this differential susceptibility. Gonadal steroids, such as estradiol, are proposed to play an important role in influencing these gender-related differences (Walf & Frye 2005, Scharfman & MacLusky 2008, Stovall & Pinkerton 2008). Estrogens have been found to modulate the functions of several physiological systems and their responses to stress in humans and animals. The stress-induced elevation of hormones of hypothalamic–pituitary–adrenal (HPA) system, ACTH and corticosterone, is higher in females than in males (Critchlow *et al.* 1963, Burgess & Handa 1992, Patchev *et al.* 1995, Dayas *et al.* 2000, Young *et al.* 2001, Serova *et al.* 2005). Ovariectomy (OVX) of adult rats reduces plasma ACTH and corticosterone levels, an effect reversed by estradiol replacement (Burgess & Handa 1992). Treatment of OVX rats with

estrogen also reduces the induction of c-Fos in the paraventricular nucleus (PVN) of the hypothalamus in response to footshock (Ter Horst *et al.* 2009). In women, cortisol levels are significantly decreased after menopause, and can be restored with estrogen replacement therapy (Helgason *et al.* 1981). Long-term estradiol supplementations lead to faster restoration of increased blood pressure during stress and tend to reduce the immobilization-induced cardiac dysfunction in OVX rats (Serova *et al.* 2005, Ueyama *et al.* 2007). Estrogens also modulate functions of catecholaminergic (CA) systems under basal conditions and in response to stress, affecting gene expression for catecholamine biosynthetic enzymes in central and peripheral noradrenergic systems (Liaw *et al.* 1992, Arbogast & Hyde 2000, Pau *et al.* 2000, Serova *et al.* 2005). We have previously shown that in OVX female rats, estradiol benzoate (EB) can differentially modulate response of HPA axis and catecholamine biosynthetic enzyme genes to single immobilization (IMO) stress in several CA regions, including the brain noradrenergic nuclei of locus coeruleus (LC) and the nucleus of the solitary tract (NTS) as well as in adrenal medulla (Serova *et al.* 2005). Several of the responses were attenuated, while some were

actually opposite those of control animals. The IMO stress-triggered elevation in plasma ACTH levels was lessened in EB-pretreated animals. Similarly, with stress in EB-treated animals, there was no further change or even decline for mRNA levels of tyrosine hydroxylase (*Th*), the initial and generally rate-limiting enzyme of CA biosynthesis, in adrenal medulla and the NTS, and dopamine- β -hydroxylase (*Dbh*) mRNA levels in the LC.

However, the responses to estrogens are tissue specific, and may depend on the specific estrogen receptor (ER) subtype expressed. The biological actions of estrogens are mediated by two different, but related, ER subtypes, ER α (ESR1) and ER β (ESR2), belonging to the nuclear hormone receptor superfamily (reviewed in McKenna *et al.* (1999a) and Klinge (2001)). Estrogens activate multiple intracellular signaling pathways depending on the receptor subtype and on its subcellular localization, which can be in the membrane or nucleus (Enmark & Gustafsson 1999, McKenna *et al.* 1999b, Patrone *et al.* 2000). The characterization of mice lacking ER α , ER β or both ERs revealed that they have overlapping and also unique roles in estrogen-dependent functions *in vivo* (reviewed in Pfaff *et al.* (2002), Matthews & Gustafsson (2003) and Harris (2007)). ER β is suggested to be crucially involved in regulating non-reproductive behaviors and brain development. Female ER β knockout (KO) mice display increased anxiety and reduced cognitive function (Krezel *et al.* 2001). The distribution of the ER subtypes is such that many of the CA-specific regions express both ER α and ER β , although the ratio varies with location and gender (Laflamme *et al.* 1998, Shughrue & Merchenthaler 2001).

Studies in cell culture revealed that transcriptional regulation of the *Th* gene by estrogens differed depending on ER subtype. In PC12 cells, 17 β -estradiol triggered an elevation of *Th* transcription with ER α and a reduction with ER β (Maharjan *et al.* 2005). In contrast to the *Th* gene, estradiol upregulates *Dbh* gene expression and promoter activity with both ER subtypes (Serova *et al.* 2002, Sabban *et al.* 2010).

It remains to be determined *in vivo* whether selective activation of ER-specific subtypes also has differential effects on CA biosynthetic enzyme gene expression. Therefore, ER-selective agonists were administered in this study. We determined both basal effects and response to stress. The studies so far were restricted to examining the effects of single exposure to IMO, while many of the harmful effects of stress are associated with prolonged or repeated exposure (reviewed in Sabban & Kvetnansky (2001), Sabban (2007) and Kvetnansky *et al.* (2009)). Here, we examine whether some of the variation in the ability of EB to modulate the response to stress depends on the selective ER subtypes, which are activated in the specific CA location. To address this issue, we have administered EB or ER subtype-selective agonists and determined their effect on the response to single stress as well as to repeated stress.

Materials and Methods

Animals

All experiments described were performed in accordance with the National Institute of Health Guide and Use of Laboratory Animals (NIH Publications no. 80-23), and were approved by the New York Medical College Animal Care and Use Committee.

Adult OVX Sprague–Dawley female rats (230–250 g) were obtained from Taconic Farms (Germantown, NY, USA) 8 days after the surgery. Purina Lab chow (#53001) and water were available *ad libitum*, and the rats were maintained four per cage under controlled conditions on a 12 h light:12 h darkness cycle at 23 ± 2 °C. Six days later, they were randomly divided into four subgroups (24 rats each), and treated with either 25 μ g/kg of EB (Sigma), 10 mg/kg of ER α agonist propyl pyrazole triol (PPT; Stauffer *et al.* 2000), 10 mg/kg of ER β agonist WAY-200070 (Malamas *et al.* 2004), or vehicle (10% DMSO/90% sesame oil, Sigma) once daily for 21 days by 50- μ l s.c. injections into the nape of the neck. PPT and WAY-200070 were synthesized by Wyeth Research, Collegeville, PA, USA.

This dose of EB has been shown in our previous studies to effectively modulate the response of noradrenergic neurons to single IMO stress (Serova *et al.* 2004, 2005) and increased plasma estradiol levels to 800 ± 61 pg/ml 3 h after the injection (Serova *et al.* 2004). These doses of PPT and WAY-200070 are based on previously published studies with mature rats (Harris *et al.* 2002, Miller *et al.* 2005). For example, a dose of 10 mg/kg PPT daily injected to rats for 21 days elicited lordosis-induced negative feedback inhibition of LH release and prevented weight gain by OVX. It also provided protection against ischemia-induced cell death in hippocampal neurons (Miller *et al.* 2005). The dose of 15 mg/kg significantly reduced hot flushes in OVX females (Harris *et al.* 2002). A similar dose of WAY-200070 or DPN was found to significantly decrease anxiety-related behavior (Lund *et al.* 2005, Weiser *et al.* 2009).

All animals were weighed on the 15th day. On the 16th day, all treatment groups were subdivided into three subgroups of eight animals each, and they continued to receive the daily drug injections as before. One subgroup was subjected to repeated daily IMO stress for 2 h each for six consecutive days (days 16–21). A second subgroup received a single 2-h IMO, 1 h after the injection on day 21. These animals were killed by decapitation on the 21st day immediately after the IMO. The third subgroup was not immobilized and was killed on the same day as the other animals from the same treatment and served as a control for the stress.

After decapitation, blood was collected into EDTA-containing prechilled tubes. Uteri were isolated and weighed. The brain was removed, and the LC, as well as the rostral–medial and caudal parts of the NTS, were immediately dissected using a tissue slicer with digital micrometer (Stoelting Co., Wood Dale, IL, USA). Frontal sections, 13.2–14.2 and 14.2–15 mm, from bregma were

taken for rostral–medial and caudal portions of the NTS respectively, and 9.2–10.4-mm frontal sections were taken for the LC. The slices were placed in ice-cold saline, and the bilateral regions of the LC and NTS were punched out and immediately frozen in liquid nitrogen.

Isolation of RNA and quantitative RT-PCR

Total RNA was isolated and analyzed as previously described (Serova *et al.* 1999, 2005). Briefly, the tissue from each animal was homogenized in RNA-Stat-60 (Tel-Test, Inc., Friendswood, TX, USA) and purified with RNAqueous-Micro RNA Isolation Kit (Ambion, Austin, TX, USA; Serova *et al.* 2004, 2005). The amount of total RNA from each sample was quantified using Ribo-Green fluorescent dye (Molecular Probes, Eugene, OR, USA).

Quantitative analysis of *Th* and *Dbh* mRNA levels was performed by real-time RT-PCR with SYBR Green I with LightCycler (Roche Molecular Biochemicals) as previously described (Serova *et al.* 2005). The RT reactions were performed separately with *Th*- or *Dbh*-specific primers 5'-TCAGGCTCCTCTGACAG-3' and 5'-AGGCTGCAAGGCTTCTGTGATGGC-3' respectively in 5-μl PCR mixture (1×AMV buffer, 10 mM dNTP, 8 units RNase inhibitor, 1.25 units AMV, 10 μM reverse primer, and 1 μg of template RNA). Twenty-microliter PCRs were set up with a final concentration of 1× LightCycler DNA Master SYBR Green I, 0.5 μM of each of the forward and reverse primers, 5 mM MgCl₂, and 2 μl of the standard cDNA or cDNA with unknown concentration. Standard curves plotted using serial dilutions from 2 ng to 0.2 pg of a *Th* or *Dbh* cDNA were used for the quantification by Fit Points Method. The following primers were used: for *Th*, 5'-TGAACCAATTCCTCATGTGG-3', 5'-TGAACCAAGTACACCGTGGAGAG-3'; for *Dbh*, 5'-CCACGCCATGCAGTTCTTCACCA-3', 5'-AGGCTGCAAGGCTTCTGTGATGGC-3'. The expected sizes of target sequences were confirmed with melting curve analysis by comparing its melting temperature with the melting temperatures of the standards as a positive control. The values for *Th* and *Dbh* mRNA were normalized to levels of total RNA.

Determination of plasma corticosterone levels

Corticosterone levels were determined by using the ¹²⁵I corticosterone RIA Kit (ICN, Costa Mesa, CA, USA) according to the manufacturer's protocol. Plasma was diluted 1:200 and incubated with ¹²⁵I-labeled corticosterone and antiserum to corticosterone at room temperature for 2 h. After centrifugation, the ¹²⁵I in the precipitates was measured in gamma counter and compared with the standard curve for quantification. The intra- and inter-assay coefficients of variation for corticosterone assays were 17.2 and 11.2% respectively.

Statistical analysis

The results were evaluated by one- and two-way ANOVA. For two-way ANOVA, we determined the significance of the main factors, treatment and stress, and their interaction, followed by Bonferroni's Multiple Comparison Test using GraphPad and InStat programs. A value of $P \leq 0.05$ was considered significant.

Results

Body and uterine weights

The body and uterine weights in the different treatment groups are shown in Table 1. As expected long-term treatment of the OVX females with EB led to significantly reduced body weight compared with the vehicle-treated group of rats. The effects of selective agonists for ERα (PPT) and ERβ (WAY-200070) on body weight were different. Injections of PPT, similar to EB, reduced body weight. In contrast, there was no change in body weight with WAY-200070. The mean weight of the uterus was about tenfold greater in EB-treated animals than in the control vehicle-treated animals. PPT treatment also led to the elevation of uterine weight, but to a lesser extent than with EB. The uterine weights of rats treated with WAY-200070 did not differ from those of the vehicle-treated group.

Changes in corticosterone levels

Plasma levels of corticosterone were significantly changed by the treatments ($F=9.14$, $P<0.0002$). Administration of EB or PPT led to about 2.5-fold higher corticosterone levels compared to treatment with vehicle ($P<0.01$ and $P<0.05$ respectively) or WAY-200070 ($P<0.01$ and $P<0.05$ respectively; Fig. 1A). The changes in response to stress for the different groups were analyzed by two-way ANOVA. There were significant effects of stress ($F_{84,2}=19.6$, $P<0.0001$) and treatment ($F_{84,3}=10.2$, $P<0.0001$), as well as interaction ($F_{84,6}=2.7$, $P<0.02$). Although the concentrations of plasma corticosterone in stressed rats in the various groups were similar, the magnitude of rise above the levels of their respective unstressed controls varied. The pattern of the

Table 1 Body and uterine weights in ovariectomized (OVX) female rats with different treatments

	Uterine weight (g)	Body weight (g)
Treatment		
Vehicle	0.08 ± 0.004	309.5 ± 3.7
EB	0.85 ± 0.191*	238.3 ± 2.4*
PPT	0.51 ± 0.054* [†]	248.1 ± 3.6*
WAY-200070	0.097 ± 0.009	295 ± 5.0

* $P<0.001$ versus vehicle, [†] $P<0.01$ versus EB.

Figure 1 Effect of long-term administration of EB or ER subtype-selective agonists to OVX female rats on plasma corticosterone levels following single or repeated immobilization stress. (A) Plasma corticosterone levels after long-term injections of vehicle, EB, PPT, or WAY-200070. Data are expressed as mean $\pm$ S.E.M. with 6–8 samples per group. * $P < 0.05$, ** $P < 0.01$ versus vehicle, + $P < 0.05$, ++ $P < 0.01$ versus WAY-200070 by Bonferroni's multiple comparison test. (B) Response to single (1 $\times$) or repeated (6 $\times$) IMO stress. Data are expressed as mean $\pm$ S.E.M. with the levels of corticosterone in the respective treatment taken as 1. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus respective unstressed controls; + $P < 0.05$, ++ $P < 0.01$ vs 1 $\times$ IMO/vehicle or 6 $\times$ IMO/vehicle by Bonferroni's multiple comparison test.

changes with single and repeated IMO was similar (Fig. 1B). With vehicle or WAY-200070, there was a significant, about sevenfold, elevation of plasma corticosterone levels after single (1 $\times$) IMO. Similar results were obtained with repeated (6 $\times$) IMO. Rats receiving injections of EB or PPT had much smaller increase of about twofold above the levels in unstressed animals with the same treatments. The responses

of EB- and PPT-treated rats to single stress were significantly ($P \leq 0.01$) different from the response of the vehicle-treated animals. Similar results were obtained with exposure to 6 $\times$ IMO ($P \leq 0.05$).

Gene expression of catecholamine biosynthetic enzymes

Locus coeruleus There was a significant effect of stress ($F_{84,2} = 11.1$, $P < 0.0001$) and treatment ($F_{84,3} = 15.3$, $P < 0.0001$), as well as interaction ($F_{84,6} = 9.9$, $P < 0.0001$) on *Dbh* mRNA levels. *Dbh* mRNA levels in control animals which received the vehicle were elevated about fourfold with single IMO, and about twofold above the levels in unstressed rats with repeated IMO (Fig. 2). Injections with EB or either of the ER agonists effectively prevented the response of *Dbh* mRNA to single IMO. Moreover, treatment with EB and PPT abolished the response to repeated IMO as well. In rats treated with WAY-200070, repeated IMO still elicited a significant rise in *Dbh* mRNA, and did not differ significantly from the response of the vehicle-treated rats. As previously shown (Serovala *et al.* 2005), we did not find changes in *Th* mRNA levels in the LC of OVX rats in response to stress (data not shown).

Nucleus of the solitary tract None of the drugs administrated significantly changed *Th* mRNA levels in the rostral–medial nor caudal NTS, except for a small, but significant, rise in *Th* mRNA in the caudal portion of the NTS in the PPT-treated group (Fig. 3A and C). In both rostral–medial and caudal regions, there were significant main effects of stress ($F_{84,2} = 3.9$, $P < 0.02$ and 14.8, $P \leq 0.0001$) and treatment ($F_{84,3} = 10.6$, $P < 0.0001$ and 17.6, $P < 0.0001$) respectively. The interaction was also significant ($F_{84,6} = 3.2$, $P < 0.01$, and 6.3, $P < 0.0001$). The pattern of changes with single IMO in the different groups was similar in both parts of the NTS (Fig. 3B and D). Single IMO triggered about threefold elevation in *Th* mRNA levels in rostral–medial NTS and 2.5-fold rise in the caudal NTS of the vehicle-treated rats. In rats treated with PPT, WAY-200070, or EB, exposure to single IMO stress did not elevate *Th* mRNA. With repeated stress, only the EB- and PPT-treated groups differed from the vehicle-treated controls.

Dbh mRNAs in the rostral–medial and caudal NTS were affected by the treatments ($F = 3.9$, $P < 0.02$, and 3.4, $P < 0.03$ respectively) with levels significantly elevated above vehicle, only in rats treated with PPT (Fig. 4A and C). In the rostral–medial NTS, no main effects or interaction was found by two-way ANOVA (Fig. 4B). In the caudal NTS, there were significant main effects of stress ($F_{84,2} = 4.2$, $P < 0.02$) and treatment ($F_{84,3} = 7.6$, $P < 0.0002$), with a significant interaction ($F_{84,6} = 2.4$, $P < 0.03$). In the caudal NTS, both single and repeated IMO stress elicited about twofold induction of *Dbh* mRNA levels in vehicle-treated rats, but not in any of the other groups (Fig. 4D).

Figure 2 Effect of long-term administration of EB or ER subtype-selective agonists to OVX female rats on *Dbh* mRNA levels in the LC following single or repeated immobilization stress. (A) Relative *Dbh* mRNA levels expressed as mean $\pm$ S.E.M. (6–8 samples per group) with levels in vehicle-treated rats taken as 1. * $P < 0.05$ versus vehicle. (B) Response to single (1×) or repeated (6×) IMO stress. Data are expressed as mean $\pm$ S.E.M. with levels of *Dbh* mRNA with respective treatment in unstressed animals taken as 1. * $P < 0.05$, *** $P < 0.001$ versus respective unstressed controls; + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ vs 1× IMO/vehicle or 6× IMO/vehicle by Bonferroni's multiple comparison test.

Discussion

This is the first study to our knowledge to investigate the ER selectivity on the regulation of gene expression of catecholamine biosynthetic enzymes in the brain noradrenergic neurons by estrogens and their role in modulation of the response to acute and chronic stress. Overall, the results indicate that the ER α is especially important in mediating the effects of estradiol on basal expression of *Th* and *Dbh* and on modulation of the response to both single and repeated stress

in OVX female rats. However, ER β is involved mostly in modulation of acute stress reactions. The findings indicate that these treatments can prevent excessive response of *Th* and *Dbh* genes to stress.

The results of this study revealed, for the first time, that long-term activation of either ER α or ER β changed the response of *Dbh* mRNA levels in the LC to acute stress. In rats injected with the ER α agonist PPT, the response to both durations of IMO stress was absent, and *Dbh* mRNA was unchanged beyond the higher basal level. Pretreatment with the ER β agonist, WAY-200070, which in contrast to PPT, did not change basal unstressed levels, also abolished the response of *Dbh* mRNA to a single IMO. However, long-term stimulation of ER β failed to reduce elevation of *Dbh* mRNA in the LC with repeated stress. This has important implications for stress-mediated behavior, and can influence vigilance and attention in potentially threatening environments. In male animals, various types of stress, including IMO, were shown to trigger marked elevations in *Th* and *Dbh* gene expression in the LC (Smith *et al.* 1991, Melia *et al.* 1992, Rusnak *et al.* 1998, Wang *et al.* 1998, Serova *et al.* 1999, Osterhout *et al.* 2005), reviewed in Sabban & Kvetnansky (2001) and Kvetnansky *et al.* (2009). In contrast, in the OVX female rats with low estradiol levels, IMO stress is not effective to trigger an elevation in the LC of *Th* mRNA, and only *Dbh* mRNA levels are increased.

The LC is sexually dimorphic with more *Th*- and *Dbh*-positive cells observed in female mice and rats (Luque *et al.* 1992, Pendergast *et al.* 2008). In female rats, both ER subtypes are found within the LC, with predominate expression of ER β (Shughrue *et al.* 1997, Mitra *et al.* 2003). However, the findings reveal that ER β is more involved with single IMO rather than with repeated IMO. The effect of an ER β agonist on the response of the LC to stress is especially important since functions of these receptors are linked to affective disorders, and decreased levels of ER β were found in persons committing suicide (Ostlund *et al.* 2003). The anti-depressant role of ER β has been demonstrated in ER β KO mice and with specific pharmacological manipulations (Walf & Frye 2008, Walf *et al.* 2009). Significantly decreased anxiety-like and depressive-like behaviors were found in OVX females injected with WAY-200070 (Weiser *et al.* 2009). Potential changes in ER subtypes expressed in the LC induced by selective agonists and stress or in combination remain to be determined. We can speculate that a chronic strong stress, such as IMO, may decrease the expression of ER β and raise the incidence of stress-related depression.

This study is also the first showing that in noradrenergic neurons of the NTS, gene expression of *Th* significantly increases in response to repeated stress, as previously seen for single IMO (Serova *et al.* 2005). *Dbh* gene expression was elevated by IMO stress with both durations in caudal NTS but in the rostral-medial NTS only with repeated IMO. The NTS is involved in the assimilation and integration of multiple viscerosensory processes, including cardiovascular control mechanisms, and also in osmoregulatory functions

that control body fluid homeostasis (Hochstenbach & Ciriello 1995, Lawrence & Jarrott 1996). Noradrenergic neurons located throughout the rostral-medial NTS and most of the caudal (commissuralis) subnuclei of the NTS are barosensitive, and respond dynamically to alterations in blood pressure as part of the homeostatic cardiovascular control process. However, their functional role is not identical. The rostral-medial portion of the NTS exerts more control on the arterial system, while the caudal NTS is more selective for the cardiac system. Thus, it will be important to perform a more detailed response of subpopulations of CA neurons of the NTS to IMO stress.

EB, as the findings indicate, can modulate the functional activity of noradrenergic neurons of the NTS during single IMO stress through ER α as well as ER β , since administration of EB and both selective agonists have similar ameliorative effects on *Th* in rostral-medial and caudal, and *Dbh* in caudal part of the NTS. A high density of ER α and ER β has been demonstrated from caudal to medial regions of the NTS (Shughrue *et al.* 1997, Laflamme *et al.* 1998, Shughrue & Merchenthaler 2001, Schlenker & Hansen 2006). However, caudal NTS expression of ER α is greater than that for ER β (Schlenker & Hansen 2006). This might be a reason why pretreatment with EB as well as PPT was especially effective in preventing or attenuating changes in *Th* and *Dbh* gene expression elicited by repeated IMO. In addition, the expression of ER subtypes might be selectively regulated by stress. Earlier studies revealed a significant elevation in ER immunoreactivity in the NTS of intact female rats after 1 h of IMO stress (Estacio *et al.* 1996).

The selectivity of the ER compounds were confirmed by changes in body and uterine weights. EB and PPT, but not WAY-200070, led to about 20% reduction in body weight and increased the uterus weight, showing their association with the activation of ER α . These data are in agreement with other studies concluding that prevention of body weight gain is associated with the activation of predominantly ER α in adipose tissue (Heine *et al.* 2000, Wegorzewska *et al.* 2008). The effect of EB and the ER α agonist on body weight may be at least partially regulated by suppression of food intake in the NTS, since gastrointestinal signals generated by ingested food are first processed within the NTS (Sutton *et al.* 2004, Travagli *et al.* 2006, Valassi *et al.* 2008). Tracing studies revealed

Figure 3 Effect of long-term administration of EB or ER subtype-selective agonists on *Th* mRNA levels in rostral-medial and caudal parts of the NTS of OVX female rats following single or repeated immobilization stress. Changes in *Th* mRNA in rostral-medial (A and B) and caudal (C and D) parts of the NTS are shown. (A and C) *Th* mRNA levels expressed as mean $\pm$ S.E.M. (6–8 samples per group) with levels of *Th* in vehicle-treated rats taken as 1. * P <0.05 versus vehicle. (B and D) Response to single (1×) or repeated (6×) IMO stress. Data are expressed as mean $\pm$ S.E.M. with levels of *Th* mRNA with respective treatment in unstressed rats taken as 1. * P <0.05, *** P <0.001 versus respective unstressed controls; + P <0.05, ++ P <0.01 vs 1× IMO/vehicle or 6× IMO/vehicle.

substantial axonal projections from the caudal NTS neurons to the immediately subjacent dorsal motor nucleus of the vagus, and that they are part of a gastro-gastric vago-vagal reflex (Rogers & McCann 1993). Thus, estrogens via ER α may modulate brainstem circuits regulating gastric function.

It is interesting to note that in several areas, basal levels of *Th* and *Dbh* were significantly elevated with PPT but not with EB treatment. It might be explained by the ying-yang relationship between ER α and ER β . Both ERs possess fairly similar binding affinities for 17 β -estradiol, 0.2 nM for ER α , and 0.6 nM for ER β (Gustafsson & Warner 2000). Compared with ER α , generally ER β has a weaker transcriptional activity, and it often functions oppositely or acts as a dominant negative regulator of estrogen signaling (Paech *et al.* 1997, Cowley & Parker 1999). When coexpressed with ER α , it causes a concentration-dependent reduction of ER α -mediated transcriptional activation and the repression of ER α -mediated effects (Hall & McDonnell 1999).

Our results also clearly show that ER α , but not ER β , is involved in estradiol-mediated elevation in corticosterone and attenuation of corticosterone response to IMO stress. Similar to our earlier studies, we demonstrated that EB could attenuate the elevation of corticosterone levels in response to single IMO (Serova *et al.* 2005). Here, we show for the first time that it can also modulate response to repeated IMO stress. In this regard, women receiving estradiol through extradermal patch (0.1 mg) for 1 month had an attenuated elevation of cortisol to single endotoxin injection (Puder *et al.* 2001). The effects of EB on corticosterone levels with and without stress were similar in rats treated with PPT, indicating the involvement of ER α . Recently, ER α was also found to mediate estrogen-mediated impairment of glucocorticoid negative feedback on the HPA axis (Weiser & Handa 2009).

ER β does not appear to mediate the corticosterone response shown in the present study as pretreatment with WAY-200070 for 21 days did not alter the rise in corticosterone with single 2-h IMO, or repeated 2-h IMO for six consecutive days. It also did not alter corticosterone levels in the absence of stress. In contrast, WAY-200070 given during 7 days is reported to reduce corticosterone levels 20 min after single forced swim test, indicating the importance of ER β (Weiser *et al.* 2009). These differences

Figure 4 Effect of long-term administration of EB or ER subtype-selective agonists to OVX female rats on *Dbh* mRNA levels in rostral-medial and caudal parts of the NTS following single or repeated immobilization stress. Changes in *Dbh* mRNA in rostral-medial (A and B) and caudal parts (C and D) of the NTS are shown. (A and C) *Dbh* mRNA levels expressed as mean $\pm$ S.E.M. (6–8 samples per group) with levels of *Dbh* in vehicle-treated rats taken as 1. * P < 0.05 versus vehicle. (B and D) Response to single (1×) or repeated (6×) IMO stress. Data are expressed as mean $\pm$ S.E.M. with levels of *Dbh* mRNA with respective treatment in unstressed rats taken as 1. * P < 0.05, ** P < 0.01 versus respective unstressed controls; + P < 0.05, ++ P < 0.01, +++ P < 0.001 vs 1× IMO/vehicle or 6× IMO/vehicle.

may be due to different duration and type of stress, as the neuronal pathways mediating the activation of the HPA axis depend on stress paradigms, stressor modality, and intensity (reviewed in Kvetnansky *et al.* (2009) and Ulrich-Lai & Herman (2009)).

The changes in plasma corticosterone with EB and PPT could be due to a direct effect, or result from modulation of the HPA axis at the levels of ACTH or CRH. Transcriptional activity of CRH promoter was found to be stimulated more strongly with ER β than with ER α , and ER β is colocalized with CRH in rat PVN (Miller *et al.* 2004). However, recent data suggest that estrogens can also regulate CRH promoter activity through ER α (Lalmansingh & Uht 2008). In addition, ER α and ER β differentially regulate transcription of the human CRH-binding protein gene leading to downregulation of the HPA axis and reduction of corticosterone levels in stress conditions.

Are the estradiol or ER agonist-mediated changes observed in the noradrenergic neurons in the response to stress mediated by effects on the HPA axis? Since we used s.c. but not local administration, we cannot rule out the possibility that their effects on gene expression in the LC and NTS might be indirect. CRH neurons innervate the LC, and can increase electrophysiological activity of noradrenergic neurons, noradrenaline synthesis, and release in prefrontal cortex and hypothalamus (Valentino & Van Bockstaele 2001). Moreover, the LC and NTS express glucocorticoid receptors (Fuxe *et al.* 1987). Glucocorticoids can influence the expression of norepinephrine biosynthetic enzymes, at least in cell culture (Lewis *et al.* 1987, McMahon & Sabban 1992, Hwang & Joh 1993, Kim *et al.* 1993). It is possible that the attenuation of the induction of IMO-triggered elevations in the LC or NTS in EB- and PPT-treated rats is partially due to the inhibition of the corticosterone response to stress. This could also be due to alterations in the activation of NTS neurons in response to stress by input from PVN, since its lesions significantly suppressed the stress-related induction of c-Fos in NTS CA cells. Furthermore, tracer deposits in the NTS retrogradely labeled substantial numbers of PVN cells that were also Fos positive after stress (Dayas *et al.* 2004). However, the effectiveness of WAY-200070 in blocking the IMO-elicited rise in TH and DBH in the noradrenergic neurons, but not in plasma corticosterone, argues against solely a glucocorticoid-mediated mechanism.

Conversely, the CA neurons from the LC as well as the NTS may contribute to the changes in the response of the HPA axis with stress observed in estradiol- or PPT-treated animals, since the hypothalamic PVN receives a major input from noradrenergic neurons in the NTS and innervation of lesser magnitude from the LC. Thus, noradrenaline can excite CRH-containing cells in the hypothalamic PVN to regulate the HPA axis (reviewed in Dunn & Swiergiel (2008) and Ulrich-Lai & Herman (2009)).

A number of important questions remain such as whether a higher concentration of WAY-200070 than used in this study might have a more potent effect on repeated IMO.

Future dose–response curves would be informative. It also remains to be determined whether chronic pretreatments are necessary to elicit the changes observed. It is still unclear whether the effects of estradiol and the ER agonists are direct or indirect and whether membrane or nuclear ERs are involved. In this regard, TH promoter activity was recently found to respond to estradiol by a membrane ER α mediated pathway (Maharjan *et al.* 2010).

In sum, the findings of this study demonstrate that estradiol and ER-selective agonists can markedly attenuate the response of norepinephrine biosynthetic enzymes in the LC and NTS and activation of the HPA axis, with ER selectivity depending on the duration of the stress. These findings demonstrate the mechanisms that likely contribute to differences in susceptibility to stress-related disorders depending on hormonal status.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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