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EFFECTS OF FEMALE GONADAL HORMONES AND LPS ON DEPRESSIVE-LIKE BEHAVIOR IN RATS

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Abstract: Considerable evidence shows an association of depression with the immune system and emphasizes the importance of gender in the etiology of the disease and the response to inflammatory stimuli. We examined the influence of immune-challenged systems on depressive-like behavior in female rats in the context of gonadal hormones. We used a neuroinflammatory model of depression elicited by lipopolysaccharide (LPS) administration on naive and ovariectomized (OVX) female rats, and examined the effects of estradiol (E2) and/or progesterone (P4) replacement therapy on animal behavior, as assessed by the forced swimming test (FST). We found that LPS and OVX increase immobility in the FST, while LPS also decreased body weight in naive female rats. Further, even though P4 application alone showed beneficial effects on the behavioral profile (it reduced immobility and increased climbing), supplementation of both hormones (E2 and P4) together to OVX rats failed to do so. When OVX rats were exposed to LPS-induced immune challenge, neither hormone individually nor their combination had any effect on immobility, however, their joint supplementation increased climbing behavior. In conclusion, our study confirmed that both LPS and OVX induced depressive-like behavior in female rats. Furthermore, our results potentiate P4 supplementation in relieving the depressive-like symptomatology in OVX rats, most likely through fine-tuning of different neurotransmitter systems. In the context of an activated immune system, the application of E2 and/or P4 does not provide any advantageous effects on depressive-like behavior.

Key words: LPS; behavior; gonadal hormones; ovariectomy; force swimming test.

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INTRODUCTION

Women, as compared to men, are twice as likely to experience depression, and their depressive episodes last longer and recur more often (Earls, 1987; Nolen-Hoeksema, 1987). From the perspective of clinical studies, the vulnerability of women to depression is associated with hormonal fluctuations during their life, particularly to gonadal steroids (Sazdanovic et al., 2013), in which estrogens have a significant role (Halbreich et al., 1986; Hamilton et al., 1988; Pavlovic et al., 2013). Indeed, the negative symptomatology associated with depression is greater in premenstrual and postpartum periods, as well as after menopause or oophorectomy, when women's 17b-estradiol (E2) levels are decreased (Torizuka et al., 2000). Moreover, the depressive symptoms of these women are ameliorated by chronic treatment with E2 (Ahonkas et al., 1999; Osterlund and Hurd, 2001). Preclinical results from animal models also suggest that E2 effects depressive-like behavior. Reduced depressive-like behavior has been found in female rats during proestrous, when E2 levels are high, compared to diestrous, when E2 levels are low (Marcondes et al., 2001; Mora et al., 1996). In addition, it has been shown that in rodents ovariectomy increases depressive-like behavior, while acute, subchronic or chronic physiological E2-replacement alleviates these symptoms (Estrada-Camarena et al., 2003). All these data suggest that alterations in E2

levels may be involved in the development of depression, even though the role of gonadal hormones is not completely understood.

Within the multifactorial origins of depression (Millan, 2009), chronic inflammation has received increasing attention as a potential contributor in the development of the disease (Dantzer et al., 2008; Heyley et al., 2005; Lackovic et al., 2013, Messay et al., 2012). Indeed, elevated biomarkers of inflammation, including inflammatory cytokines and acute-phase proteins, have been found in depressed patients (Haroon et al., 2012). It has also been shown that gender is a risk factor for depression upon the administration of some inflammatory stimuli (Pavlovic et al., 2011). Thus, in order to explore the effects of female gonadal hormones and inflammation in the etiology of depression, we utilized a neuroinflammatory model of depression elicited by lipopolysaccharide (LPS) administration to naive and ovariectomized (OVX) female rats. We also examined the effects of E2 and/or progesterone (P4) supplementation on depressive-like behavior in OVX rats.

MATERIALS AND METHODS

Animals and treatment

The experiments were performed on adult 3-month-old female Wistar rats (body mass 250-350 g). All animals were housed at 20±2°C, with a 12h light/dark cycle (lights on at 07:00 h), with food (commercial rat pellets) and drinking water available *ad libitum*. The experimental methods were approved by the Ethical Committee for the Use of Laboratory Animals of the VINCA Institute of Nuclear Sciences, according to the guidelines of the EU-registered Serbian Laboratory Animal Science Association (SLASA).

Estrous cycle of naive animals

Vaginal smears were collected daily and microscopically analyzed for determination of the estrous cycle stage one week before the start of the experiment. Only female rats with normal 4-5 days of estrous cycle were included in the study. The vaginal smears were examined 24 and 48 h after the last LPS injection (before the forced swimming pretest and forced swimming test) and it was shown that LPS did not alter the estrous cycle phase distribution.

Surgical procedure

Ovariectomy was performed under 5% chloral hydrate (400 mg/kg) anesthesia. Briefly, a single midline incision was made in the ventral area, the oviducts were exposed and the ovaries removed (Filova et al., 2013). The complete extraction of the ovaries was corroborated by visual inspection. OVX rats were used 2-3 weeks after surgery.

Table 1. Body mass (grams) in naive and OVX rats measured before and after 7-day treatment with saline (SAL), lipopolysaccharide (LPS), and hormones (E2 – 17 β – estradiol and P4 – progesterone). Data and presented as mean ± SD $p<0.05$ (* before and after treatment, # vs Ctrl-SAL).

day	Ctrl-SAL	**LPS**	**OVX**
Before treatment	265.9±33.9	**263.5±25.9**	**347.5±28.8#**
After treatment	275.3±36.1	**244.1±20.7***	335.0±25.2

day	**OVX**	**OVX+E2**	**OVX+P4**	**OVX+E2P4**
Before treatment	315.2±22.7	327.3±38.5	**305.0±18.1**	335.8±20.0
After treatment	337.1±32.8	335.7±44.7	**330.9±18.5***	330.3±25.6
	OVX+LPS	**OVX+LPS+E2**	**OVX+LPS+P4**	**OVX+LPS+E2P4**
Before treatment	317.5±24.4	273.8±37.7	286.2±23.3	**305.4±24.6**
After treatment	307.7±24.3	256.3±30.7	276.3±24.5	**268.6±18.6***

Paradigm and treatments

In order to analyze whether LPS effects on female behavior could be modified by 17 β-estradiol (E2) and/or progesterone (P4), two separate experiments were performed. In the first experiment, naive and OVX rats were treated intraperitoneally (i.p.) with saline solution (SAL) and lipopolysaccharide (LPS) and assigned to the following groups: control (SAL, n=14), LPS group (500 μg/kg, n=14) and OVX group treated with SAL (OVX+SAL, n=10). All groups were treated for the next 7 days. Dose and duration of LPS treatment were determined in our previous experiments. The applied LPS (Escherichia coli 055: B5, No. L-2880, Sigma-Aldrich, St. Louis, MO, USA) was previously dissolved in sterile, pyrogen-free physiological saline and administered at a dose of 500 μg/kg of rat body mass. a fresh solution of LPS was prepared on the day of the injection.

In the second experiment, we tested the individual effects of the hormones, E2 and P4, as well as their joint effect (E2P4) in combination with LPS on OVX females. OVX rats were divided into 8 experimental groups: OVX-control-control group (OVX+SAL+OIL, n=8), OVX-control-E2 group (OVX+SAL+E2, n=8), OVX-control-P4 group (OVX+SAL+P4, n=8), OVX-control-E2P4 group (OVX+SAL+E2P4, n=8), OVX-LPS-control group (OVX+LPS+OIL, n=8), OVX-LPS-E2 group (OVX+LPS+E2, n=8), OVX-LPS-P4 group (OVX+LPS+P4, n=8) and OVX-LPS-E2P4 group (OVX+LPS+E2P4, n=8). The hormones E2 and P4 (both from Sigma Chemical Co., St Louis, MO) were dissolved in sesame oil (Milosevic et al., 2012) and administered subcutaneously (sc) every day, parallel with LPS injection (500 μg/kg, as described in the first experiment). The treatment lasted for 7 days. Dosages of E2 (10 μg/kg) and P4 (4 mg/kg) were chosen on the basis that they produce moderate physiological plasma levels, both for E2 (Walf et al., 2005) and P4 (Lianeza et al., 2009).

Forced swimming test (FST)

To test depressive-like behavior we used the forced swimming test (FST) described previously by Porsolt et al. (1977). The FST consisted of two sessions, pre-test and test. Twenty-four h after the last injection the rats were subjected to a pre-test and placed individually for 15 min in a Plexiglas cylinder (40 cm height, 20 cm in diameter) filled with water to a depth of 30 cm at 24±0.5°C. The water depth was adjusted to a height that did not allow the animals to touch the tank bottom with their hind paws or tails and under conditions where escape was not possible. After 15 min they were dried and placed in their home cage. Twenty-four h after their pre-test, the animals were placed for a 5-min test session in the swimming apparatus, and each test session was recorded using a video camera in a dimly illuminated room. A time sampling technique was used whereby the predominant behavior in each 5 s period of the 300 s test was recorded. The behaviors measured were immobility (i.e., floating and only making the movements necessary to keep the rat's head above water), swimming (i.e., horizontal movements throughout the swim chamber, which also included crossing into another quadrant), and climbing (i.e., making intense upward-directed movements of the forepaws along the side of the cylinder). Uncontrollable reflex movements during periods of immobility, such as shivering or wiping of water away from the eyes, were considered as floating (Spasojevic et al., 2008).

Statistical analysis

Data are presented as a mean ± SD. In order to establish significant differences in body weight at the beginning of the experiment and after treatments, data were analyzed by Student's (two-tailed) t-test. Two-way analysis of variance (ANOVA) was used to assess the effects of E2 and/or P4 on behavior in the forced swimming test in LPS- and saline-treated animals. Three-way ANOVA was used to analyze the effects of LPS, E2 and/or P4 treatments on behavior. Only when there was a significant main effect and/or interaction effect in the ANOVA were post-hoc analyses (Tukey's test) performed. The statistical significance was accepted at $p<0.05$.

RESULTS

The effect of LPS treatment on body mass

The effects of LPS and OVX treatments on body mass changes are presented in Table 1. Body mass was signif-

icantly decreased by LPS and OVX treatment in respect to the control group ($p<0.05$). In the second experiment, the trend of decrease in body mass was detected in all LPS-treated OVX groups, with significant change only in the LPS+E2P4-treated OVX group ($p<0.05$).

The effect of LPS treatment on FST behavior of naive and OVX rats

In Fig. 1, both LPS and OVX treatments increased immobility (LPS $F=21.54$, OVX $F=66.63$, $p<0.05$) and decreased swimming behavior compared to the naive group treated with saline (LPS $F=6.67$, OVX $F=107.46$, $p<0.05$). In addition, the climbing behavior was significantly increased in OVX rats compared to the saline group ($F=67.09$, $p<0.05$).

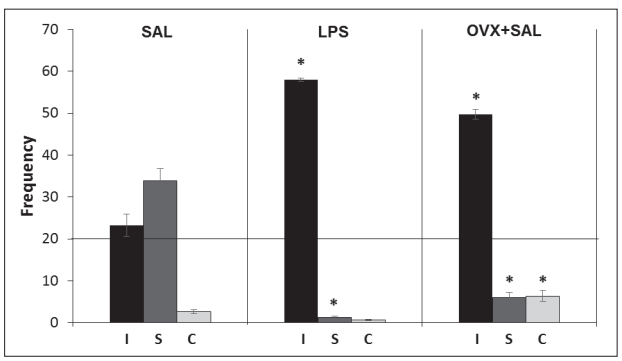

Fig. 1. Changes in immobility, swimming and climbing (frequency) in the forced swimming test (FST) in naive and ovariectomized (OVX) rats treated for 7 days i.p. with saline (SAL) and lipopolysaccharide (LPS). Data and presented as mean ± SD, $p < 0.05$ (* vs SAL).

The effects of LPS, E2 and/or P4 treatments on FST behavior of OVX rats

In Fig. 2a, two-way ANOVA revealed that P4 significantly decreased immobility ($F=14.59$, $p<0.05$) and increased climbing behavior ($F=16.41$, $p<0.05$) of OVX rats in comparison to the OVX-SAL group. Furthermore, combined treatment of E2P4 significantly increased immobility (E2 x P4 interaction, $F=57.81$, $p<0.05$) while decreasing swimming (E2 x P4 interaction, $F=48.71$, $p<0.05$) and climbing (E2 x P4 interaction, $F=4.53$, $p<0.05$) in respect to the OVX-SAL group. E2P4 combined treatment also significantly decreased climbing behavior in respect to the other two OVX-SAL groups treated with individual hormones, E2 and P4 ($p<0.05$) (Fig. 2a), while decreased swimming compared to the P4-alone treatment ($p<0.05$).

As can be seen in Fig. 2b, two-way ANOVA showed that only the E2P4 combined treatment significantly increased climbing behavior in comparison to all the other OVX-LPS groups (E2 x P4 interaction, $F=16.36$, $p<0.05$). Three-way ANOVA revealed significant changes in behavior between the OVX-SAL and OVX-LPS groups only with the combined E2P4 treatment (Fig. 2a and b). Namely, E2P4 joint treatment significantly decreased immobility (LPSxE2xP4 interaction, $F=40.78$, $p<0.05$) with a concomitant increase in swimming (LPSxE2xP4 interaction, $F=22.25$, $p<0.05$) and climbing (LPSxE2xP4 interaction, $F=19.08$, $p<0.05$) in the OVX-LPS group in comparison to the OVX-SAL group.

DISCUSSION

Based on the complexity and heterogeneity of depression, it is likely that several interacting systems underlie its pathogenesis. Clinical studies indicate that inflammatory processes, at least in certain clinical subpopulations, are associated with depression. It was documented that some depressed patients exhibited a disturbed peripheral immune system (Dowlati et al., 2010; Schiepers et a., 2005), and that depression was manifested as a comorbidity in patients suffering from conditions characterized by a systemic inflammation (Anderson et al., 2001; Dickens et al., 2002; Khawaja et al., 2009;). Moreover, therapeutic stimulation of the immune system leads to depression in hepatitis C patients-(Bonaccorso et al., 2001), particularly women (Pavlovic et al., 2011).

A well-established animal model to study behavioral and physiological responses under activation of the immune system is the administration of bacterial LPS. However, experiments on the effects of LPS administration on female rats are very scarce. To address this question and to evaluate the contributions of female gonadal hormones and the immune system in the etiology

of depression we used an experimental paradigm where naive and ovariectomized rats were injected with 500 μg/kg LPS7 for 7 consecutive days, with or without parallel treatment with estradiol and progesterone.

The first finding of this study was that repeated LPS injections reduced body mass. This result is in agreement with previous reports showing that high doses of LPS induced weight loss (Lugarini et al., 2002; Hrupka et al., 2001; Plata-Salaman et al., 1993). Moreover, it is documented that infection and inflammation in animal models significantly decreased food intake (McHugh et al., 1993), and that LPS decreased body weight gain by inducing anorexia. On the other hand, our data from OVX rats support the notion that estrogen deficiency leads to body weight gain and excessive feeding, which increase the risk of metabolic syndrome (Albert et al., 1991; Okada et al., 1993).

The behavioral profile of the LPS-treated female rats differed from that found in the controls. Namely, LPS treatment increased immobility and decreased swimming. The effects of LPS on female behavior are in agreement with the observation of male rodents indicating an induction of depressive-like behavior after i.p. administration of LPS (Dantzer et al., 2008). The profile of the LPS-induced behavioral changes in the FST is worth noting because antidepressants that enforce serotonergic transmission primarily increase swimming, while antidepressants that enhance noradrenergic transmission predominantly increase climbing (Cryan et al., 2005; Detke et al., 1995). Analagou to this, we can speculate that the depression-like behavior caused by subchronic LPS administration in female rats involves impairment of serotonergic but not noradrenergic pathways.

Ovariectomy (OVX) is utilized as a model of examining the effect of E2 deprivation in animal models of anxiety and depression (Frye et al., 2004; Galea et al., 2001). We found that OVX significantly increased immobility and climbing and decreased swimming, which confirmed previous findings. The profile of OVX-induced behavioral changes in the FST indicated impairment in both serotonergic and noradrenergic transmis-

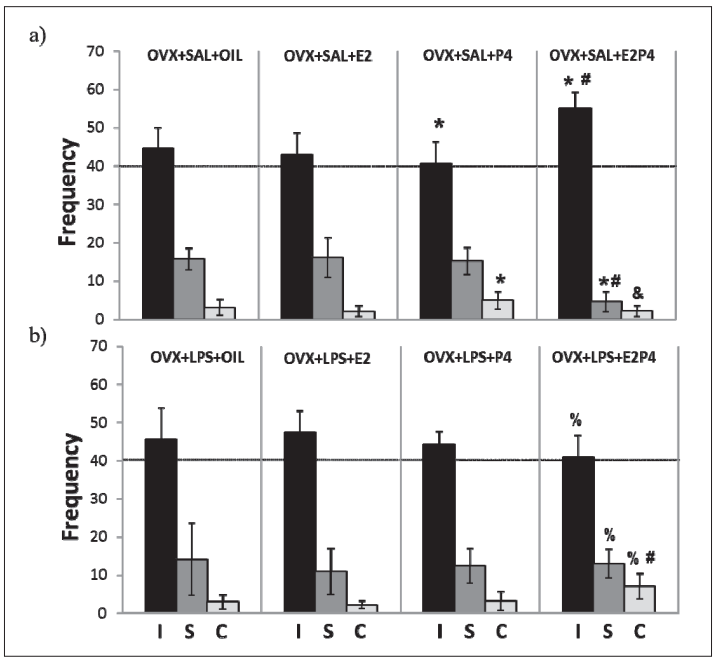

Fig. 2. Changes in immobility, swimming and climbing (frequency) in the forced swimming test (FST) in ovariectomized (OVX) rats treated for 7 days i.p. with saline (SAL), lipopolysaccharide (LPS), and hormones (solely E2 – 17 β – estradiol, solely P4 – progesterone or in combination E2P4). Data and presented as mean ± SD, $p < 0.05$ (* vs. adjacent vehicle group, # E2P4 vs. other groups, & E2P4 vs. P4, and % LPS vs. OVX adjacent group).

sion. This is not surprising, since it has been reported that ovarian hormones may interact with specific serotonergic and noradrenergic transmission (Birzniece et al., 2002; Mize and Alper, 2000). Mechanisms by which estrogen affects the serotonin transmission include the degradation of monoamine oxidase, the enzyme that catabolizes serotonin (Luin et al., 1975), and alteration of intraneuronal serotonin transport (Sherwin, 1997). On the other hand, a decrease in estrogen leads to a norepinephrine increase (Douma et al., 2005), which in our case could be responsible for the enhancement in climbing. Overall, our results confirmed that the absence of ovarian hormones contributes to the behavioral profile of OVX rats by modulating both serotonergic and noradrenergic transmission.

To elucidate further the role of ovarian hormones and LPS treatment in the etiology of depressive-like behavior we applied LPS, E2 and/or P4 in OVX rats.

Our results regarding chronic E2- and P4-replacement therapy in OVX rats are unexpected and to some extent contradict each other. Namely, we found a positive effect of P4 administration on the behavioral profile of OVX rats, while at the same time the absence of effect after E2 application and a negative effect after E2P4 application on depressive symptomatology of OVX rats. To clarify, P4 application alone significantly reduced immobility and increased climbing behavior, thereby alleviating depressive-like symptoms in OVX rats. These results are in accordance with a number of papers suggesting that the supplementation of ovarian hormones in OVX rats relieves depressive-like behavior (Bekku et al., 2006; Bernardi et al., 1989; Estrada-Camarena et al., 2003; Frye and Walf, 2002; Rachman et al., 1998; Stoffel and Craft, 2004). In addition, this opposite effect of P4 on immobility and climbing may imply a compensatory mechanism in the fine-tuning between different neurotransmissions, such as serotonergic and noradrenergic, which governs the different components of behavior, whereby P4 can relieve depressive-like symptomatology. In contrast to P4, rather unexpected effects of E2 and the E2P4 combination were obtained. E2 alone had no effect on any of the behavioral components, while E2P4 increased immobility. The lack of E2 effect could be explained by dosage and/or regimen of E2 application. Namely, our chronic E2 administration coincides with some studies reporting that longer a E2-replacement regimen and/or higher E2 dosages may not reduce depressive-like behavior, similar to our case (Galea et al., 2002; Martinez-Mota et al., 2000, Morgan and Pfaff, 2001, 2002; Stoffel and Craft, 2003). On the other hand, the increased immobility upon joint E2P4 application in OVX rats was contrary to expectations and could be in part explained through an opposite effect of the ovarian hormones used singly or in combination on serotonin autoreceptors and postsynaptic receptors in the brain (Birzniece et al., 2001). In particular, it was documented that ovarian hormones applied alone or in combination differentially influence the 5-HT_{1A} receptor in the hippocampal subregions and the raphe nuclei. Such different modulations of the 5-HT_{1A} receptor may elucidate the behavioral profile of OVX rats treated with a combination of ovarian hormones.

Another unexpected result of our study concerned hormone supplementation with E2 or P4 in OVX rats when exposed to immune challenge. When LPS-OVX rats were given individual hormones, no changes were found in behavior. In addition, when the E2P4 combination was applied, no immobility changes were found, but rather increased climbing behavior. Additionally, E2P4 treatment of OVX-LPS rats markedly changed the behavioral profile, decreased immobility and increased swimming and climbing in comparison to the OVX-E2P4 group.

Altogether, our study confirmed that both LPS and OVX induce depressive-like behavior in female rats. Furthermore, our results potentiate P4 supplementation in the relief of depressive-like symptomatology in OVX rats through a fine-tuning of different neurotransmissions, such as serotonergic and noradrenergic, that govern various components of behavior. Supplementation of rats with either E and/or P did not produce beneficial effects on the response to immune challenge with LPS hormone.

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Božović and Miroslav Adžić participated in designing the study, experimental work, obtaining and analyzing the results. Jelena Djordjević and Miroslav Adžić participated in drafting the article and revising it critically. All authors contributed to and have approved the final manuscript.

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