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Reproductive Experience Modifies the Effects of Estrogen Receptor Alpha Activity on Anxiety-Like Behavior and Corticotropin Releasing Hormone mRNA Expression

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Abstract

Previous studies have demonstrated that prior reproductive experience can influence anxiety-like behaviors, although neural mechanisms underlying this shift remain unknown. Studies in virgin females suggest that activation of the two estrogen receptor subtypes, ER α and ER β , have differing effects on anxiety. Specifically, ER β activation has been shown to reduce anxiety-like behaviors, while ER α activation has no significant effect. The purpose of the present study was to examine the possible roles of ER α and ER β subtypes in parity-induced alterations in anxiety-like behavior, as tested on the elevated plus maze (EPM). Groups of ovariectomized, age-matched, nulliparous and primiparous females were tested on the EPM following administration of the ER α agonist 4,4',4''-(4-Propyl-{1H}-pyrazole-1,3,5-tryl)trisphenol (PPT; 1 mg/kg), the ER β agonist Diarylpropionitrile (DPN; 1 mg/kg) or vehicle (DMSO). All drugs were administered once daily for 4 days prior to testing as this dosing paradigm has previously been used to demonstrate anxiolytic effects of DPN in virgin rats. In addition, as exposure to the EPM is a psychological stressor, physiological markers of the stress response were measured in both plasma (corticosterone) and brain (corticotropin releasing hormone; CRH) post-EPM testing. Unexpectedly, the ER α agonist PPT selectively increased the time spent exploring the open arms of the EPM in non-lactating, primiparous females, with no significant effects of DPN observed in either nulliparous or primiparous subjects. All females administered PPT and tested on the EPM demonstrated significantly reduced corticosterone secretion when compared to vehicle-treated controls. In addition, significant effects of both reproductive experience and PPT administration on CRH mRNA expression were observed in both the paraventricular nucleus and amygdala using qPCR. These findings indicate that reproductive experience modulates the effects of ER α activation on both EPM behavior related to anxiety and CRH gene expression.

Introduction

The experiences of pregnancy, parturition and lactation/mothering induce dramatic remodeling of the neuroendocrine axis. Many of these changes are stimulated by rising levels of estrogens secreted from the developing placenta. At birth, the loss of the placenta results in a precipitous decline in circulating estrogens. These dramatic fluctuations in

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circulating estrogens likely affect neural processing. Indeed, numerous effects of estrogens on neuronal function as well as synaptic remodeling have been documented (Flanagan-Cato, 2000; Goodman et al., 2004; King and Seiler, 1988; Kurunczi et al., 2009; Luine et al., 1998; Parducz et al., 2006; Spencer et al., 2008; Zurkovsky et al., 2006; Zurkovsky et al., 2007). Moreover, effects on neural processes induced by the profound loss of circulating estrogens following ovariectomy have also been reported (Beltran-Campos et al., 2011; Das et al., 2002; King and Seiler, 1988; Vaucher et al., 2001). Few studies, however, address the potential long-term changes that may occur in the brain as a consequence of the dramatic hormone fluctuations that occur naturally during pregnancy and lactation.

Previous studies report significant alterations in the expression of estrogen receptor alpha (ER α) in select brain regions as a function of reproductive experience (Byrnes et al., 2009). These effects were only observed on the afternoon of proestrus, suggesting that the increasing levels of estradiol observed during this phase of the cycle may play a role in modulating the effects of reproductive experience on ER α expression. In addition to effects on ER α expression, shifts in anxiety-like behavior as a function of reproductive experience have also been demonstrated (Byrnes and Bridges, 2006b; Love et al., 2005; Walf and Frye, 2008), with non-lactating, parous females demonstrating decreased anxiety-like behavior. Thus, shifts in both anxiety-like behavior and ER α expression are observed following reproductive experience.

Several studies suggest differential roles for the two ER subtypes (ER α and ER β) in the modulation of anxiety-like behavior (Imwalle et al., 2002; Lund et al., 2005; Weiser et al., 2009). Many of these studies have relied on the use of knockout mice. For example, studies in ER α knockout mice observed decreased anxiety-like behavior as well as deficits in reproductive and social behaviors (Choleris et al., 2003). In addition, studies examining genotype effects between wild type (WT) or ER β knockout mice, observed anxiolytic effects of estradiol administration in WT but not ER β knockouts (Walf et al., 2008; Walf et al., 2009). Finally, studies utilizing selective ER α and ER β agonists have reported significant anxiolytic effects of ER β agonists, with no effects on anxiety-like behavior observed following ER α agonist administration (Lund et al., 2005; Walf and Frye, 2005; Weiser et al., 2010). Together, these findings suggest that ER α activation may be anxiogenic, while ER β activation may be anxiolytic. Exposure to high levels of estrogens during pregnancy, followed by their abrupt decline postpartum, could induce significant alterations in ER function. Whether shifts in ER modulation of anxiety-like behavior play a role in the long-term effects of reproductive experience on anxiety is unknown.

In addition to effects on anxiety-like behavior, ER activation also modulates the hypothalamic-pituitary-adrenal stress axis via effects in both the paraventricular nucleus (PVN) and the central nucleus of the amygdala (Isgor et al., 2003; Serova et al., 2010; Weiser and Handa, 2009; Weiser et al., 2010). Given the interaction between stress and anxiety, differences in anxiety-like behavior in response to ER agonist may be mediated in part by changes in the stress response. Thus, the current study examines the effects of reproductive experience on anxiety-like behavior as measured using the elevated plus maze (EPM) following sub-chronic (4 days) administration of select ER agonists. In addition, as EPM testing is a psychological stressor, plasma corticosterone and expression of CRH mRNA within the PVN and amygdala were examined following EPM testing.

Material and Methods

Subjects and Mating

Female Sprague-Dawley rats (151–175g) were purchased from Charles River Laboratories (Kingston, NY) and housed in our animal facility. One week after arrival, half of the females

were mated with males from our colony. Two weeks after mating, females were individually housed in polypropylene cages (45 × 25 × 20 cm) and monitored for the onset of parturition. On postpartum day 1 (day after parturition), litters were culled to 10 pups (5 males and 5 females). All litters were weaned on postpartum day 21. All other females served as virgin, age-matched controls (nulliparous). Sample sizes ranged from 10 to 12 per group. For all experiments, animals were maintained in temperature- (21–25° C) and light- (14:10 light-dark cycle) controlled rooms and food and water were available *ad libitum*. Animals were maintained in accordance with the National Research Council (NRC) Guide for the Care and Use of Laboratory Animals (© 1996, National Academy of Science), and methods were approved by the Institutional Animals Care and Use Committee of Tufts University.

Experimental Procedures

Beginning 3 weeks after primiparous female litters were weaned, primiparous and age-matched, nulliparous subjects were ovariectomized under isoflurane anesthesia. One week later, females were injected subcutaneously with either the ER α agonist PPT (1 mg/kg.), the ER β agonist DPN (1 mg/kg) or vehicle (DMSO). All injections were administered once daily between 1000–1100 h on 4 consecutive days, and body weights were recorded daily. Thirty minutes after injection on Day 4, females were tested on the EPM. This dosing regimen was based on previous studies demonstrating significant agonist effects on EPM in female rats (Lund et al., 2005) and the selected doses have been demonstrated to be equipotent to 0.1 mg/kg 17 β estradiol (Frasor et al., 2003; Harrington et al., 2003; Harris et al., 2002) at their respective receptors. Five minutes after EPM testing (i.e. 10 minutes after being exposed to the EPM), all females were sacrificed by brief CO₂ (30 sec) followed by decapitation. Trunk blood was collected into heparinized tubes, centrifuged and plasma stored at –20°C for future radioimmunoassay analysis. Brains were rapidly frozen in –20°C methylbutane and stored at –80°C until being processed for gene expression.

Elevated Plus Maze Behavior

EPM testing was conducted between 1030–1130 h in a quiet behavioral testing room under standard vivarium lighting. At the time of testing, animals were placed individually into the center of a fully automated EPM (Hamilton-Kinder; Poway, CA) consisting of two open arms (38 × 5 cm) and two closed arms (38 × 5 × 15 cm) with a central intersection (5 cm × 5 cm). The apparatus was elevated 75 cm above the floor. Movement through the maze was detected by 48 equally spaced photocells embedded in each arm of the apparatus. Each test lasted 5 minutes during which animals were able to explore the maze. Ethanol (70%) was used to clean the apparatus between individual test sessions. All data were automatically collected and quantified using MotorMonitor® software (Hamilton-Kinder). Measures included overall activity (beam breaks), time spent, distance travelled and entries into open and closed arms as well as at the intersection. In addition, the number of pokes into the open arm and the number of head dips were also calculated. Increased time in the open arms was interpreted as reflecting reduced anxiety-like behavior (Lister 1990; Pellow et al. 1985).

Corticosterone Radioimmunoassay

Trunk blood was collected into heparinized tubes, centrifuged, and plasma stored at –20°C until assayed for corticosterone. Plasma corticosterone levels were measured by radioimmunoassay using commercially available kits (Diagnostics Products Corporation, Los Angeles, CA) according to the respective protocol. The detection limit of the assay was 0.02 ng/ml and all samples were run in duplicate with an intraassay variation of 3.2%.

Gene Expression Assays

To collect samples for analysis of CRH mRNA, frozen brains were mounted on a cryostat and bilateral micropunches were taken from the PVN (0.5 mm) and lateral amygdala (1 mm; central and basolateral nuclei). Bilateral tissue punches were then homogenized in lysis buffer and total RNA extracted using the RNeasy kit from Qiagen (Valencia, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized using the RETROscript kit from Applied Biosystems (Carlsbad, CA). PCR was performed using an AB 7500 (Applied Biosystems) under standard amplification conditions: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 60 s at 60°C. All PCR primers were TaqMan® Gene Expression Assays purchased from Applied Biosystems. The amplification efficiency of each of these assays has been validated by Applied Biosystems and averages 100% (± 10). Assay ID and accession numbers were as follows: CRH – Rn01462137_m1 and NM_031019.1; GAPDH – Rn99999916_s1 and NM_017008.2.

Quantification of gene transcription

Final quantification of mRNA was obtained using the comparative cycle threshold (CT) method (User Bulletin #2, Applied Biosystems). Data are reported as relative transcription or the N-fold difference relative to a calibrator cDNA. In brief, the housekeeping gene for the rat brain tissue, GAPDH, was used as an internal control against which each target signal was normalized, this is referred to as the Δ CT. Validation studies confirmed that the raw CT values of GAPDH did not vary by treatment group, confirming GAPDH as an appropriate housekeeping gene. The Δ CT was then normalized against the calibrator (i.e. the lowest gene transcription for the target gene in each brain region) and data are presented as $\Delta\Delta$ CT. The calibrator and target genes were run in separate wells.

Statistical Analyses

All data were analyzed using a two-way factorial ANOVA with reproductive experience and drug as independent factors. Post hoc analyses were conducted using the Tukey's test and significance was defined as $p \leq 0.05$.

Results

Administration of the ER α agonist PPT resulted in a significant decrease in body weight in both nulliparous and primiparous females as determined by the change in body weight from the first to the final day of the injection regimen (Main Effect Drug: $F_{[1,61]} = 54.5$, $p < 0.001$; see Figure 1). As expected, no effects of DPN on body weight were observed.

Significant interactions between reproductive experience and drug were found on a number of EPM behaviors. As shown in Table 1, PPT-treated, primiparous females made more entries ($F_{[2,61]} = 3.81$, $p < 0.05$), spent more time ($F_{[2,61]} = 3.71$, $p < 0.05$), and travelled greater distances on the open arms of the maze ($F_{[2,61]} = 4.13$, $p < 0.05$), when compared to both Vehicle- and DPN-treated, primiparous females (all posthoc comparisons, p 's < 0.05). In addition to raw values, we also determined the percent of time females spent in the open arm, which is the most common measure of anxiety-like behavior on the EPM. As shown in Figure 2, there was a significant interaction, ($F_{[2,61]} = 3.71$, $p < 0.05$), with primiparous, PPT-treated females spending a greater percentage of time on the open arm compared to all other groups. No significant effects of PPT on EPM behaviors were observed in nulliparous females. In addition, neither reproductive experience nor drug treatment altered overall locomotor activity. Finally, no significant effects of DPN were observed in nulliparous or primiparous females.

Analysis of corticosterone taken 10 minutes after subjects were placed on the EPM revealed additional effects of PPT (Main Effect Drug: $F_{[2,61]} = 4.09$, $p < 0.05$). These data are shown in Figure 3. Levels of corticosterone were slightly, yet significantly, lower in PPT-treated females when compared to Vehicle- and DPN-treated females collapsed across RE condition (both p 's ≤ 0.05).

As no significant effects of reproductive experience on anxiety-like behavior were observed in DPN-treated animals, CRH expression was only examined in PPT- and VEH-treated females. Significant effects of reproductive experience on CRH mRNA expression were observed in both the PVN and lateral amygdala. As shown in Figure 4 (Panel A), there was a significant reproductive experience by drug interaction ($F_{[1,19]} = 4.71$, $p < 0.05$) in the PVN. Primiparous, PPT-treated females had significantly higher CRH expression when compared to both PPT-treated nulliparous and VEH-treated primiparous females (both p 's < 0.05). As shown in Figure 4 (Panel B), a reproductive experience by drug interaction was also observed in the lateral amygdala ($F_{[1,19]} = 4.72$, $p < 0.05$). However, in this brain region, only PPT-treated, nulliparous females demonstrated increased CRH expression when compared to VEH-treated, nulliparous controls ($p < 0.05$) with no effects of PPT observed in primiparous females.

Discussion

These findings reveal that reproductive experience alters the behavioral and physiological effects of $ER\alpha$ activation. Primiparous females spent significantly more time on the open arm of the EPM following administration of PPT, an effect comparable to that observed following the administration of the benzodiazepine diazepam under similar testing procedures (Byrnes and Bridges, 2006a). Thus, in primiparous females selective activation of $ER\alpha$ has effects similar to those observed in response to a classic anxiolytic. PPT administration had no effect on EPM behavior in nulliparous females. These data are consistent with findings from a number of other laboratories demonstrating a lack of effect of PPT administration on anxiety-like behaviors in virgin females (Jacome et al., 2010a; Lund et al., 2005; Patisaul et al., 2009; Walf and Frye, 2005). In addition to differences in EPM behaviors, reproductive experience also modified the effects of PPT administration on CRH expression in both the PVN and lateral amygdala. No effects of reproductive experience were observed with regard to changes in body weight or attenuation of stress-induced corticosterone secretion following sub-chronic PPT administration. Thus, reproductive experience can significantly impact the effects of $ER\alpha$ activation on both anxiety-like behavior and CRH expression in brain regions that are known to influence stress and anxiety.

Effects of reproductive experience on anxiety-like behavior have been reported previously, however these effects have been inconsistent. While several groups have reported parity-related reductions in anxiety-like behavior (Byrnes and Bridges, 2006b; Walf and Frye, 2008; Wartella et al., 2003), others studies have not found effects of reproductive experience on these behaviors (Pawluski et al., 2009). Moreover, while we have not observed significant effects of reproductive experience on EPM behavior in ovariectomized, vehicle-treated females, others have reported decreased anxiety-like behavior on the EPM in ovariectomized, multiparous females (Walf and Frye, 2008). In the current study we did not observe any significant effects of reproductive experience on EPM performance in ovariectomized, VEH-treated animals. These effects are similar to those that we have previously reported (Byrnes and Bridges, 2006b).

In contrast to the reported effects of the $ER\beta$ agonist DPN on anxiety-like behavior, we did not observe any effects of DPN administration on EPM behavior in either nulliparous or

primiparous females. Several recent findings have also failed to observe anxiolytic effects of DPN in either male (Patisaul et al., 2009) or female rats (Jacome et al., 2010a), while still reporting significant effects on other behaviors, such as object recognition. Interestingly, more recent papers from the laboratory that originally reported decreased anxiety-like behavior following 1 mg/kg DPN, now utilized a 2 mg/kg dose of DPN to elicit similar anxiolytic effects. Thus, it is certainly possible that higher doses of DPN in the current study may have elicited an anxiolytic response, although it should be noted that a recent study utilizing a sub-chronic dose of 3 mg/kg DPN did not observe a significant anxiolytic response (Jacome et al., 2010b). It is not uncommon for estrogenic effects to demonstrate a U-shaped response curve (Calabrese and Baldwin, 2001) which, when coupled with the variations in duration and timing of ER agonist administration, lead to numerous disparate results in the literature. Thus, while no effects of DPN were observed at the current dose, it is certainly feasible that effects on anxiety-like behavior may be observed using a different dosing paradigm. Clearly, studies examining dose-response relationships with regard to the effects of DPN on anxiety-like behavior as well as studies examining the impact of variations in testing procedures on any observed anxiolytic effects are warranted.

Our findings with regard to corticosterone secretion following PPT administration are similar to those recently reported following immobilization stress (Serova et al., 2010), with PPT administration blunting stress-induced corticosterone secretion, while increasing corticosterone secretion under basal condition. Such effects could be due to ER α -mediated alterations in negative feedback systems that are activated during exposure to a stressor. In the current study, we observed a significant reduction in EPM-stimulated corticosterone secretion when compared to both vehicle- and DPN-treated subjects. This effect was not altered by prior reproductive experience. As all of the animals were tested on the EPM, we did not determine whether any shift in response to PPT as a function of reproductive experience could be observed under basal conditions. We have previously examined both baseline and EPM-stimulated corticosterone (30 min post-test) in reproductively experienced females with no significant differences observed between primiparous and nulliparous females (Byrnes and Bridges, 2006a). In the current study, there does not appear to be an association between the effects of PPT on corticosterone secretion and anxiety-like behavior on the EPM. In fact, there is little evidence that corticosterone levels predict behavior on the EPM (File et al., 1994) and recent findings have dissociated both stress- and drug-induced corticosterone secretion and anxiety/fear behavior (Albrechet-Souza et al., 2007; Hodgson et al., 2008; Marquez et al., 2006). Indeed, the classic anxiolytic diazepam significantly increases corticosterone secretion in both stressed and unstressed rats at doses that effectively decrease anxiety (Barlow et al., 1979). It should be noted, however, that stress-induced corticosterone secretion is a dynamic process. The current study only examined corticosterone levels at one time-point and therefore cannot exclude potential differences in corticosterone secretion as a function of either reproductive experience or ER agonist exposure which may be observed at later time points. However, the current data suggest that the decreased anxiety-like behavior observed in primiparous females following sub-chronic PPT administration are not related to blunted corticosterone secretion around the time of testing.

In addition to differences in anxiety-like behavior, reproductive experience significantly altered PPT-mediated regulation of CRH gene expression. Whether these differences played a role in the anxiolytic effects observed in PPT-treated, primiparous females remain unknown. Previous studies have observed increased CRH gene expression within the central amygdala following estrogen administration which were coincident with increased fear behavior (Jasnow et al., 2006). Moreover, the effects of stress on CRH activity in this brain region have been well-documented (Hand et al., 2002; Hsu et al., 1998). However, it has been suggested that acute increases in CRH activity within the central amygdala do not

directly affect anxiety-like behavior (Merali et al., 2003). Instead, CRH gene expression in the lateral amygdala may be related to the stress or fear associated with the task. In the current study, PPT augmented CRH gene expression in nulliparous females with no significant effect in primiparous females. Whether these findings suggest decreased fear or stress in PPT-treated primiparous females is unclear given that vehicle-treated primiparous females had similar levels of CRH mRNA and yet spent significantly less time exploring the open arms of the EPM. Nonetheless, these findings suggest that reproductive experience can modify CRH gene expression in response to ER α under the current testing conditions.

CRH gene expression in the PVN has a well-established role in the neural stress response and is critical for the stimulation of adrenocorticotrophic hormone (ACTH). More recent studies have demonstrated that direct infusions of PPT into the PVN result in a rapid increase in c-Fos activation in CRH neurons (Thammacharoen et al., 2009). It is important to consider, however, that PVN CRH also plays a critical role in stress-induced suppression of gonadotropins via projections to the medial preoptic area (MPOA) (Li et al., 2003). Thus, increased PPT-mediated CRH gene expression in primiparous females may suggest enhanced suppression of gonadotropins in response to stress in reproductively experienced females. Thus, while the pituitary stimulation of ACTH and subsequent corticosterone secretion from the adrenals may be similar in both nulliparous and primiparous females, the regulation of reproductive processes by ER α , such as stress-induced suppression of LH secretion may be primed by prior experience. As such, changes in CRH gene expression in primiparous females may be unrelated to activation of the HPA axis and rather may reflect an enhanced efficiency regarding the regulation of reproductive function under stressful conditions. Thus, while it is difficult to speculate on the relationship between the anxiolytic effects of PPT in primiparous females and the observed differences in CRH gene expression, the demonstration that reproductive experience may interact with estrogenic regulation of this system could have broader implications on a number of processes modulated by this neuropeptide.

Overall, these findings demonstrate significant differences in the behavioral effects of ER α activation as a function of reproductive experience in female rats. Anxiety-like behaviors, as assessed in the EPM, are mediated by the conflict between the motivation to explore new spaces and the fear associated with the open arms. It is not possible to discern whether this switch in the effects of ER α activation in parous females is due to enhanced motivation to explore, decreased fear or some combination of the two. Interestingly, ER α activation has been associated with a number of social behaviors, including maternal behavior. It is certainly possible that activation of this receptor in the primiparous females enhanced motivational factors associated with prior mothering, similar to the retrieval behavior observed postpartum. Additionally, one might speculate that the effects of PPT on feeding behavior (i.e. decreased meal size and a subsequent reduction in body weight) could interact with prior reproductive experience to increase foraging behaviors, thereby resulting in increased exploration of the entire maze, including the open arms. Alternatively, it is equally plausible that PPT administration reduces fear behavior, similar to the reduced rear and anxiety observed in late pregnant and postpartum females (Agrati et al., 2008; Lonstein, 2005; Neumann et al., 1998). To discern these effects, future studies using tasks that discriminate between the effects of PPT on motivation versus fear should be conducted in parous females. Overall, these studies indicate that the response of females to the administration of estrogen receptor agonists is significantly altered by parity. In women, a number of anxiety-related disorders are associated with periods marked by significant variations in estrogen levels (e.g. premenstrual period, postpartum period, menopause). Moreover, hormone replacement therapy has been found to lessen symptoms of anxiety in post-menopausal women. As the majority of these subjects are parous females, it is possible that some of these effects may be due to activity at the ER α subtype, which would not have

been predicted based on prior animal studies using virgin females. Thus, these data serve as a reminder of the complexity of estrogenic regulation of brain and behavior.

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Highlights

- Determined the effects of parity on estrogen receptor agonist modulation of anxiety in rats.
- ER alpha agonist was anxiolytic in primiparous but not nulliparous rats.
- CRH gene transcription following ER alpha agonist was altered by parity.

Figure 1. The effect of repeated administration of VEH, PPT (1.0 mg/kg), or DPN (1.0 mg/kg) on the mean ($\pm$ SEM) body weight change. # $p < 0.05$ as compared to VEH- and DPN-treated females collapsed across reproductive experience. N's = 10–12 per group.

Figure 2.

The effect of repeated administration of VEH, PPT (1.0 mg/kg), or DPN (1.0 mg/kg) on percent time spent on the open arms of the EPM. * $p < 0.05$ as compared to VEH- and DPN-treated, primiparous females and $p < 0.01$ as compared to PPT-treated, nulliparous females. N's = 10–12 per group.

Figure 3.

The effect of repeated administration of VEH, PPT (1.0 mg/kg), or DPN (1.0 mg/kg) on plasma corticosterone post-EPM testing. # $p < 0.05$ as compared to VEH- and DPN-treated females collapsed across reproductive experience. N's = 10–12 per group.

Figure 4.

The effect of repeated administration of VEH or PPT (1.0 mg/kg) on CRH mRNA expression in the PVN (top panel) or lateral amygdala (bottom panel). * $p < 0.05$ as compared to PPT-treated, nulliparous females and VEH-treated, primiparous females. # $p < 0.05$ as compared to VEH-treated, nulliparous females. N's = 5 per group.

Effects of selective ERalpha and ERbeta agonists on EPM behaviors in age-matched, nulliparous and primiparous females.

Table 1

Dose	Reproductive Experience		locomotor activity	head dips	open arm distance (cm)	open arm time (s)	open arm entries	closed arm distance (cm)	closed arm time (s)	closed arm entries	intersection distance (cm)	intersection time (s)	intersection entries
veh	Nulliparous	Mean	669.3	10.0	838.5	45.5	15.1	3387.7	225.0	21.5	540.6	25.6	31.5
		±SEM	32.3	1.9	161.4	9.2	2.6	191.9	9.5	3.0	51.0	2.9	3.5
	Primiparous	Mean	656.8	9.8	766.2	43.4	13.9	3445.6	228.0	22.2	531.8	25.2	30.4
		±SEM	48.2	1.7	218.0	9.8	2.4	198.0	11.6	2.9	59.3	3.1	3.1
dpm	Nulliparous	Mean	630.5	10.2	796.5	49.7	17.9	3206.3	214.7	22.7	612.7	31.0	34.6
		±SEM	28.2	1.6	169.7	10.3	2.4	177.6	11.6	1.9	71.2	3.2	3.2
	Primiparous	Mean	669.9	10.2	830.9	47.6	14.8	3347.7	217.3	24.5	657.8	32.0	34.3
		±SEM	29.3	1.5	172.2	9.6	2.2	173.7	12.3	3.4	80.7	4.9	4.1
ppt	Nulliparous	Mean	636.2	8.2	609.5	38.1	12.8	3372.0	227.8	24.5	588.4	30.4	31.8
		±SEM	28.8	0.8	96.9	6.9	1.5	196.4	5.1	2.9	66.5	3.9	3.2
	Primiparous	Mean	686.2	12.3	*1570.5	*94.4	*21.8	2662.6	*167.3	18.9	717.8	34.2	35.5
		±SEM	37.9	1.8	292.1	22.2	2.7	296.2	20.9	2.4	72.7	4.8	3.2

* p < 0.05 as compared PPT-treated, nulliparous females