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Effect of di(2-ethylhexyl) phthalate on the neuroendocrine regulation of reproduction in adult male rats and its relationship to anxiogenic behavior: Participation of GABAergic system

S Carbone[1,2], OJ Ponzo[1], N Gobetto[1], YA Samaniego[1], R Reynoso[1], JA Moguilevsky[3] and RA Cutrera[2]

Abstract

The endocrine disruptor di-(2-ethylhexyl) phthalate (DEHP) is used in a variety of consumer products made with polyvinyl chloride and also in the manufacture of medical devices. DEHP disrupts reproductive tract development in an antiandrogenic manner and also may induce neurobehavioral changes. The aim of this study was to investigate the effects of chronic postnatal exposure to DEHP (30 mg/kg body weight/day, orally from birth to day 60) on the neuroendocrine regulation of the gonadal axis and its impact on the anxiety-like behavior in adult male rats, as well as the probable participation of the GABAergic system in these effects. DEHP produced a significant increase in plasmatic luteinizing hormone and follicle stimulating hormone, as well as significant testosterone decrease, accompanied with a decrease in hypothalamic gamma-aminobutyric acid (GABA) concentration. On the other hand, DEHP increased the anxiety-like behavior in the elevated plus maze test, evidenced by a significant decrease in the percentages of time spent in the open arms and the frequency in the open arm entries and a significant increase in the percentage of time spent in closed arms. Neuroendocrine and behavioral effects were reversed by GABA agonists, muscimol (2 mg/kg i.p.) and baclofen (10 mg/kg i.p.). In conclusion, chronic DEHP postnatal exposure induced a disruption in the neuroendocrine regulation of the testicular axis in young adult male rats, and this effect was correlated with an anxiety-like behavior. Since GABA agonists reversed these effects, the results suggest that GABA could participate in the modulation of reproductive and behavioral DEHP effects.

Keywords

Phthalate, gonadotropin, anxiety-like behavior, gamma-aminobutyric acid, baclofen, muscimol

Introduction

The endocrine disruptor (ED) di-(2-ethylhexyl) phthalate (DEHP) is used in a variety of consumer products made with polyvinyl chloride and also in the manufacture of medical devices.[1–3] The potential DEHP toxic risk in humans is high and increases during pregnancy, due to maternal transmission, and during breastfeeding through breast milk.[2,4] The higher exposure to phthalates in infants and children[4–6] is concerning, given that at this stage, the impairment of the normal developing would have an impact later

[1]Laboratorio de Endocrinologí, Departamento de Fisiologí, Facultad de Medicina, Universidad de Buenos Aires. Buenos Aires, Argentina.
[2]Laboratorio de Neurobiologí y Ritmos, Instituto de Fisiologí y Biofísica Bernardo Houssay (IFIBIO), Universidad de Buenos Aires and CONICET, Buenos Aires, Argentina.
[3]Facultad de Ciencias Médicas, Universidad Favaloro. Buenos Aires, Argentina.

Corresponding author:
OJ Ponzo, Laboratorio de Endocrinología, Departamento de Fisiología, Facultad de Medicina, Universidad de Buenos Aires. Paraguay 2155 7 Piso (C1121ABG), Ciudad de Buenos Aires, Argentina.
Email: oponzo@fmed.uba.ar

in adulthood. Exposure to DEHP early in life impairs the testis ability to secrete testosterone and causes disorders in male reproductive tract of animals (e.g. undescended testis, retained nipples, malformations in ventral prostate, seminal vesicle, and the epididymis).[7–10]

Epidemiological evidences have indicated a higher incidence of inborn genital malformations and spermatogenic dysfunction in boys born from women exposed to phthalates during pregnancy.[11,12] DEHP exerts its antiandrogenic effect by a direct action on gonads through different mechanisms (e.g. decreasing steroidogenesis-related factors and interacting with peroxisome proliferator–activated receptors).[7,13,14] Also, a possible central action on male reproductive axis was suggested.[15,16]

It has been reported that EDs could alter the homeostasis of the neuroendocrine system, initiating the disruptive action on the central nervous system, specifically at hypothalamus level.[17,18] DEHP exposure could lead to imbalance of the hypothalamic–pituitary–gonadal (HPG), thyroid, and adrenal axes in female rats.[19–21] In utero and lactational exposure to DEHP could disrupt testicular axis in pre and peripubertal rats, altering gonadotropin secretion and the hypothalamic concentration of neurotransmitters, such as gamma-aminobutyric acid (GABA).[15,16,22] It is known that GABA signaling pathway is mediated through $GABA_A$ and $GABA_B$ receptors,[23] which can also mediate the neuroendocrine and behavioral effects of testosterone.[24,25]

On the other hand, DEHP can also alter behavior by operating mainly on the brain regions that respond to gonadal hormones.[26] In fact, exposure to DEHP during development modifies aromatase activity in hypothalamus of male rats[27] and consequently the conversion of testosterone to estradiol, thus altering sexual differentiation of the brain and affecting anxiety-like behavior.[28] DEHP could act on anxiety in different ways depending on the treatment, age, and sex of the animals.[29–32] It has been reported that postnatal exposure to DEHP can induce anxiogenic effect in pre and peripubertal male rats but not in females in the same stages of sexual maturation.[33] Moreover, it has been proposed that the decrease in testosterone levels induced by postnatal exposure to DEHP could be one possible mechanism underlying DEHP anxiogenic-like behavior in immature male rats.[33]

It is known that testosterone and estradiol (the product of its aromatization) play a key role in the development of GABAergic system in the postnatal hypothalamus,[34] and also androgens and GABA are involved in the modulation of anxiety.[35,36]

On this basis, the aim of this study was to investigate the effect of chronic postnatal exposure to DEHP on the neuroendocrine regulation of the gonadal axis and its impact on anxiety-like behavior in adult male rats. In addition, our purpose was to evaluate the possible participation of the GABAergic system in DEHP effects.

Materials and methods

All animal procedures were performed following the protocols of the National Institute of Health—Guide for the Care and Use of Laboratory Animals. The approval to conduct the study was granted by the Institutional Committee for the Care and Use of Laboratory Animals (CICUAL: Comité Institucional para el Cuidado y Uso de Animales de Laboratorio) of the School of Medicine, Universidad de Buenos Aires.

Animals

Wistar rats used were provided by the Department of Physiology, School of Medicine, UBA, Argentina. Animals were kept under a controlled environment (temperature 22–24°C; lights on from 7.00 h to 19.00 h) and they had free access to food and filtered water, until the time of killing. All animals were fed with balanced food for laboratory rodents (Cooperation, ACA-16014007, Asociación de Cooperativas Argentinas—División de Nutrición Animal). The diet contained 15% of soy, but as the food used and the quantity of food intake by control and DEHP-treated groups were similar, we assumed that all animals were exposed to equivalent levels of food-borne phytoestrogens. Moreover, the same lots of diet were provided to animals from all groups at the same time, during the course of the study, to control across groups for possible variation in the diet content. We used ultrapure-filtered water (obtained from EDS-Pack, Millipore Merck, installed in the Milli-Q water system) that was presumed to be free of phthalates and other EDs. To minimize additional exposures to substances that may act as EDs, rats were housed in stainless steel cages with wood beddings and water was supplied in glass bottles.

Drugs and doses

DEHP (1 g/ml, >99% pure; Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, USA) was administered in drinking water. DEHP solution was

made up fresh daily by adding 200 µl of DEHP to 1 l of filtered water to reach a concentration of 0.2 mg/ml and sonicating for 30 min to ensure a permanent and homogenized solution. An oral route in DEHP administration was chosen intending to mimic best the most common route of human exposure to the ED. The estimated average DEHP dose of exposure was 30 mg/kg body weight (BW)/day, based on the daily intake of DEHP solution and the weight of the related animal. Liquid consumption was measured calculating the difference between the amount of liquid placed in the bottle every day and the remaining amount on the following day to assess the intake. It was assumed that all DEHP solution missing in the bottle had been consumed by the animals. Assessments did not contemplate possible leakage or evaporation of the solution or potential loss of DEHP activity during the 24-h period. No significant differences between the amount of liquid consumed by dams and pups receiving DEHP and those which did not receive this chemical were found. The dose was chosen based on prior studies published by us, in which we demonstrated alterations in the reproductive axis and in the behavior in immature male rats exposed to DEHP at a dose of 30 mg/kg BW/day but not in animals exposed to a lower dose (3 mg/kg BW/day).[15,22] For testing neurobehavioral changes in rodents, higher DEHP doses have been reported in the literature.[28–31]

Muscimol (5-aminomethyl-3-hydroxysoxazole (Sigma Chemical Co., St. Louis, Missouri, USA), a $GABA_A$ agonist, was administered at 2 mg/kg (i.p.) 90 min before the behavioral test. Baclofen (β-p-chlorophenyl-GABA (Sigma Chemical Co.), a $GABA_B$ agonist, was administered at 10 mg/kg (i.p.) 120 min before the behavioral test. The route of administration, dose, and time after injection to evaluate the effects of GABA agonists were selected according to previous studies.[37–39]

Experimental design

Pregnant dams were placed individually in metallic cages and upon delivery, pups were sexed; male pups were separated and distributed with one surrogate dam (n = eight male pups per dam). On postnatal day (PND) 1, surrogate dams with their male pups were randomly assigned into control (C) and DEHP exposure groups. Dam's exposure to DEHP began on PND 1 and continued until weaning. On PND 21, pups of each group continued receiving the same treatment until PND 60. On this day, control and DEHP-exposed male pups were randomly assigned to the following treatments: (1) C + S: controls that were given water and injected with saline; (2) DEHP + S: animals that were given DEHP and injected with saline; (3) C + M: animals that were given water and injected with muscimol; (4) DEHP + M: animals that were given DEHP and injected with muscimol; (5) C + B: animals that were given water and injected with baclofen; and (6) DEHP + B: rats that were given DEHP and injected with baclofen. After receiving the GABA agonist or the vehicle, animals were submitted to the behavioral test, and finally, all animals were killed by decapitation between 16:00 pm and 17:00 pm.

EPM test

The elevated plus maze (EPM) test is a widely used behavioral assay for rodents, and it has been validated to assess the antianxiety effects of pharmacological agents and steroid hormones and to define mechanisms underlying anxiety-related behavior.[40] The EPM apparatus consists of two open arms (10 × 50 cm), alternating in right angles with two closed arms (10 × 50 × 10 cm), delimiting a central area. The maze was elevated 50 cm above the floor. Before starting the test, animals were individually placed in a rectangular plastic glass area (40 × 40 cm) for 5 min in order to habituate them to the test environment. After that, the rats were placed in the central area of the maze, facing one of the closed arms, and were allowed to explore it for 5 min. The maze's arms were equally illuminated so that the animals did not perceive lighting differences. Behavioral tests were performed from 12:00 h to 14:00 h. We used 10% ethanol to clean each arm of the maze and to remove olfactory cues every time between trials. Each rat was tested only once. The animal's behavior was videotaped and the number of entries and the time spent in both, open and closed arms, were measured by an observer. The parameters measured were frequency of entries in the open arms (FEO), time spent in the open (TSO) and closed (TSC) arms, and number of total entries in both open and closed arms (TE). These parameters were calculated following a four-paw criterion; entry into the EPM arms was defined as the animal placing all four paws in that particular part of the maze. FEO, TSO, and TSC were expressed in percentages that were calculated as ([open arm entries] / [total number of entries] × 100), ([open arm time] / [total time] × 100), and ([closed arm time] / [total time] × 100), respectively, as described previously.[41] The EPM rests on the conflict between the tendency of rodents to explore a novel environment

and the natural aversion to open spaces. Based on this fact, the EPM test estimates the anxiety-like behavior of the animal by the reduction in %FEO and %TSO, as well as by increase in TSC. Also, the parameter of TE provides a built-in control measure for locomotor activity indicating hyperactivity or sedation.[42]

Hormonal assays

After killing, blood was collected from the trunks and the samples centrifuged for 10 min at 2500 r/min, and the serum separated and stored at −70°C for hormonal determination. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) serum were determined in duplicate using the double antibody radio immunoassay technique.[43] The material for this assay was provided by the National Hormone and Peptide Program of the National Institutes of Diabetes and Digestive and Kidney Diseases (Harbor-UCLA Medical Center, Torrance, California, USA). Results were expressed in nanograms per milliliter of serum, in accordance with the referenced preparation (rat LH RP-1 and rat FSH RP-1). All samples were analyzed in the same assay, and intra-assay coefficient of variation was 6%. Total serum testosterone concentrations were measured by a competitive immunoassay provided by VITROS, (Immunodiagnostic Products Testosterone Reagent Pack; Ortho Clinical Diagnostics by Johnson & Johnson Company, New Jersey, USA). The technique depends on competition between testosterone present in the sample with a horseradish peroxidase (HRP)-labeled testosterone conjugate for a limited number of binding sites on a biotinylated antibody (anti-testosterone). The bound HRP conjugate is measured by a luminescent reaction. The amount of HRP conjugate bound is indirectly proportional to the concentration of testosterone present. Duplicate volumes of 50 µl serum were used for each sample. Intra-assay coefficient of variation was 3.1%. Values were expressed as nanomoles per milliliter of serum.

Hypothalamic dissection and incubation

The anterior preoptic and medial-basal (APO-MB) hypothalamic areas were dissected, as previously described,[15,22] and each APO-MB hypothalamic area was placed in a chamber containing 500 µl of Earle's saline medium with glucose (1 mg/ml), pH: 7.4. Each chamber was incubated in a Dubnoff shaker at 37°C with constant shaking (60 cycles/min) under an atmosphere of 95% oxygen and 5% carbon dioxide. After 30 min of preincubation, the medium was removed by aspiration and discarded. Equal volume of fresh medium was immediately added to start incubation for 60 min. Then the incubation medium was collected and immediately frozen at −70°C for GABA determination.[44]

GABA measurement

GABA was determined in APO-MB hypothalamic areas by high-performance liquid chromatography after derivatization with phenyl isothiocyanate and ultraviolet detection at 254 nm, as previously described.[45] The drugs used did not interfere with the derivatization process. Intra-assay coefficient of variation was 4.0%. The detection limit was 5 pmol. The mobile phase consisted of 0.57 M sodium acetate buffer (pH: 6.5) containing 10% acetonitrile (Sintorgan, Buenos Aires, Argentina). GABA standard was purchased from Sigma Chemical Co. The results are expressed as pmol/100 µl of medium.

Statistical analysis

Results were expressed as the mean ± standard error of the mean. It must be noted that our statistical analysis was based on individual offspring numbers. As we did not keep track of the surrogate dam for each of the pup, the surrogate dam was not used as the unit. Therefore, we did not consider the potential effects of the surrogate dam in the statistical analysis, as pups were exposed to this chemical through her milk. Hormones and GABA were analyzed non-parametrically with the Kruskal–Wallis test followed by Dunn's multiple comparisons test. The behavioral data were analyzed by one-way analysis of variance with the post hoc Tukey–Kramer multiple comparisons test. Correlation analysis was performed with the non-parametric Spearman's rank correlation coefficient, which analyzes the statistical dependence between two variables. In all the cases, the statistical software used was GraphPad InStat 3 (version 3.00 for Windows 95, GraphPad Software, San Diego CA, USA). and a difference was considered statistically significant when $p < 0.05$.

Results

Effect of chronic postnatal exposure to DEHP and GABA agonists on neuroendocrine regulation

As can be seen in Figure 1, DEHP increased both plasmatic LH ($p < 0.01$) and FSH ($p < 0.01$) compared to the control group. As expected, $GABA_A$ and $GABA_B$ agonists decreased gonadotropin levels with

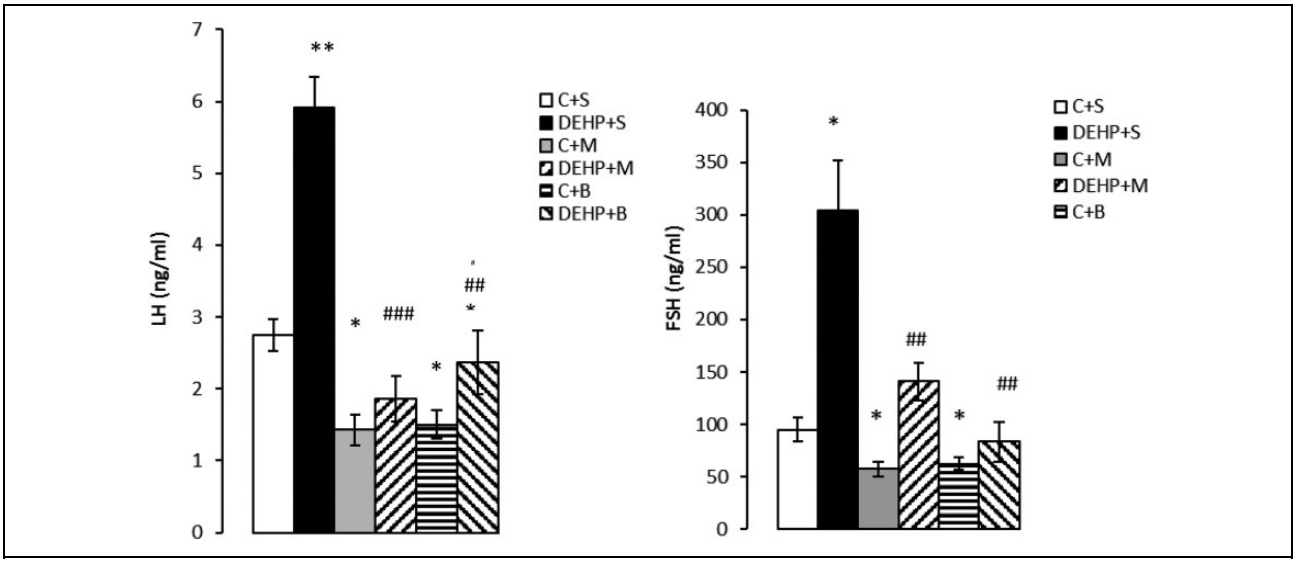

Figure 1. Effect of chronic postnatal exposure to DEHP and GABA agonists on serum LH and FSH in young adult male rats. Each value represents the mean ± SEM of eight animals. *$p < 0.05$ versus C + S; **$p < 0.01$ versus C + S; #$p < 0.05$ versus DEHP + S; ##$p < 0.01$ versus DEHP + S; ###$p < 0.001$ versus DEHP + S. C: control; S: saline; DEHP: di-(2-ethylhexyl) phthalate; M: muscimol; B: baclofen; GABA: gamma-aminobutyric acid; LH: luteinizing hormone; FSH: follicle stimulating hormone; SEM: standard error of the mean.

respect to controls ($p < 0.05$). The stimulatory effect of DEHP was reversed by muscimol, producing a decrease of both plasmatic LH ($p < 0.001$) and FSH ($p < 0.01$). Similarly, baclofen reversed the increase produced by DEHP in plasmatic LH and FSH ($p < 0.01$), leading their values to be similar to those measured in the control group.

These changes were accompanied by a decrease in plasma levels of testosterone in animals exposed to DEHP ($p < 0.01$). This effect was reversed by both muscimol and baclofen ($p < 0.01$), reaching testosterone values similar to the control group (Figure 2).

As can be seen in Figure 3, DEHP exposure significantly decreased hypothalamic GABA concentration ($p < 0.01$) compared to the control group. Muscimol and baclofen increased GABA concentration ($p < 0.05$) and also reversed DEHP effect ($p < 0.01$).

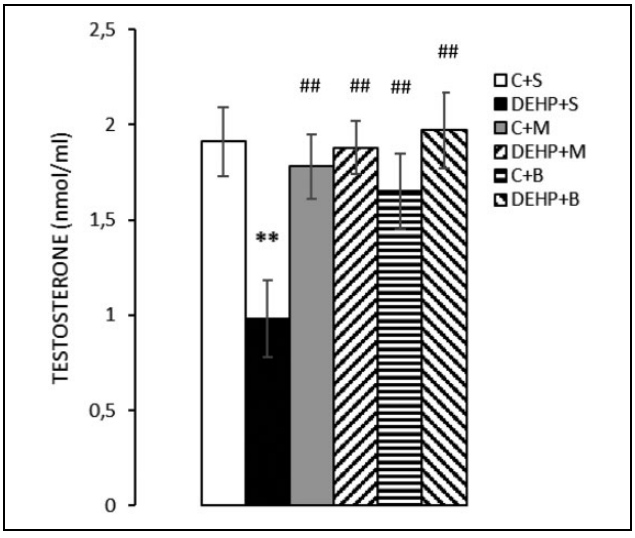

Figure 2. Effect of chronic postnatal exposure to DEHP and GABA agonists on serum testosterone in young adult male rats. Each value represents the mean ± SEM of eight animals. **$p < 0.01$ versus C + S; #$p < 0.05$ versus DEHP + S; ##$p < 0.01$ versus DEHP + S. C: control; S: saline; DEHP: di-(2-ethylhexyl) phthalate; M: muscimol; B: baclofen; GABA: gamma-aminobutyric acid; SEM: standard error of the mean.

Effect of chronic postnatal exposure to DEHP and GABA agonists on anxiety-like behavior

Significant differences between treatments were found in %TSO (F (5, 42) = 16.367, $p < 0.0001$), %FEO (F (5, 42) = 8.385, $p < 0.0001$), and %TSC (F (5, 42) = 8.547, $p < 0.0001$). On the other hand, no significant differences in TE were found between groups (F (5, 42) = 0.8025, $p = 0.5543$).

As shown in Figure 4, DEHP significantly decreased %TSO ($p < 0.05$) and %FEO ($p < 0.01$) and also increased %TSC ($p < 0.01$). Rats treated only with muscimol or baclofen did not show significant differences in %TSO, %FEO, and %TSC compared to the control group. However, animals treated with baclofen alone exhibited less anxiety status compared

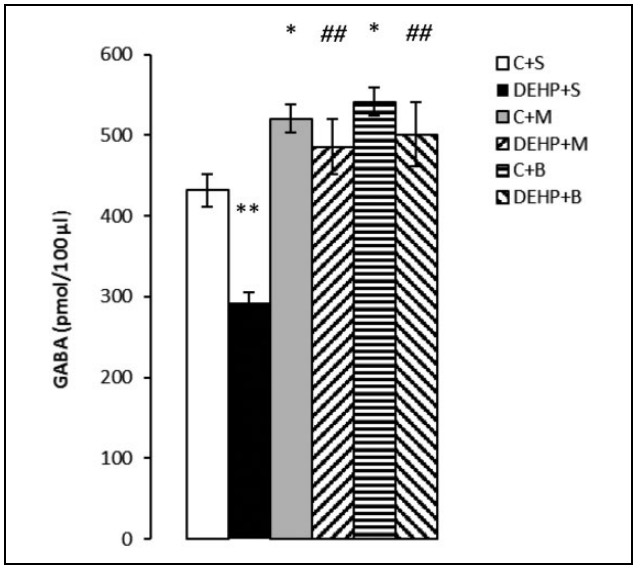

Figure 3. Effect of chronic postnatal exposure to DEHP and GABA agonists on GABA concentration in young adult male rats. Each value represents the mean ± SEM of eight animals. *$p < 0.05$ versus C + S; **$p < 0.01$ versus C + S; ##$p < 0.01$ versus DEHP + S. C: control; S: saline; DEHP: di-(2-ethylhexyl) phthalate; M: muscimol; B: baclofen; GABA: gamma-aminobutyric acid; SEM: standard error of the mean.

to DEHP + S group (%TSO, $p < 0.01$; %FEO, $p < 0.01$; and %TSC, $p < 0.001$). The same results can be seen with muscimol alone (%TSO, $p < 0.05$; %FEO $p < 0.05$; and %TSC, $p < 0.05$). $GABA_A$ agonist muscimol co-administered to DEHP reversed the effects (%TSO, $p < 0.05$; %FEO, $p < 0.01$; and %TSC, $p < 0.001$), leading these parameters to be similar to values detected in unexposed rats. Similarly, $GABA_B$ agonist baclofen counteracted DEHP effects (%TSO, $p < 0.001$; %FEO, $p < 0.001$; and %TSC, $p < 0.001$), leading the %FEO and %TSC to be similar to values measured in the control group. Moreover, a significant ($p < 0.001$) increase in %TSO with respect to unexposed rats was detected.

Gonadotropin levels showed a significant ($p < 0.05$) inverse correlation with anxiety-like behavior parameters (%TSO, %FEO, and %TSC), whereas testosterone and GABA correlated significantly ($p < 0.05$ and $p < 0.001$, respectively) in a positive way (Table 1).

Discussion

In the present work, we investigated the effects induced by chronic postnatal exposure to DEHP on the neuroendocrine control of testicular axis in young adult male rats. Given that testosterone can modulate anxiety-related behavior[24,25] and that DEHP has anti-androgenic effect,[7] we also studied the probable impact of DEHP on this behavior. Further, we investigated whether the GABA system may be involved in reproductive neuroendocrine changes and in the anxiety-like behavior induced by DEHP.

Effect of chronic postnatal exposure to DEHP and GABA agonists on neuroendocrine regulation

Our results demonstrated a significant increase in plasmatic LH and FSH levels as well as a decrease in serum testosterone level in young adult male rats exposed postnatally to DHEP. Previously, similar results were observed in prepubertal male rats exposed to DEHP during gestational and lactational periods.[22] We also showed that postnatal exposure to DEHP significantly decreased the concentration of GABA in APO-MB hypothalamic areas. It is known that GABA plays an important role in the central regulation of the HPG axis during sexual maturation and the adulthood.[46] In male rats, GABA has an inhibitory effect, focusing its actions in the APO-MB hypothalamus, where GnRH neurons are located.[47] Therefore, the decrease in GABA detected in our experiments could indicate that DEHP exposure was able to induce an overstimulation in the hypothalamus, by decreasing GABA inhibitory tone on GnRH, which in turn could stimulate LH and FSH secretion by the pituitary. This could be one of the possible mechanisms involved in the increase in plasmatic gonadotropin levels observed.

In accordance with other authors,[16,48–50] results showed a decrease in serum testosterone provoked by DEHP. It is known that DEHP can act in testis, decreasing the steroidogenic capacity of Leydig cells to synthesize testosterone.[48,49] In this manner, DEHP could affect in turn the negative feedback mechanism at the pituitary gland and predominantly at the hypothalamus, via the neurotransmitters that are responsive to steroids.[51] Therefore, this could be other probable mechanism to explain the changes of GABA in APO-MB hypothalamic areas and the enhancement of LH detected in our experiments.

We also detected a significant increase in plasmatic FSH levels. It has been reported that phthalate esters increase FSH and decrease inhibin B in correlation with Sertoli cell toxicity.[52] In this way, DEHP could alter the negative feedback mechanism on FSH. Therefore, the significant increase in FSH induced by chronic exposure to DEHP could be caused by the stimulatory effect of DEHP on hypothalamic–

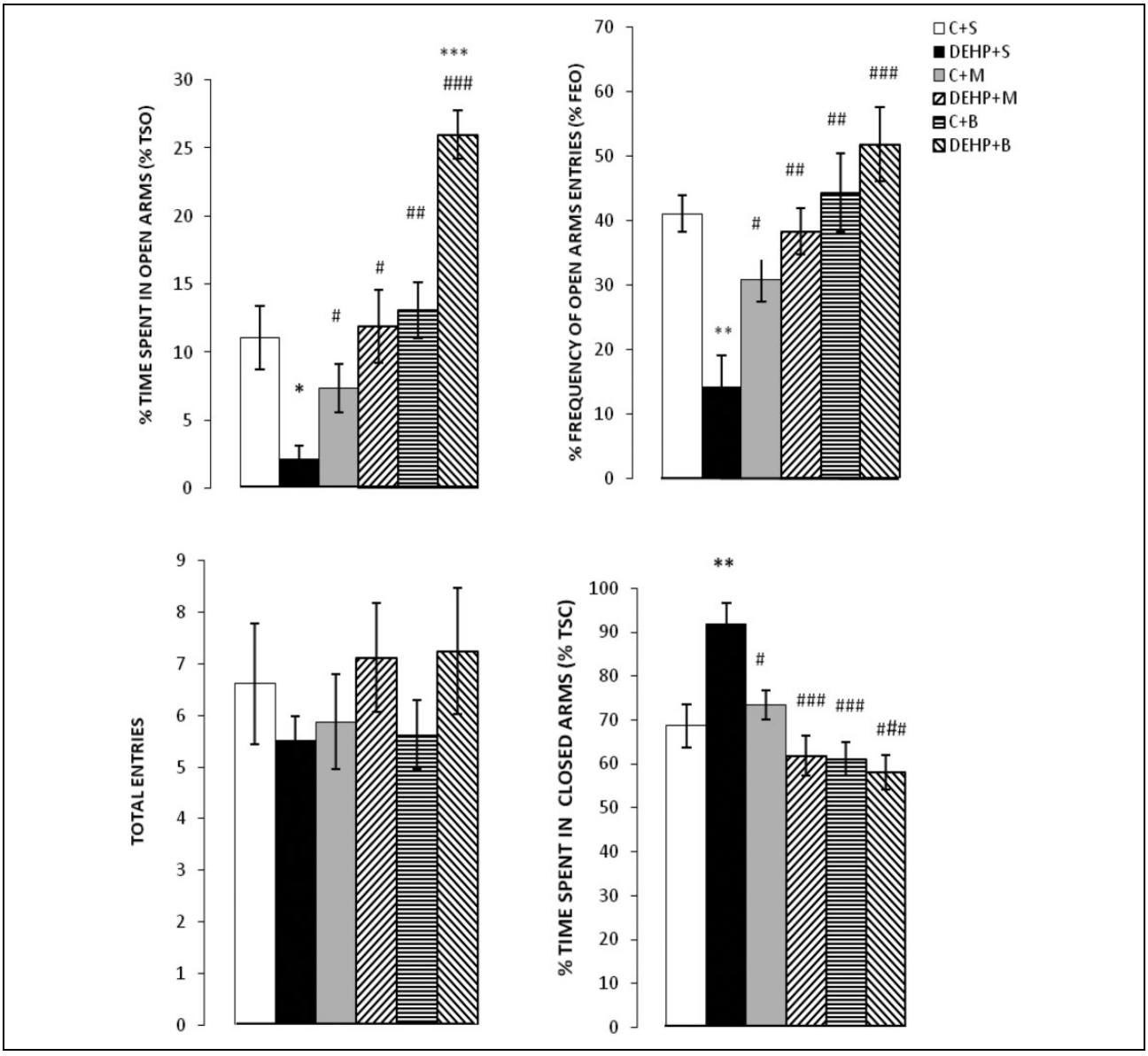

Figure 4. Effect of chronic postnatal exposure to DEHP and GABA agonists on anxiety-like behavior in young male rats. Each value represents the mean ± SEM of eight animals per group. One-way ANOVA: %FEO (F (5, 42) = 8.385, $p < 0.0001$); %TSO (F (5, 42) = 16.367, $p < 0.0001$); %TSC (F (5, 42) = 11.586 8.561, $p < 0.0001$); and TEs (F (5, 42) = 0.8025, $p = 0.5543$). Tukey–Kramer test: $^{*}p < 0.05$ versus C + S; $^{**}p < 0.01$ versus C + S; $^{\#}p < 0.05$ versus DEHP + S; $^{\#\#}p < 0.01$ versus DEHP + S; $^{\#\#\#}p < 0.001$ versus DEHP + S. C: control; S: saline; DEHP: di-(2-ethylhexyl) phthalate; M: muscimol; B: baclofen; GABA: gamma-aminobutyric acid; SEM: standard error of the mean; ANOVA: analysis of variance; FEO: frequency of entries in the open arms; TSO: time spent in the open arm; TSC: time spent in the closed arm; TE: total entry.

pituitary axis and also by a reduction of the testosterone down regulation of FSH, possibly due to the direct effect of phthalates in testis.

It is known that within the hypothalamus, GABA acts predominantly as an inhibitory transmitter through $GABA_A$ and $GABA_B$ receptors, which mediate inhibitory postsynaptic transmission in GnRH neurons.[53] Therefore, we examined whether activation of the GABAergic system with selective agonists of $GABA_A$ (muscimol) and $GABA_B$ (baclofen) receptors might reverse neuroendocrine effects of DEHP. Our results showed that GABA agonists were able to counteract the increase in plasmatic LH and FSH levels as well as the decrease in hypothalamic GABA induced by DEHP, thus decreasing the inhibitory tone of GABA in the hypothalamic–pituitary

Table 1. Correlation between hormones and GABA with parameters of anxiety-like behavior in young adult male rats exposed to DEHP.[a]

	%TSO	%FEO	%TSC	TE
LH	$\rho = -0.8713$; $p = 0.0072$[b]	$\rho = -0.7952$; $p = 0.0218$[b]	$\rho = 0.7545$; $p = 0.0368$[b]	$\rho = -0.0566$; $p = 0.8820$
FSH	$\rho = -0.7807$; $p = 0.029$[b]	$\rho = -0.7306$; $p = 0.0450$[b]	$\rho = 0.7381$; $p = 0.0458$[b]	$\rho = -0.1151$; $p = 0.7033$
T	$\rho = 0.8051$; $p = 0.0218$[b]	$\rho = 0.85031$ $p = 0.0100$[c]	$\rho = -0.9048$; $p = 0.046$[b]	$\rho = -0.0818$; $p = 0.8401$
GABA	$\rho = 0.835$; $p = 0.0100$[c]	$\rho = 0.8264$; $p = 0.0154$[b]	$\rho = -0.7381$; $p = 0.050$[b]	$\rho = -0.0962$; $p = 0.8401$

ρ: Spearman correlation coefficient; DEHP: di-(2-ethylhexyl) phthalate; LH: luteinizing hormone; FSH: follicle stimulating hormone; T: testosterone; GABA: gamma-aminobutyric acid; %TSO: percentage of time spent in the open arms; %FEO: percentage of frequency of entries in the open arms; %TSC: percentage of time spent in the closed arms; TE: number of total entries in both open and closed arms.

[a] Correlation is significant at $p < 0.05$ level.

[b] $p < 0.05$.

[c] $p < 0.01$.

axis. It is difficult to explain how muscimol and baclofen can reverse GABA levels in DEHP-exposed animals. It has been suggested that neonatal androgen exposure plays an important role in the expression and/or activation of different GABA receptor subtypes in hypothalamus of prepubertal male rats.[54] On this basis, we think that probable changes in GABA receptors induced by DEHP anti-androgenic action during sexual maturation could be counteracted by GABA receptor agonists, by inducing receptors activation and changes in GABA levels.

In summary, our findings showed that chronic postnatal exposure to DEHP disrupts the hypothalamic–pituitary–testicular axis in young adult male rats, and GABA agonists can reverse the neuroendocrine effects of this ED (see Figure 5).

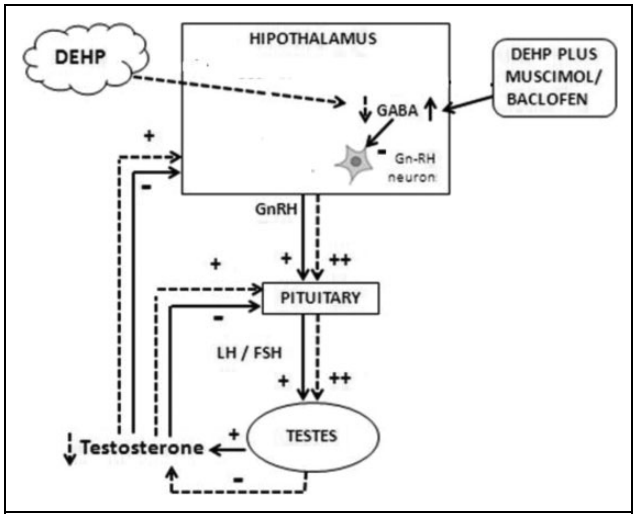

Figure 5. Schematic description of how hypothalamic–pituitary–testicular axis of young adult male rats could be altered by postnatal chronic DEHP exposure and the probable effects of GABAergic system activation by muscimol and baclofen on neuroendocrine disruption induced by the chemical. The solid line represents the normal operation of testicular axis and also the effect of GABA agonist in DEHP-exposed rats. The broken line indicates the neuroendocrine disruption induced by DEHP. DEHP: di-(2-ethylhexyl) phthalate; GABA: gamma-aminobutyric acid.

Effect of chronic postnatal exposure to DEHP and GABA agonists on anxiety-like behavior

It is known that multiple interactions between different endogenous substances, such as hormones, neurosteroids, and neurotransmitters, can play an important role in the genesis of anxiety.[55] The second objective of this work was to study the impact of disruption of the reproductive axis induced by DEHP on anxiety-like behavior in adult male rats and the possible reversion of this effect by GABA agonists. Our results showed an anxiogenic effect of DEHP, as evidenced by a significant increase in TSC and a decrease in both TSO and FEO percentages in the EPM test. These results were consistent with the findings reported in immature rats and mice exposed perinatally to DEHP (10, 30, 50, and 200 mg/kg).[28,33] On the contrary, Liang et al. and Park et al. have not found significant changes in anxiety-like behavior of adult rats after sub-chronic exposure to DEHP at high doses (150, 450, and 1350 mg/kg) and in adult mice exposed at doses of 60, 80, and 540 mg/kg.[29,30] Similarly, Tang et al. have not found significant differences using the open field test to assay anxiety-like behavior of mice treated with DEHP (500 mg/kg for 10 days).[56] In addition, in ours experiments, the increase in anxiety induced by DEHP correlated inversely with the decrease in GABA ($p < 0.01$) and testosterone ($p < 0.05$) and positively with gonadotrophin levels ($p < 0.05$).

Although the animals treated only with muscimol or baclofen did not show significant changes in anxiety-like behavior, these agonists reversed the anxiogenic effect induced by DEHP, leading the percentages of TSO, FEO, and TSC to be similar to those detected in the control group. Our results in animals injected only with muscimol without DEHP are in accordance with a previous study, reporting that muscimol (1 mg/kg i.p.) does not produce anxiolytic effect in adult rats under physiological conditions in the EPM test.[39] Similarly, the results in rats treated only with baclofen are in agreement with that reported by other authors.[57] It is important to note that baclofen is able to induce sedation, hypothermia, and muscle relaxation which may interfere with its use in behavioral tests related to anxiety.[58] Considering that TE provides an approximate measure for the locomotor activity, we believe that the lack of changes in this parameter could indicate that the side effects of baclofen (e.g. sedation) do not affect the anxiety-like behavior observed in animals exposed to DEHP and treated with this GABA agonist.

In short, the EPM test in young adult male rats chronically exposed to DEHP from birth showed an increase in anxiety-like behavior, which was counteracted by GABA agonists. This effect was not observed in animals not exposed to DEHP.

Our work did not include an analysis of probable histological alterations that could be associated with changes in hypothalamic GABA system, as well as the study of the interaction with other neurotransmitters involved in the neuroendocrine regulation of the gonadal axis and behavior. More experiments are necessary to evaluate these issues.

Conclusions

Our results show that chronic postnatal exposure to DEHP was able to disrupt the neuroendocrine regulation of the testicular axis in young adult male rats. This effect was correlated significantly with its antiandrogenic action and with an increase in anxiety-like behavior. We also demonstrate that GABA agonists, muscimol and baclofen, can reverse DEHP neuroendocrine reproductive effect, as well as its anxiogenic action, supporting the notion that GABAergic system may be one of the neurotransmitter systems that participates in DEHP effects. More experiments are needed to investigate, in particular, the related mechanisms.

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