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ORIGINAL PAPER

Reproductive axis response to repeated lipopolysaccharide administration in peripubertal female rats

Nancy Cardoso · Pablo Arias · Berta Szwarcfarb · Osvaldo Ponzo · Silvia Carbone · Jaime Moguilevsky · Pablo Scacchi · Roxana María Reynoso

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Abstract Immune system disorders are often accompanied by alterations in the reproductive axis. Several reports have shown that administration of bacterial lipopolysaccharide (LPS) has central inflammatory effects and activates cytokine release in the hypothalamus where the luteinizing hormone releasing hormone (Gn-RH) neurons are located. The present study was designed to investigate the effect of repeated LPS administration on the neuroendocrine mechanisms of control of the reproductive axis in peripubertal female rats (30-day-old rats). With this aim, LPS (50 μg/kg weight) was administered to the animals during 25, 27 and 29 days of age and sacrificed on 30 day of life. Gn-RH, γ−amino butyric acid (GABA) and glutamic acid (GLU), two amino acids involved in the regulation of Gn-RH secretion, hypothalamic content were measured. LH and estradiol serum levels were also determined and the day of vaginal opening examined. The results showed a significant increase in Gn-RH and GLU content ($p<0.0001$), shared by a reduction of GABA one ($p<0.0001$). LH and estradiol serum levels were decreased ($p<0.01$, $p<0.001$) and delay in the day of vaginal opening was also observed in treated animals. Present results show that repeated LPS administration impaired reproductive function, modifying the neuroendocrine mechanisms of control of the axis in peripubertal female rats.

Keywords Gn-RH · Glutamic acid · GABA · Lipopolysaccharide · Puberty

N. Cardoso · P. Arias · B. Szwarcfarb · O. Ponzo · S. Carbone · J. Moguilevsky · P. Scacchi · R. M. Reynoso (✉)
Laboratory of Endocrinology, Department of Physiology, School of Medicine, University of Buenos Aires, Buenos Aires, Argentina
e-mail: rreynoso@fmed.uba.ar
e-mail: rroxam@yahoo.com.ar

Introduction

Clinical reports have shown that infections and inflammatory diseases are often accompanied by impaired reproductive function [7, 59]. Experimental evidence obtained in rats, cows, sheep, and monkeys demonstrate that immune activation induces different reproductive alterations, including sexual cycle disruption [2, 33, 43, 61]. From these reports, it stands clear that several cytokines produced by immune cells act within the hypothalamus and/or on the pituitary to produce the pattern of pituitary hormone secretion that characterizes infection, in particular the activation of the hypothalamic-pituitary-adrenal axis, and the inhibition of growth hormone, thyrotropin and gonadotropin secretion [3, 18, 25]. By administering endotoxin or lipopolysaccharide (LPS), a well-characterized model of immune/inflammatory challenge [37, 57], different authors have demonstrated a disruption of the reproductive capability, most probably by intermediary

molecules comprising proinflammatory cytokines, prostaglandins, endogenous opioid peptides and several neurotransmitters [1, 11, 22, 23, 42, 60].

It is well established that gonadotropin-releasing hormone (Gn-RH) is the fundamental hypothalamic hormone involved in the activation of pituitary gonadotropins during sexual maturation and the onset of puberty. The pubertal increase in Gn-RH secretion is prompted by changes in transsynaptic and glial inputs to the Gn-RH neuronal network. Studies conducted by different groups have identified these inputs as being both facilitatory and inhibitory. The former use excitatory amino acids, glutamic and aspartate acid (GLU, ASP), and the recently identified neuropeptide kisspeptin for neurotransmission/neuromodulation [8, 32, 46]. γ−amino butyric acid (GABA) and opioid peptides provide the inhibitory inputs [54, 55]. The excitatory amino acids GLU and ASP stimulate Gn-RH release gradually from prepuberty until the onset of puberty, while GABA exerts an inhibitory tone in peripubertal and adult female rats [30]. GABAergic neurons acting via GABA-A receptors provide a major inhibitory transsynaptic influence controlling GnRH secretion during peripubertal development [27, 28]. A key component of hypothalamic development involves alterations in GABA levels, receptors and synapses. Not only GABA subunit expression patterns in the hypothalamus change with development, but the nature of the effect of GABA undergoes a developmental switch from excitatory to inhibitory. In early development, studies suggest that GABA stimulates neural developmental processes, and later in development (e.g., puberty), it appears to play inhibitory roles including the mediation of negative feedback effects of steroids on reproductive physiology [24]. Moreover, in some studies using tissue fragments that include the entire medial basal hypothalamus and the preoptic region, blockade of GABA-A receptors was shown to increase Gn-RH release from immature hypothalamus but decreased it from mature hypothalamus [12]. Evidence for this hypothesis has been provided by a number of different techniques, although there has been some disagreement in the literature. In this sense, electrophysiological studies actually suggest that GABA may exert excitatory effects on GnRH electrical properties that are potentially mediated through actions of GABA through a more complex neural network. A reconciliation of these results has not been made to date but differences may be attributable to direct vs. indirect actions of GABA on GnRH cells and GABAergic result may vary depending upon the target tissue and the neural environment [9, 29, 62].

It has been demonstrated that LPS administration modifies neurotransmitter release in adult male rats. In this sense, previous reports showed that, in vitro, LPS inhibited hypothalamic Gn-RH output, modifying the release rate of taurine, glutamate and GABA, leading to an alter function of the reproductive axis [11, 13].

Previous observations indicate that immune neuroendocrine interactions exhibit modifications during sexual maturation. It has been reported the existence of age-related differences in the effect of LPS on Gn-RH and gonadotropin release in male rats, but there are no evidence of modifications of the axis in female rats under repeated LPS administration [48]. Considering these data, the aim of the present work was to study the effect of repeated LPS administration on the neuroendocrine mechanisms involved in the regulation of the reproductive axis in peripubertal female rats.

Material and methods

Animals

Female Wistar rats from the Department of Physiology, School of Medicine, University of Buenos Aires of 25 days of age (50-60 g body weight), housed in group cages (eight to ten per cage), under standardized conditions, light from 7.00 to 19.00 h, temperature 22-24°C, food and water ad libitum were used in these experiment.

Drugs

Lipopolysaccharide (LPS), *Salmonella typhosa*, Sigma Chemical Co., St Louis, Mo USA, was used dissolved in sterile saline solution.

Experimental design

LPS 50 μg/kg body weight was injected, via ip to treated animals (n=8-10) and control animals (n=8-10) with saline solution on 25, 27 and 29 days of age.

After ip administration of LPS, the animals showed signs of inflammation, such as piloerection, lethargy and milk shaking. The animals were sacrificed on 30 days of age (group 1). Another group of animals (n=8–10), control and treated with LPS, were used to control the day of vaginal opening from 30 days of age. These animals were examined every day from 30 days of age in order to determine the day of vaginal opening (group 2). At 30 days of age, the animals of group 1 were sacrificed by decapitation. Troncal blood was collected, the samples were centrifuged and serum was frozen until hormone determination. Hypothalamic samples containing anterior preoptic area (APOA) and medio basal hypothalamus (MBH) were immediately dissected with the help of a stereomicroscope. The samples were cut to a depth of 3-4 mm and were laterally bordered by the hypothalamic sulci, rostrally, 3 mm anterior to the optic chiasm and caudally, by the mammillary bodies. The thickness of each sample was less than 2 mm. These tissues were frozen at –70°C to determine Gn-RH, GLU and GABA content.

Animal care was carried out according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington DC 2005, and USA).

Determinations

Gn-RH determination: Tissues were homogenized in perchloric acid 0.1 N, tissue weight/acid ratio: 1:20. Homogenates were centrifuged at 2,000 rpm, for 30 min at 4°C. The supernatants were stored at −70°C until the time of Gn-RH determination. Gn-RH was measured in the supernatants in duplicate by RIA using a highly specific antibody generously provided by Dr. Hubertus Jarry (University of Göttingen, Germany). Intra and inter assay coefficients of variation were lower than 9% and 10% respectively, sensitivity was 0.2 pg/tube and the curve was linear up to 100 pg. The results were expressed as pg/mg tissue.

Amino acids determination The tissues for amino acid determination were homogenized in ClH acid 0.6 N, relation tissue weight/acid 1:20. The concentrations of GLU and GABA in the tissues were determined by HPLC after derivatization with phenylisothiocyanate and UV detection at 254 nm. The drugs used did not interfere in the derivatization process. Mean inter- and intra-assay coefficients of variation were 4.0 and 5.6% respectively. The detection limit was 10 pmol for GLU and 5 pmol for GABA. The mobile phase consisted of 0.57 M sodium acetate buffer (pH 6.5) containing 10% acetonitrile (Sintorgan, Buenos Aires, Argentina). GLU and GABA used as standards were from Sigma Chemical Co St Louis, Mo, USA. Results were expressed as pmoles/mg tissue.

LH and estradiol determinations and control of the day of vaginal opening Serum LH levels were determined in duplicated using a double antibody radioimmunoassay technique. The material for the assay was provided by NIAMDD rat pituitary program. Intra assay coefficient of variation was 5.6%. Reference standard was RP2. Results were expressed as ng/ml. Serum estradiol levels were determined in duplicated using a commercially available kit from Diagnostics System Laboratories, INC. Intra assay coefficient of variation was 6%. Results were expressed as pg/ml.

Vaginal opening Control and treated animals were examined every day from 30 days of age in order to determine the day of vaginal opening.

Statistical analysis

The results were expressed as means±SEM. The differences between the means of two groups were calculated by Student's "t" test. $p<0.05$ was considered significant. Analyses of data on vaginal opening values were performed by Fisher's exact test.

Results

In order to analyze LPS effect on the neuroendocrine mechanism of control of the reproductive axis, Gn-RH, GLU and GABA content were evaluated. Both Gn-RH and GLU content increased significantly after LPS administration (Table 1, $p<0.0001$). Conversely, GABA content was decreased by treatment (Table 1, $p<0.0001$). These results may indicate that repeated LPS administration acting at the hypothalamic level

Table 1 Effect of repeated LPS administration on Gn-RH, GLU and GABA content in peripubertal female rats

	Gn-RH (pg/mg tissue)	GLU (nmol/mg protein)	GABA (nmol/mg protein)
Control	1.8±0.05	250±15	35±0.1
LPS	2.7±0.4****	425±68****	10±0.05****

Each value represents the mean±SEM of 8–10 determinations
****$p<0.0001$ vs. control

produced a decrease in Gn-RH and GLU release and an increase in GABA one. As it can be taken from Fig. 1, both LH and estradiol were markedly decreased by repeated LPS administration ($p<0.05$, $p<0.001$). Moreover, as a result of the immune activation exerted during days 25-29, a 4-day delay in vaginal opening (VO) arises, as clearly shown in Fig. 2: by postnatal day 32, 50% of saline-injected animals have shown VO, as compared to 0% in the treated group. This process, reflecting the impact of growing estrogen levels on vulvar/vaginal tissues, is completed by 100% of control animals by day 34; at this time point none of the LPS-treated rats has even initiated VO. These differences, as well as those arising from the comparison of the proportion of animals showing VO at day 33 and 35, are statistically significant.

Discussion

As we mentioned above, there are clinical and experimental evidences that infections and inflammatory diseases are often accompanied by impaired reproductive function [56, 59]. Previous reports have shown that immune system activation can modify the activity of the hypothalamic-pituitary-gonadal system [10]. Present results showed that repeated LPS administration enhances Gn-RH and GLU content, suggesting a decrease in their release, concurrently with a decrease in GABA one, that may be understood as an increase in its release. These modifications in neurotransmitters and Gn-RH contents may be responsible of an impaired function of the axis. In this sense, in a recent report, we have shown that acute exposure to LPS also depresses reproductive function in prepubertal female rats, by decreasing GABA content and increasing Gn-RH one [41]. Observations of Feleder et al. [11, 13, 14, 16] indicate that LPS inhibits Gn-RH and GLU release, but stimulates taurine and GABA secretion from APOA-MBH of adult male rats, described previously as a region where GABA exerts an inhibitory effect [30]. The authors speculate that these effects may be explained by the stimulation of cytokines of a neuronal and/or glial source which may interact with the excitatory and inhibitory amino acids to control Gn-RH release. They later described that IL-1, one of the cytokines released by LPS, significantly increased taurine and GABA release without changes in GLU. They conclude that the cytokine may regulate Gn-RH release indirectly via

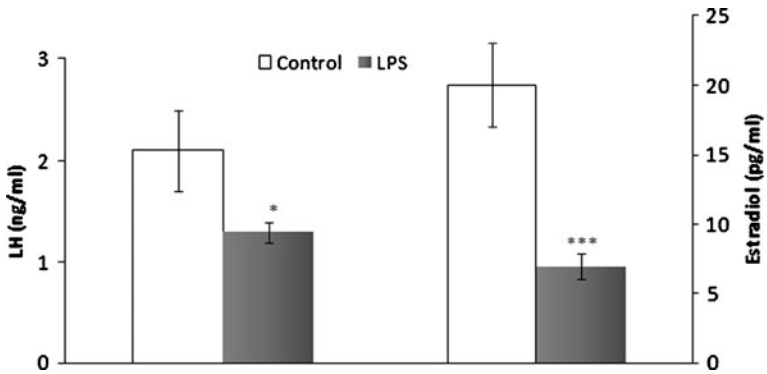

Fig. 1 Effect of repeated LPS administration on LH and estradiol serum levels in peripubertal female rats. Each column represents the mean±SEM of eight to ten determinations. *$p<0.05$ vs. control, ***$p<0.001$ vs. control

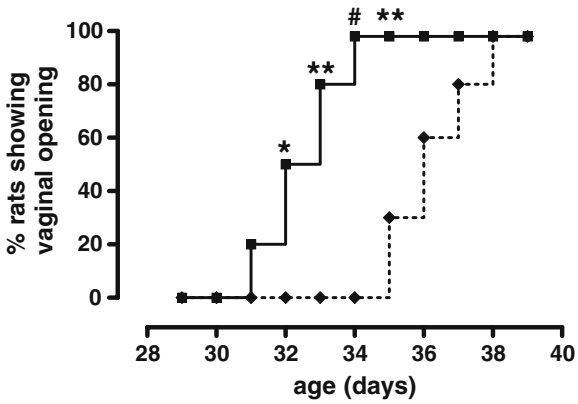

Fig. 2 Percentage of control rats showing vaginal opening (n= 10, *full line*) and LPS-treated animals (n=10, *dotted line*). *p< 0.05, **p<0.005, #p<0.0001 (control versus LPS treated)

its effect on the amino acid neurotransmitter system, leading to a decrease in the secretion of the hypothalamic peptide. It has been shown that, in vitro, IL-1β diminishes hypothalamic Gn-RH release, simultaneously increasing hypothalamic GABA output [14, 17]. Moreover, in 30-day-old male mice, the activation of the hypothalamus-pituitary-adrenal axis by LPS treatment is increased [49] and it has been shown that the corticotropin-releasing hormone (CRH) inhibits Gn-RH release [44]. Besides, a coliberation of GABA with CRH at the median eminence has been described in adult male rats [26]. Hence, it could be further speculated that increased GABA output would result from augmented activation of CRH neurons.

However, kisspeptins participation may not be discarded. Kisspeptin binding to its G-protein-coupled receptor KISS1R, which is expressed by Gn-RH neurons, stimulates Gn-RH release and activation of the mammalian reproductive axis, being critical for puberty and the regulation of reproduction [6, 53]. Kisspeptin can act directly on Gn-RH neurons and/or indirectly via synaptic input from other neurons [45]. In this sense, some evidence has been produced recently suggesting interactions between kisspeptin, GLU and GABA pathways. Pielecka-Fortuna et al.; [34] have recently reported that kisspeptin increased frequency and amplitude of GABAergic postsynaptic currents (PSCs) and also increased frequency of glutamatergic excitatory PSCs in Gn-RH neurons from ovariectomized and treated with estradiol implants (OVX + E) mice. Kisspeptin did not affect either GABAergic or glutamatergic transmission to Gn-RH neurons in cells from OVX mice, indicating that the effects on transmission are estradiol dependent. In contrast to stimulatory effects on GABAergic PSC frequency during negative feedback, kisspeptin had no effect during positive one. Moreover, it has been suggested recently that the activation of GABA-B receptors in Gn-RH neurons from OVX + E adult female rats, may provide increased inhibitory tone during estrogen-negative feedback states that is attenuated by kisspeptin during positive feedback. The findings mentioned above suggest a kisspeptin role in regulating the hypothalamic amino acids secretion [63].

Another Gn-RH secretion regulator is leptin, a cytokine secreted by adipocytes, which plays an important role in the regulation of body weight. It has been suggested that an increase in serum leptin is associated with the onset of puberty. In previous studies, we have demonstrated that leptin administration increases Gn-RH release in female rats during sexual maturation [36]. We have also reported that leptin modifies GLU and GABA release via nitric oxide (NO) in peripubertal female rats [40]. Moreover, experimental evidence suggests that hypothalamic Kiss1 neurons operate as a central conduit delivering metabolic information onto the centers governing reproductive function, through a putative leptin-kisspeptin-Gn-RH pathway [53]. Smith et al.; [47] reported that around 40% of kisspeptin neurons in the mouse possess leptin receptors, this implicates kisspeptin neurons as a mediator of metabolic control of the hypothalamic-pituitary-gonadal axis and puberty. Considering previous findings, it may be proposed a pathway whereas leptin may stimulates Kiss1 and NO release which in turn may modify GABA and GLU tone leading to changes in Gn-RH secretion.

Recently, the hypothalamic Kiss1 system has been suggested as potential target for transmitting immune-mediated repression of the gonadotropic axis during acute inflammation. Studies of Castellano et al.; [4] have shown that LPS administration to adult male rats induced a dramatic fall in LH and testosterone. These findings were accompanied by a significantly decrease in kisspeptin-immunoreactivity in the arcuate nucleus, a key center for the neuroendocrine control of reproduction, suggesting that suppressed gonadotropic function following inflammatory challenges might involve a reduction in absolute responsiveness

to kisspeptin. Iwasa et al.; [21] have also reported that LPS injection decreased hypothalamic KiSS-1 mRNA expression as well as plasma LH levels in ovariectomized rats.

Weight loss and anorexia frequently accompany infection, probably secondary to cytokine release during endotoxemia shock [35]. Among several factors, the anorexigenic protein leptin is release during endotoxemia. Grunfeld et al.; [20] showed a significant stimulation of leptin secretion by LPS, confirming the existence of an acute effect of LPS on the adipocyte function. However, we have recently demonstrated, (observations not published), that repeated LPS administration during 3 days failed to modify leptin serum levels in peripubertal female rats suggesting in agreement with other authors tolerance phenomena of the adipocyte. In this sense, Chateaurd et al.; [5] provide evidence that the adipocyte may develop tolerance to repeated endotoxemia, as suggested by the finding that an acute stimulation of leptin secretion was observed only on the first experimental day, without modifications of its serum levels on day 3 and 5 after LPS administration. Therefore, it seems probable that under repeated LPS administration, leptin exerts its effect on early stages of endotoxemia.

Considering these findings, we may speculate that repeated LPS administration may induce a decrease in Kiss1 release which may modify GABAergic and glutamatergic tone altering Gn-RH secretion. Moreover, an augmented CRH output and IL-1β release may also be considered as other factors modifying amino acids release as was previously demonstrated [14, 17].

These changes at the hypothalamic level may be also responsible of the decrease of LH serum levels. Several studies have shown that LPS, administered peripherally or centrally to adult male rats decreases LH release in a significant manner [10, 38].

Another finding that showed the effect of repeated LPS administration on the axis is the delay of the day of vaginal opening and the decreased of serum estradiol levels. Estradiol synthesis and secretion are regulated by gonadotropins, which are under hypothalamic control. The decrease in LH release may impair estradiol ovarian synthesis. Moreover, it has been described that LPS has a direct effect on the ovary, Taylor et al. [50, 51] demonstrated that LPS administration to immature rats inhibited ovarian estradiol secretion in response to human chorionic gonadotropin. Vaginal opening in the female rodents is the initial sign of estrogenic rise that accompanies the onset of puberty and first ovulation [31]. In our animals vaginal opening occurs on day 32-33 of age. In present experiment, the decrease in estradiol level was reflected as it was expected, in the delay of the day of vaginal opening.

In summary, the onset of puberty is the result of reactivation of Gn-RH pulse generator which is under the inhibitory effect of several negative inputs [15, 19, 30, 55]. After removal of them, positive inputs known to stimulate Gn-RH release such as GLU, kisspeptin and leptin [39, 52, 58] among others, become operative and allows puberty to go on. In our research, we demonstrated that repeated LPS administration impairs reproductive axis during the peripubertal stage of life in female rats. We may hypothesize that LPS may induce a decrease in Kiss1 secretion which in conjunction with IL-1 β and CRH may alter the normal balance between the inhibitory and excitatory amino acids involved in Gn-RH regulation leading to a decrease in its release.

The inhibition of the reproductive capability in peripubertal female rats after repeated LPS treatment can be considered as part of all the effects of an activated immune system.

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