Effects of early life overnutrition and hyperandrogenism on spatial learning and memory in a rat model of polycystic ovary syndrome

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Abstract

Polycystic Ovary Syndrome (PCOS) is a complex disorder characterized by endocrine and metabolic abnormalities such as obesity and insulin resistance. PCOS is also associated with psychiatric disorders and cognitive impairment. The animal model of PCOS was induced by treating rats with 5α-dihydrotestosterone (5α-DHT) and additionally modified to induce adiposity by litter size reduction (LSR). Spatial learning and memory were assessed using the Barnes Maze test, and striatal markers of synaptic plasticity were analyzed. Striatal insulin signaling was estimated by the levels of insulin receptor substrate 1 (IRS1), its inhibitory phosphorylation at Ser307, and glycogen synthase kinase-3α/β (GSK3α/β) activity. Both LSR and DHT treatment significantly decreased striatal protein levels of IRS1, followed by increased GSK3α/β activity in small litters. Results of the behavioral study showed that LSR had a negative effect on learning rate and memory retention, whereas DHT treatment did not induce impairment in memory formation. While protein levels of synaptophysin, GAP43, and postsynaptic density protein 95 (PSD-95) were not altered by the treatments, DHT treatment induced an increase in phosphorylation of PSD-95 at Ser295 in both normal and small litters. This study revealed that LSR and DHT treatment suppressed insulin signaling by downregulating IRS1 in the striatum. However, DHT treatment did not have an adverse effect on learning and memory, probably due to compensatory elevation in pPSD-95-Ser295, which had a positive effect on synaptic strength. This implies that hyperandrogenemia in this setting does not represent a threat to spatial learning and memory, opposite to the effect of overnutrition-related adiposity.

Keywords: polycystic ovary syndrome, dihydrotestosterone, postnatal overnutrition, Barns maze test, striatum, insulin resistance, synaptic plasticity

Introduction

Polycystic Ovary Syndrome (PCOS) is a complex disorder characterized by both endocrine and metabolic disturbances (Conway et al., 2014). Although it is primarily recognized as a hyperandrogenic state, with obesity as a comorbid condition, a significant proportion of women with PCOS have insulin resistance and hyperinsulinemia (Vazquez et al., 2015). It is known that PCOS is characterized by a vicious cycle between excess androgens and insulin resistance, where hyperandrogenemia favors the development of abdominal obesity and insulin resistance. In turn, hyperinsulinemia further stimulates androgen excess (Azziz et al., 2006). Current literature data suggest that women with PCOS have poor cognitive performance on tests of verbal memory, executive function, and visuospatial ability (Perovic et al., 2022). It was noted that the cognitive functions of women with PCOS are impaired due to changes in hormone levels, such as testosterone and insulin, with a general notion that elevated testosterone in those women induces subtle cognitive impairments and that antiandrogen treatments improve several aspects of cognitive performance (Barnard et al., 2007; Barry et al., 2018). However, studies in healthy (Burkitt et al., 2007) and postmenopausal women (Davison et al., 2011) showed that testosterone administration can also have positive effects on cognitive functions. Animal studies on rodents revealed that androgens have beneficial effects on cognition in aged female mice (Benice and Raber, 2009b), as well as in aged and adult male rats (Bimonte-Nelson et al., 2003; Vazquez-Pereyra et al., 1995). Yet, a detailed survey of the literature showed that data about the effects of hyperandrogenism on cognitive functions in humans and rodents, during early life, childhood, or adolescence are still missing.

Additionally, the negative effect of obesity on learning and memory was recognized, with decreased insulin sensitivity identified as a key link between obesity and impaired cognitive functions (Stingl et al., 2012; Wang et al., 2016). Insulin in different brain regions, besides affecting metabolic control, is also involved in synaptogenesis and neuronal plasticity, while brain insulin resistance is associated with cognitive dysfunction (Lee et al., 2016). Studies of the influence of obesity on diverse aspects of memory formation (Heyward et al., 2012; McNay et al., 2010; Stranahan et al., 2008) have pointed out that brain insulin resistance plays a significant role in spatial memory impairment (McNay et al., 2010). There are several mechanisms of insulin resistance in the cells (Pirola et al., 2004) including changes in insulin receptor, insulin receptor substrates (IRSs) and its phosphorylated forms, and downward signaling pathways

regulated by phosphatidylinositol-3-kinase (PI3K)/AKT, GSK3α/β, and MAPK/ERK1/2 (Riehle and Abel, 2016). However, downregulation of IRS-1 protein level was accepted as an additional mechanism for modulation of the insulin signaling (Mayer and Belsham, 2010).

In this study, we used a well-established animal model of PCOS induced by treatment of young rats with 5α-dihydrotestosterone (5α-DHT). It has been previously confirmed that continuous postnatal treatment of female rats with 5α-DHT induces reproductive and metabolic disturbances that faithfully mirror PCOS in humans (Manneras et al., 2007; Walters, 2016). In our model, DHT-treated animals were acyclic and in the diestrous phase of the estrous cycle, with leukocytes dominating the vaginal smears. Masses of ovaries and uteri were significantly decreased and ovaries were dominated by atretic follicles and lacked corpora lutea (Mićić et al., 2022; Nikolic et al., 2015). We additionally challenged the model with early postnatal overnutrition, gained by litter size reduction, to add the factor of prepubertal obesity to the model. Both factors and their combination have been shown to be significant for the development of systemic insulin resistance including hyperinsulinemia and increased HOMA index (Mićić et al., 2022).

In order to assess spatial learning and memory capacity, the Barns Maze test was performed. Since research on sex differences in learning and memory processes has revealed that female rats mainly use a striatum-dependent response learning strategy during spatial navigation (Hawley et al., 2012) focus of our investigation was on the striatum, which has been recently accepted as a key player in flexible goal-directed behavior (Gahnstrom and Spiers, 2020; Rusu and Pennartz, 2020). Thus, the aim of the present study was to evaluate striatal insulin signaling and its contribution to spatial learning and memory parameters, as well as the underlying markers of synaptic plasticity, synaptophysin, growth-associated protein 43 (GAP43), postsynaptic density protein 95 (PSD-95), and its phosphorylated form (pPSD-95-Ser295) in the aforementioned rat model of PCOS.

Materials and methods

Animals and treatment

In this study, all litters consisted of female Wistar rat pups in order to avoid the effect of sex litter composition on the reproductive or behavioral characteristics of the animals (Brain and Griffin, 1970; Monclus et al., 2014). Moreover, to minimize the impact of litter effects and litter-

to-litter variations female pups from 9 litters were randomly allocated to 7 litters of different sizes on the second postnatal day. Pups were grouped into small litters (SL, three pups per litter) and normal litters (NL, 10 pups per litter) as described previously (Mićić et al., 2022). Litter size manipulation was used to induce early postnatal overnutrition in the SL group. On the 22nd postnatal day, pups were separated from lactating dams, randomly divided and implanted subcutaneously in the back of the neck with hormonal pellets containing 7.5 mg 5α-DHT (Innovative Research of America, USA) or placebo pellets lacking bioactive substance (Innovative Research of America, USA). Four experimental groups were made (n=6 animals per group): animals from normal litters implanted with placebo pellets (NL-Placebo); animals from normal litters implanted with 5α-DHT pellets (NL-DHT); and corresponding two groups with animals from small litters (SL-Placebo and SL-DHT). After pellet implantation, animals were housed three per cage, under standard 12 h light/dark cycle (lights on at 7:00 a.m.), and constant temperature (22 ± 2°C) and humidity. Animals had *ad libitum* access to standard laboratory chow (Veterinary Institute "Subotica", Serbia) and tap water. At the end of 90 day treatment, rats were sacrificed by rapid decapitation with a guillotine (Harvard Apparatus, Holliston, MA, USA) in the diestrus phase of the estrus cycle. Visceral adipose tissue (VAT, consisting of retroperitoneal and perirenal depots) was immediately excised and weighed. The protocols were compliant with the guidelines of the Serbian Laboratory Animal Science Association (SLASA) and EEC Directive 2010/63/EU for the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Siniša Stanković", University of Belgrade (No. 01-02/19).

Behavioral tests

Apparatus and testing room

Spatial learning and memory were assessed with the Barnes maze, as described (Gawel et al., 2019). This apparatus consists of a rotatable circular wooden panel (120 cm in diameter x 1 cm) pasted with grey a water-tight coating, mounted on a stand 85 cm above the ground. The platform has 18 evenly spaced holes (10 cm in a diameter), whose upper rims are 5 cm from the edge of platform. The holes may be closed from below, creating 17 false-bottomed holes and one escape hole leading to removable black acrylic escape box (27×18×12 cm) under the circular

platform. An opaque cylinder (25 cm in a diameter, 20 cm high) was used as a start box for placing animals at the center of the maze at the beginning of each trial. The natural rat aversion towards open space was emphasized by the presence of three equally positioned LED floodlights, 1 m above the edges of the circular platform, which resulted in an average light intensity of about 1000 lux at the level of the platform. The platform was surrounded with a white curtain 60-80 cm from its edge, where four high contrast spatial cues were fixed at the level up to 30 cm above the maze surface (yellow cross, red triangle, violet quadrant, orange circle), avoiding placing of cues directly behind the escape hole. Behavior was recorded by an overhead HiRes Web-camera positioned 1.8 m above the center of the platform. Video files were captured and saved for later scoring.

Experimental procedure

Rats were acclimated to the testing room for 1 h before starting each experiment. The Barnes maze consisted of three phases: habituation (Day 0), training (Days 1-4), and probing (Day 5). To avoid the effects of cyclic sex steroids fluctuations on different phases of the learning process, all rats began training at the same phase of estrus cycle (diestrus). After each rat was tested the whole maze was cleansed with 30% ethanol, and maze was rotated to avoid intra-maze odor cues. To reduce anxiety, rats were allowed to become familiar with their surroundings through habituation. This was performed for 3 min 24 h before the first acquisition trial, during which the rat was allowed to freely explore it. They were gently guided into the escape box afterwards, and allowed to stay there for 1 min before being returned to home cage. During the training phase, rats learned to find escape hole. They were trained in two trials (separated by 2-3 hours) per day for four consecutive days. Each animal started its trial under the central start box for 30 seconds, and after it was removed, rat was allowed to explore the maze for up to 3 min. If the rat reached the escape box during this period, it could explore it for 1 min, before being returned to its home cage. If the rat did not find the safe shelter within 3 min, it was guided slowly by the experimenter to the escape box and was allowed to stay there for 1 min before being returned to the home cage. On Day 5, rats were tested for remembering the location of the escape hole. During the 90 seconds of the probe trial, the previous escape hole and all other holes were closed.

The main parameter registered during Days 1-5 was latency, defined as a period of time from the beginning of the trial until the moment when the animal entered the escape box (during the training phase), or when animal by its head reached the closed escape hole for the first time (during the probe trial). On Day 5, additional parameter representing memory retention was registered: the percent of time spent in the Target zone (defined as the upper half of the maze sextant that contained the former escape and two adjacent holes). This parameter was calculated in order to diminish the dissimilarity in the mobility (total distance traveled and/or duration of immobility) among the groups, which could have effect on latency values, and thus represents more sensitive measure of performance than latency *per se*. Additional parameters: total errors (number of false holes visited before correct hole), hole deviation score (number of holes between first approached and the correct one) and assessment of search strategy (random, serial or direct) on the Day 5, were also estimated.

The general locomotion, reflecting the locomotor activity of rats on Day 5, was detected by the distance traveled and the total time of immobility during the 90 seconds. Actual speed during mobility phase was calculated as adjusted speed (distance traveled/mobility time).

Initial analyses of the behavioral parameters were performed by AnyMaze software (ANY-maze Video Tracking System 4.30, Stoelting Co., USA). The experiment was conducted by an experimenter who was blind to the experimental conditions and study hypotheses.

Preparation of whole cell extract

After decapitation, brains were removed and striata were precisely dissected. Brains were removed from the skull and rinsed in ice-cold 0.9% NaCl to remove excess surface blood. Brains were placed on ice with the dorsal side on a glass plate covered with filter paper. After cutting off the olfactory bulb, the second incision was made in the section containing the frontal cortex. The ventral and dorsal striatum of two striatal halves were dissected along the natural boundaries from the genus corpus callosum and adjacent capsula externa, and from the septum. All striatal parts of one brain were collected, frozen in liquid nitrogen, and stored at -80°C until use.

To obtain whole cell protein extracts, tissues were homogenized in 10 vol. (w/v) of ice-cold RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet NP40, 0.1% SDS, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 2 mM DTT with protease and phosphatase inhibitors) using a glass/teflon (Potter-Elveiheim) homogenizer. The homogenates were sonicated on ice (3 x 5 s at

10 MHz, Hielscher Ultrasound Processor) followed with extraction for 30 minutes at 0^{o}C prior to 30 min centrifugation at 20000 x g. The resulting supernatants were stored at -70^{o}C.

Western blot analysis

Concentration of isolated proteins was determined by Lowry method, using bovine serum albumin (BSA) as a standard. The samples were boiled in an equal volume of 2x Laemmli buffer for 5 minutes, and 10 to 50 µg of proteins were subjected to electrophoresis on 8% or 10% sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidenedifluoride membranes (Immobilon-P, Merck, USA). The membranes were blocked by phosphate-buffered saline (PBS, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 2.7 mM KCl, 0.14 M NaCl, pH 7.2) containing 5% non-fat dry milk or 2% bovine serum albumin at room temperature for one hour, and then incubated overnight at 4°C with following primary rabbit polyclonal antibodies: anti-PSD95 (#2507s, Cell Signaling; 1:1000), anti-phospho-PSD95-Ser295 (#45737s, Cell Signaling; 1:1000), anti-GAP-43 (sc-10786, Santa Cruz Biotechnology; 1:15000), anti-IRS1 (#2382s, Cell Signaling; 1:1000), anti-phospho-IRS1-Ser307 (ab5599, Abcam; 1:500), anti-AKT (#9272s, Cell Signaling; 1:1000), anti-phospho-AKT-Ser473 (#4060s, Cell Signaling; 1:1000), anti-β-actin (PA1-183, Thermo Fisher Scientific; 1:2000) and anti-GAPDH (#2118, Cell Signaling; 1:10000) which were used as an equal loading controls. Anti-synaptophysin (MA5-14532, Thermo Fisher Scientific; 1:200) was rabbit monoclonal antibody, while anti-GSK3α/β (sc-7291, Santa Cruz Biotechnology; 1:1000), anti-GSK3α-Ser21 (sc-365483, Santa Cruz Biotechnology; 1:1000), and anti-GSK3β-Ser9 (sc-373800, Santa Cruz Biotechnology; 1:1000) were mouse monoclonal antibodies. After extensive washing, membranes were incubated for 90 min with rabbit horseradish peroxidase conjugated secondary antibody (#7074, Cell Signaling; 1:2000). The immunoreactive proteins were visualized by the chemiluminiscence method by iBright FL1500 Imaging System and quantitative analysis was performed using iBright Analysis Software (Thermo Fisher Scientific, USA).

Statistical analyses

All data are given as mean ± standard error of the mean (SEM). Results from physiological parameters, behavioral tests and Western blot data were analyzed by two-way analysis of variance (ANOVA) in order to evaluate the effects of litter size reduction and DHT treatment, and their interaction. Further intergroup differences were analyzed by the Tukey *post hoc* test

only when there was significant interaction between factors. For the analyses of behavioral test results for latency to reach escape hole during four days of acquisition training, three-way ANOVA was used to determine the effects of time, litter size reduction and DHT treatment, and their interactions, followed by *post hoc* Holm-Sidak's multiple comparisons test. Effect sizes were calculated with partial eta squared coefficients (η_p^2) and interpreted as small effect (η_p^2=0.01), medium effect (η_p^2=0.06), and large effect (η_p^2 =0.14). The differences between groups were considered significant at p<0.05. Statistical analyses were performed using STATISTICA 7.0 (StatSoft Inc., USA) and GraphPad Prism 8 (San Diego, USA) software.

Results

Physiological parameters and analysis of insulin signaling pathway in the striatum

A two-way ANOVA revealed significant effects of litter size reduction (F(1, 20)=13.48, p<0.01, η_p^2 =0.40) and DHT treatment (F(1, 20)=5.5, p<0.05, η_p^2 =0.22) on body mass (**Fig. 1A).** The adiposity index, calculated as the VAT mass to body mass ratio, was significantly affected by litter size reduction (F(1, 20)=19.69, p<0.001, η_p^2 =0.50) (**Fig. 1B**).

Since it is accepted that a nexus exists between insulin resistance and neuropsychological outcomes, we analyzed the protein levels of IRS1 and its inhibitory phosphorylation at Serine 307 in the striata of the treated animals. As shown in **Fig. 2A,** while pIRS1-Ser307 has not been affected by the treatments or their combination, all three factors, litter size reduction, DHT treatment, and their interaction had significantly decreased protein levels of IRS1 in this brain region (litter size: F(1, 20)=8.76, p<0.01, η_p^2 =0.30; DHT treatment: F(1, 20)=6.57, p<0.05, η_p^2 =0.25; litter size x DHT treatment: F(1, 20)=7.68, p<0.05, η_p^2 =0.28). Further *post hoc* analysis revealed that the protein level of IRS1 was significantly decreased in all treated groups compared to normal litter placebos (**p<0.01 NL-DHT, SL-Placebo and SL-DHT *vs.* NL-Placebo). In addition, pIRS1-Ser307/IRS1 ratio was affected by both treatments and their interaction (litter size: F(1, 20)=6.45, p<0.01, η_p^2 =0.24; DHT treatment: F(1, 20)=10.27, p<0.01, η_p^2 =0.34; litter size x DHT treatment: F(1, 20)=10.54, p<0.01, η_p^2 =0.35). Further *post hoc* analysis revealed that the pIRS1-Ser307/IRS1 ratio was significantly increased in all treated groups compared to normal litter placebos (**p<0.01 NL-DHT, SL-Placebo and SL-DHT *vs*. NL-Placebo).

Furthermore, striatal insulin sensitivity was estimated by the protein level of AKT and its stimulatory phosphorylation at Serine 473. As shown in **Fig. 2B,** statistical analyses revealed that neither of the treatments nor their interaction affected the protein levels of total pAKT-Ser473, AKT, or their ratio.

Additionally, activity of a downstream effectors of insulin signaling pathway, GSK3α/β was estimated by the protein levels of GSK3α and GSK3β isoforms and their inhibitory phosphorylation at Serine 21 and Serine 9, respectively. A two-way ANOVA revealed significant effects of litter size reduction on pGSK3α-Ser21 ($F(1, 20)=7.24$, $p<0.05$, $\eta_p^2=0.27$), and its ratio to total pGSK3α-Ser21/GSK3α/β ratio ($F(1,20=10.93$, $p<0.01$, $\eta_p^2=0.35$), as well as on pGSK3β-Ser9/GSK3α/β ($F(1,20)=7.51$, $p<0.05$, $\eta_p^2=0.28$) (**Fig. 3**).

Spatial learning and memory testing

The Barnes maze was used to evaluate spatial learning and memory. The registered behavioral parameters were statistically analyzed to reveal the influence of litter size reduction and DHT treatment, and their interaction on memory performance.

A three-way ANOVA analysis was performed to evaluate the impacts of three parameters (time, litter size reduction, and DHT treatment) on the latency to reach the escape hole during four days of acquisition training. There was a very significant effect of time expressed as a general decrease in the average group latencies during the 4-day training period ($F(3, 80)=10.08$, $p<0.001$, $\eta_p^2=0.27$) (**Fig. 4**). Litter size reduction significantly increased latency ($F(1, 80)=3.99$, $p<0.05$, $\eta_p^2=0.05$), while the effect of DHT treatment and the interaction of these two factors were above the level of significance ($F(1, 80)=3.12$, $p=0.08$, $\eta_p^2=0.04$ and $F(1, 80)=3.76$, $p=0.06$, $\eta_p^2=0.04$ respectively). Furthermore, interaction of all three factors on latency was statistically significant ($F(3, 80)=2.70$, $p<0.05$, $\eta_p^2=0.09$). Results of *post hoc* test have shown that the average latency of the SL-DHT group in the first day of training was significantly higher compared to NL-Placebo ($^{*}p<0.05$), SL-Placebo ($^{\$\$}p<0.01$), and NL-DHT group ($^{\#\#}p<0.01$). Additionally, latencies of the SL-DHT group in the following days of training (days 2, 3 and 4) were significantly lower than the latency in the first day of training (1 vs. 2 day $^{++}p<0.01$; 1 vs. 3 day $^{+++}p<0.001$; 1 vs. 4 day $^{+++}p<0.001$), suggesting the highest learning speed in this group.

The average latencies during the probe trial were determined as a period of time from the beginning of the trial until the moment when the head of each animal in the group first reached the closed escape hole. Two-way ANOVA revealed that animals raised in small litters showed an increased latency period ($F(1, 20) = 5.22$, $p<0.05$, $\eta_p^2 =0.21$) (**Fig. 5A**).

Evaluation of the memory retention during the probe trial was additionally estimated by the time that animals spent in the proximity of the closed escape hole – Target zone, and presented as the percent of time spent in this zone during the trial (**Fig. 5B**). Two-way ANOVA revealed that there was statistically significant negative effects of increased caloric intake of animals raised in small litters on this parameter ($F(1, 20)=12.41$, $p<0.01$, $\eta_p^2 =0.38$), while DHT treatment and combination of both factors did not have effect. This result indicates that rats raised in small litters (SL-placebos) spent less time in the proximity of the closed escape hole. Other parameters of cognitive performance, i.e. number of total errors, hole deviation score, and searching strategy, were not affected by litter size reduction and DHT-treatment (data not shown).

The overall mobility of rats during the probe trial was also assessed by registering three parameters: the duration of immobility (**Fig. 6A**), the total distance traveled (**Fig. 6B**), and the adjusted speed (**Fig. 6C**). Two-way ANOVA showed a significant effect of litter size reduction ($F(1, 20)=7.27$, $p<0.05$, $\eta_p^2 =0.27$), and DHT treatment ($F(1,20)=6.90$, $p<0.05$, $\eta_p^2 =0.26$) on the duration of immobility. As shown in **Fig. 6A**, duration of immobility was increased in placebos raised in small litters compared to normally raised ones but decreased in DHT animals raised in small litters. On the other hand, only DHT treatment had an effect on distance traveled (**Fig. 6B**; DHT treatment: $F(1, 20)=5.41$, $p<0.05$, $\eta_p^2 =0.21$). Statistical analyses revealed that neither of the treatments nor their interaction affected the speed of rats during movement (**Fig. 6C).**

Synaptic plasticity markers in the striatum

In order to evaluate synaptic plasticity, we analyzed the protein levels of PSD-95 and its phosphorylation at Serine 295 (pPSD-95-Ser295) in the striata of the treated animals. As shown in **Fig. 7A,** a two-way ANOVA revealed a significant effect of DHT treatment on the phosphorylation of pPSD-95-Ser295 ($F(1, 20)=9.17$, $p<0.01$, $\eta_p^2 =0.31$), as well as on the ratio of phosphorylated pPSD-95-Ser295 to total PSD-95 ($F(1, 20)=16.70$, $p<0.001$, $\eta_p^2 =0.45$).

Furthermore, we analyzed protein levels of presynaptic proteins synaptophysin and GAP-43, and a two-way ANOVA revealed that litter size reduction and DHT treatment alone, as well as their interaction, did not affect their expression (**Fig. 7B** and **7C,** respectively).

Discussion

In this study, we evaluated whether insulin resistance and synaptic plasticity in the striatum underlie spatial learning and memory capacity in the animal model of PCOS that was additionally challenged by postnatal overnutrition gained by litter size reduction.

It is known that insulin exerts a beneficial effect on memory formation in rats and humans through a complex regulation of insulin receptor-mediated signal transduction pathways (Lee et al., 2016; McNay et al., 2010). In line with this, insulin resistance is identified as an important risk factor for cognitive impairment associated with metabolic syndrome (Kim and Feldman, 2015; McNay et al., 2010; Stranahan et al., 2008). In our previously published paper we have shown that the combination of postnatal overfeeding and DHT treatment led to decreased systemic insulin sensitivity and hyperinsulinemia, which represents a compensatory mechanism in order to maintain normal plasma glucose level (Mićić et al., 2022). In this study, insulin receptor substrate 1 (IRS1) as a key component of this pathway (Rhea et al., 2022), was downregulated after DHT treatment, as well in overweight animals, which may indicate impaired insulin action in the striata of all treated animals. In line with this, the degradation of IRS1 has been identified as an important mechanism of insulin resistance that may be caused by prolonged insulin stimulation (Mayer and Belsham, 2010; Zhande et al., 2002). Moreover, observed increased ratio of phospho-IRS1 at Serine 307 to total IRS1 indicates impaired insulin signaling in the striata after applied treatments. This phosphorylation of IRS1 at Serine 307 represents a part of negative feedback pathway involved in regulation of insulin signaling, and is recognized as first step in insulin-stimulated degradation of IRS-1 (Boura-Halfon and Zick, 2009). On the other hand, the activity of AKT, a downstream effector of the insulin signaling pathway, was not altered by either of the treatments applied in this study. Another kinase involved in the insulin/PI3K/AKT/GSK3α/β cascade, GSK3α/β, was also assessed in this study. We observed decreased inhibitory phosphorylation of GSK3α/β at Serine 21 and Serine 9 in small-litter raised animals, inducing increase of GSK3α/β activity in the striata of these animals (Liu and Yao, 2016). The observed impairment at the level of GSK3α/β, without corresponding changes in

AKT activity, could be explained by a stepwise disruption of the PI3K/AKT/GSK3α/β axis following overstimulation of the insulin signaling pathway, with GSK3α/β insensitivity occurring before AKT insensitivity (Liu and Yao, 2016). However, in addition to insulin resistance, increased GSK3α/β activity has been implicated in several neurological disorders and is considered an important factor contributing to cognitive decline (Salcedo-Tello et al., 2011).

Previous studies pointed out that brain insulin resistance plays an important role in spatial memory impairment (McNay et al., 2010; Stranahan et al., 2008). We observed reduction in protein level of IRS-1 induced by both DHT treatment and postnatal overnutrition that can be considered as a negative factor for spatial learning and memory formation. Learning and memory parameters were assessed in our study using a distributed Barnes maze training protocol. The place learning in dry-land Barnes and Morris water mazes is commonly thought to be hippocampus-dependent (Gawel et al., 2019), but studies consistently suggest that the striatum is also involved in the processing and storage of spatial information (Gahnstrom and Spiers, 2020; Miyoshi et al., 2012; Rusu and Pennartz, 2020). A recent study showed that memory acquired during distributed training with extra-maze cues in the Morris water maze test was dependent on the dorsolateral striatum, which goes in line with version of the Barnes maze with distant (remote) extra-maze cues used herein. Also, an aversive stimulus in the form of a bright light above the platform was used, which creates a stressful environment, and stress has been shown to shift hippocampus-dependent learning towards striatum-dependent one (Vogel et al., 2017). Finally, a constant position of the target hole was used during the acquisition trial, and it has been shown previously that continuous training directs the processing of spatial information toward inflexible habitual behaviour, that is highly dependent on the dorsolateral striatum (Packard and McGaugh, 1996). Summarizing the results of the present behavioral study, it appears that postnatal overnutrition of animals raised in small litters has a generally negative effect on learning rate as well as on memory retention. Namely, the performance during the trial (Day 5) revealed that rats raised in small litters had the highest primary latency to reach the previous escape hole and they spent the least time in the proximity of the escape hole, according to the lowest percentage of time spent in the Target zone during the 90 seconds of the trial. The test used in this study also indicated a negative effect of postnatal overnutrition on the mobility of rats' during the trial, primarily by stimulating their immobility, whereas the effects of postnatal overnutrition and DHT treatment on their ambulation appeared to be in the opposite

direction. However, although rats raised in small litters spent a shorter period of time in movement, the distance they traveled during the probe trial remained unchanged compared to NL-placebos. Judging from the calculated speed during movement, raising in small litters did not impair their locomotor activity. The observed behavioral effects of postnatal overnutrition and resulting adiposity developed in these animals are in accordance with several studies in animals (Heyward et al., 2012; Stranahan et al., 2008) and humans (Stingl et al., 2012; Wang et al., 2016) which showed that obesity may contribute to impaired cognitive and motor functions (Davidson et al., 2014). Indeed, increased caloric intake in the early postnatal period led to the development of adiposity, judged by the VAT/body mass ratio, and these rats were also characterized by hyperinsulinemia and decreased systemic insulin sensitivity (Mićić et al., 2022).

However, it cannot be excluded that other factors, such as changes in mother-pup interaction due to manipulation of litter size, could have an influence on the observed behavioral changes. In the study by Enes-Marques and Giusti-Paiva (2018), a reduction in litter size resulted in improved maternal care, which in turn led to improved affective and stress-related responses in pups of both sexes (Enes-Marques and Giusti-Paiva, 2018). Apart from this study, another one has shown that small litter size raised animals had impaired spatial memory in strain-dependent manner (Salari et al., 2018). Thus, besides metabolic malprogramming induced by early life overfeeding, differential maternal care may also be involved in the observed behavioral changes.

Several studies have correlated spatial memory impairment in metabolically compromised animals with disturbed insulin signaling in both the periphery and the brain (Abbott et al., 2019; Arnold et al., 2014; McNay et al., 2010; Xu et al., 2018). Indeed, insulin is known to positively affects synaptic rearrangements responsible for memory consolidation and flexibility (Spinelli et al., 2019). It should be emphasized that placebo animals raised in small litters in our study displayed the worst spatial memory performance, although all experimental groups had decreased total IRS1 levels in the striatum. However, the absence of memory impairment measured as latency time in the trial in DHT-treated animals could be attributed to the beneficial effects of androgen treatment in spatial memory performance tasks (Benice and Raber, 2009b). Thus, it could be speculated that in our study, postnatal overnutrition impaired the formation of spatial reference memory, whereas DHT treatment restored it to the control levels. In contrast to the effects of postnatal overnutrition, androgen treatment appeared to have no significant influence on memory retention. Additionally, DHT treatment stimulated animal locomotion on

the maze platform at Day 5, which may be correlated with the active search for the escape. It is noticeable that such hyperlocomotion induced by DHT treatment in young female rats is in accordance with the outcome of the study performed in male mice, which suggested elevated motor activity stimulated by testosterone *via* dopaminergic receptors (Jardi et al., 2018). In different learning and memory paradigms, the distinct effects of testosterone or DHT supplementation on cognitive performance have already been reported in aged female mice (Benice and Raber, 2009b), adult and aged male rats (Bimonte-Nelson et al., 2003; Vazquez-Pereyra et al., 1995) but not in young female rats. DHT, as a non-aromatizable metabolite of testosterone, enhances only some aspects of memory, like spatial working memory of castrated mice in the Morris water maze (Benice and Raber, 2009a) and passive avoidance-related memory without affecting spatial reference memory of aged female mice (Benice and Raber, 2009b). Accordingly, our study showed no distinct effect of DHT on memory retention (short-term spatial reference memory). Although further studies with larger sample sizes are needed to statistically support this assumption, it may be that DHT facilitates learning by enhancing memory consolidation, as others have suggested (Frye and Lacey, 2001). One possible explanation for the absence of improved performance in DHT treated animals in a probe trial is that beneficial effects of androgens on spatial memory may be detected only when higher doses of DHT are used compared to those used in our study and/or when other behavioral assays are employed. Nevertheless, as already discussed, DHT-treated animals raised in small litters did not have impaired memory-associated parameters in a probe trial, implying that beneficial effects of androgens can be revealed only in animals with already impaired spatial performance. Similar protective effect of DHT on spatial memory performance was reported in aged female mice expressing the apolipoprotein ApoE4, which predisposes them towards cognitive impairment (Raber et al., 2002).

The effects of applied treatments on the levels of three synaptic proteins which are involved in synapse formation and regulation of synaptic strength (Holahan, 2017; Kwon and Chapman, 2011; Taft and Turrigiano, 2014), synaptophysin, GAP43, and PSD-95, were investigated, as well as the pPSD-95-Ser 295, which was found to promote its synaptic accumulation (Vallejo et al., 2017). The results showed that neither postnatal overnutrition nor DHT treatment, nor their combination, induced any significant changes in total protein levels of synaptophysin, GAP43, and PSD-95 in the striata of the treated animals. On the other hand, DHT treatment induced an

increase in phosphorylation of PSD-95 at serine 295 in the striatum of treated animals. These results imply that the dynamics of synaptic structure had been affected by the DHT treatment, whereas the molecular structure of striatal synapses appeared unaltered by any of the applied treatments. Notably, it was shown that phosphorylation of PSD-95 at Ser295 enhances the ability of PSD-95 to strengthen the excitatory synapse in cultured hippocampal neurons, which is the mechanism that may transform a silent synapse into a functional one (Kim et al., 2007). Moreover, *ex vivo* (Hatanaka et al., 2015) and *in vivo* (Naghdi et al., 2005) studies have shown the ability of androgens (DHT and testosterone) to improve hippocampal synaptic plasticity and memory through androgen receptor-mediated activation of kinase networks and non-genomic signaling pathways, i.e., independent of protein synthesis. However, it should be kept in mind that most of the studies investigating the effects of androgens on synaptic characteristics were conducted on hippocampal neurons and that further experiments aimed at exploring the effects of androgens on the synaptic properties of striatal neurons are needed.

In conclusion, this study revealed that both adiposity-related early life overnutrition and DHT-treatment suppressed insulin signaling through increased ratio of inhibitory phosphorylation at serine 307 of IRS1 to IRS1, with a simultaneous downregulation of IRS1 in the striatum. Additional disruption in the activity of GSK3α/β insulin downstream kinase, was observed in small litter raised animals. However, this was translated into cognitive deterioration only in the absence of DHT. Namely, DHT treatment did not have an adverse effect on learning and memory, probably due to a compensatory increase in pPSD-95-Ser295, which had a positive effect on synaptic strength. According to our results, in this model of hyperandrogenism-induced PCOS accompanied by adiposity, hyperandrogenism *per se* does not represent a significant threat to spatial learning and memory, opposite to the effect of overnutrition-related adiposity. This suggests that in PCOS, both hormonal profile and body weight, are important factors in preventing cognitive decline.

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Figure legends

Fig. 1. Physiological parameters.

(A) Body mass, and (B) VAT mass to body mass ratio. The results are shown as means ± SEM (n=6 animals per group). A two-way ANOVA was performed to determine the statistical effects of litter size reduction and DHT treatment, as well as their interaction. A value of $p<0.05$ was considered statistically significant.

Fig. 2. IRS1 and AKT in the striatum.

Representative Western blots and relative quantification of (A) pIRS1-Ser307, total IRS1, and their ratio; (B) pAKT-Ser473, total AKT, and their ratio in the striata of the DHT-treated and placebo rats raised in normal and small litters. β-actin was used for the normalization of immune-positive bands of target proteins. The results are shown as mean ± SEM (n=6 animals per group). A two-way ANOVA was performed to determine the statistical effects of litter size reduction and DHT treatment, as well as their interaction. A value of $p<0.05$ was considered statistically significant. The *post hoc* Tukey test was used to analyze inter-group differences. The symbol used denotes a statistically significant difference between groups (**$p<0.01$ vs. Normal litter placebo).

Fig. 3. GSK3α/β in the striatum

Representative Western blots and relative quantification of (A) pGSK3α–Ser21, total GSK3α/β, and their ratio; (B) pGSK3β–Ser9, total GSK3α/β, and their ratio in the striata of the DHT-treated and placebo rats raised in normal and small litters. GAPDH was used for the normalization of immune-positive bands of target proteins. The results are shown as mean ± SEM (n=6 animals per group). A two-way ANOVA was performed to determine the statistical effects of litter size reduction and DHT treatment, as well as their interaction. A value of $p<0.05$ was considered statistically significant.

Fig. 4. Learning rate.

Female rats were trained to learn the location of the escape box for 4 days with 2 sessions per day and latencies to enter the escape box within 3 min training were monitored. Data are presented as average daily group latencies during four days of acquisition training ± SEM of n =6 per group. The effects of time, litter size reduction, and DHT treatment, as well as their

interaction, were determined by three-way ANOVA, followed by the Tukey *post hoc* test. Symbols denote a statistically significant difference compared to the mean values of latencies of NL-Placebo (*$p<0.05$), SL-Placebo ($^{\$\$}p<0.01$), NL-DHT ($^{\#\#}p<0.01$), and SL-DHT ($^{++}p<0.01$, $^{+++}p<0.001$) group measured during the first day of training.

Fig. 5. Memory retention.

(A) Latency to the first reach closed escape hole during the probe trial. On Day 5 the previous escape hole was closed and during the 90 seconds of probe trial rats were let to search for its location. The period of time from the beginning of the trial until the moment when the animal's head reached the closed escape hole for the first time was registered. (B) Percent of time spent in the Target zone during the probe trial. The upper half of the maze sextant that contained the former escape and two adjacent holes was defined as the Target zone. The time spent in the Target zone was measured and the percent of time spent in the zone during the probe trial was calculated. The data are presented as the mean ± SEM of n =6 per group. Effects of litter size reduction and DHT treatment, as well as their interaction, were determined by two-way ANOVA.

Fig. 6. Locomotor activity.

(A) Total time of immobility during the probe trial. On the Day 5 the previous escape hole was closed and the time of immobility during the probe trial was measured using Any Maze software. Immobility is defined as the absence of any vertical or horizontal movements. (B) Total distance traveled during the probe trial. The total length of the path covered by an animal in motion during the probe trial was measured with Any Maze software. (C) Adjusted speed. Speed during the movement is calculated as a ratio of total distance traveled and mobility time. The data are presented as the mean ± SEM of n =6 per group. Effects of litter size reduction and DHT treatment, as well as their interaction, were determined by two-way ANOVA.

Fig. 7. PSD-95, synaptophysin, and GAP43 in the striatum.

Representative Western blots and relative quantification of (A) pPSD-95-Ser295, total PSD, and their ratio; (B) synaptophysin; (C) GAP43 in the striata of the DHT-treated and placebo rats raised in normal and small litters. β-actin and GAPDH were used for the normalization of immune-positive bands of target proteins. The results are shown as mean ± SEM (n=6 animals

per group). A two-way ANOVA was performed to determine the statistical effects of litter size reduction and DHT treatment, as well as their interaction. A value of $p<0.05$ was considered statistically significant.

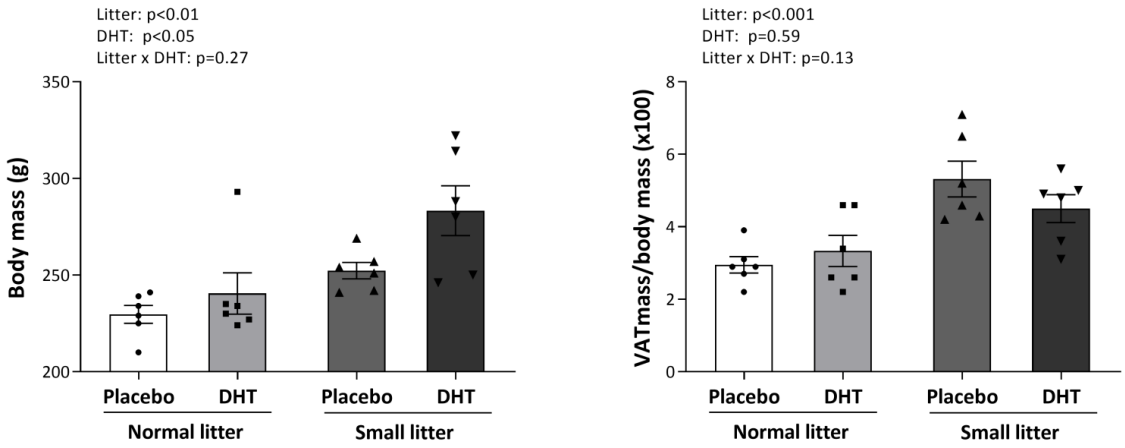
Litter: p<0.01
DHT: p<0.05
Litter x DHT: p=0.27
Body mass (g)
350
300
250
200
Placebo
DHT
Placebo
DHT
Normal litter
Small litter
Litter: p<0.001
DHT: p=0.59
Litter x DHT: p=0.13
VATmass/body mass (x100)
8
6
4
2
0
Placebo
DHT
Placebo
DHT
Normal litter
Small litter

Fig.1

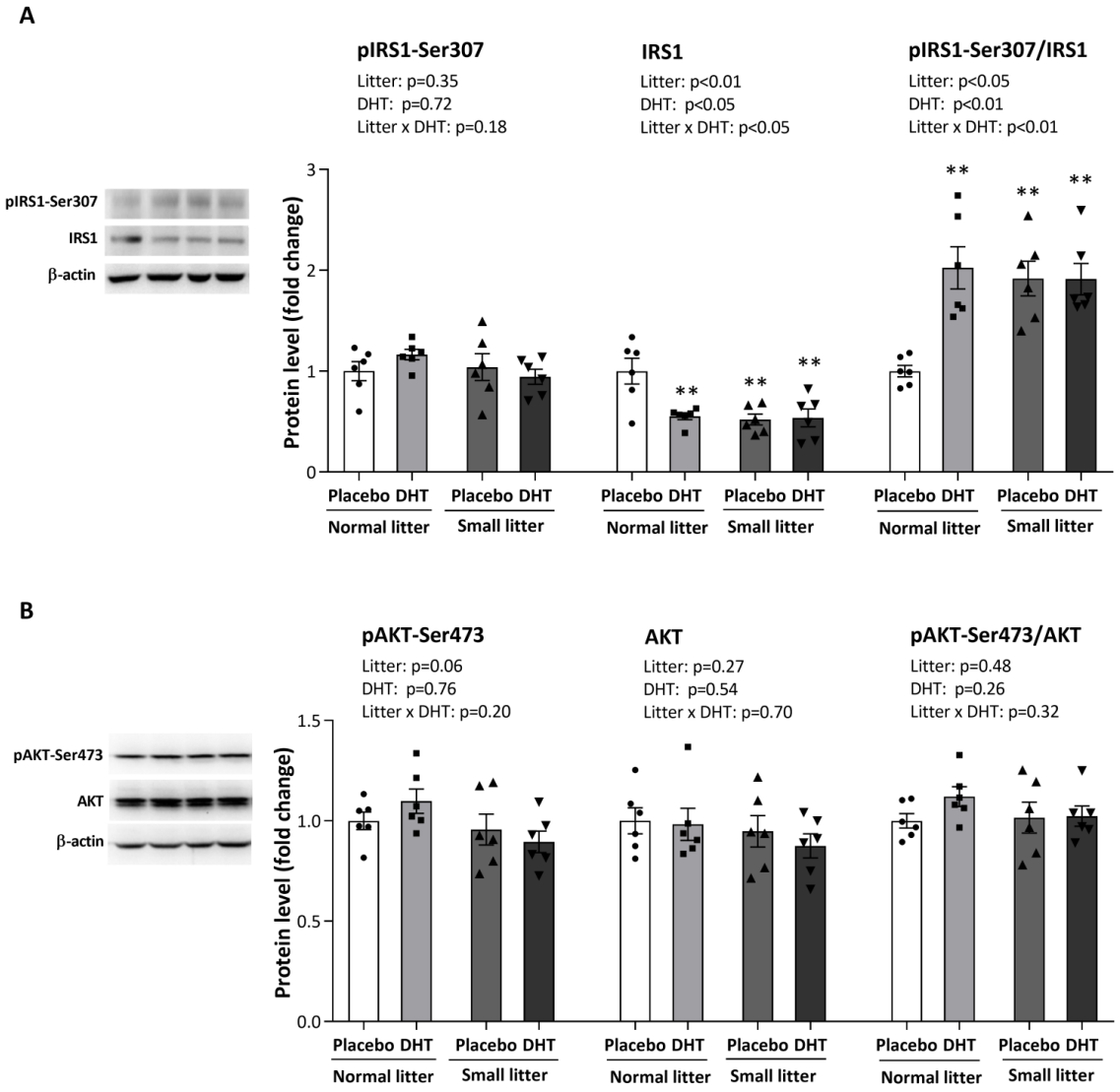
A
pIRS1-Ser307
IRS1
β-actin
pIRS1-Ser307
Litter: p=0.35
DHT: p=0.72
Litter x DHT: p=0.18
IRS1
Litter: p<0.01
DHT: p<0.05
Litter x DHT: p<0.05
pIRS1-Ser307/IRS1
Litter: p<0.05
DHT: p<0.01
Litter x DHT: p<0.01
Protein level (fold change)
Placebo DHT
Normal litter
Small litter
B
pAKT-Ser473
AKT
β-actin
pAKT-Ser473
Litter: p=0.06
DHT: p=0.76
Litter x DHT: p=0.20
AKT
Litter: p=0.27
DHT: p=0.54
Litter x DHT: p=0.70
pAKT-Ser473/AKT
Litter: p=0.48
DHT: p=0.26
Litter x DHT: p=0.32

Fig. 2

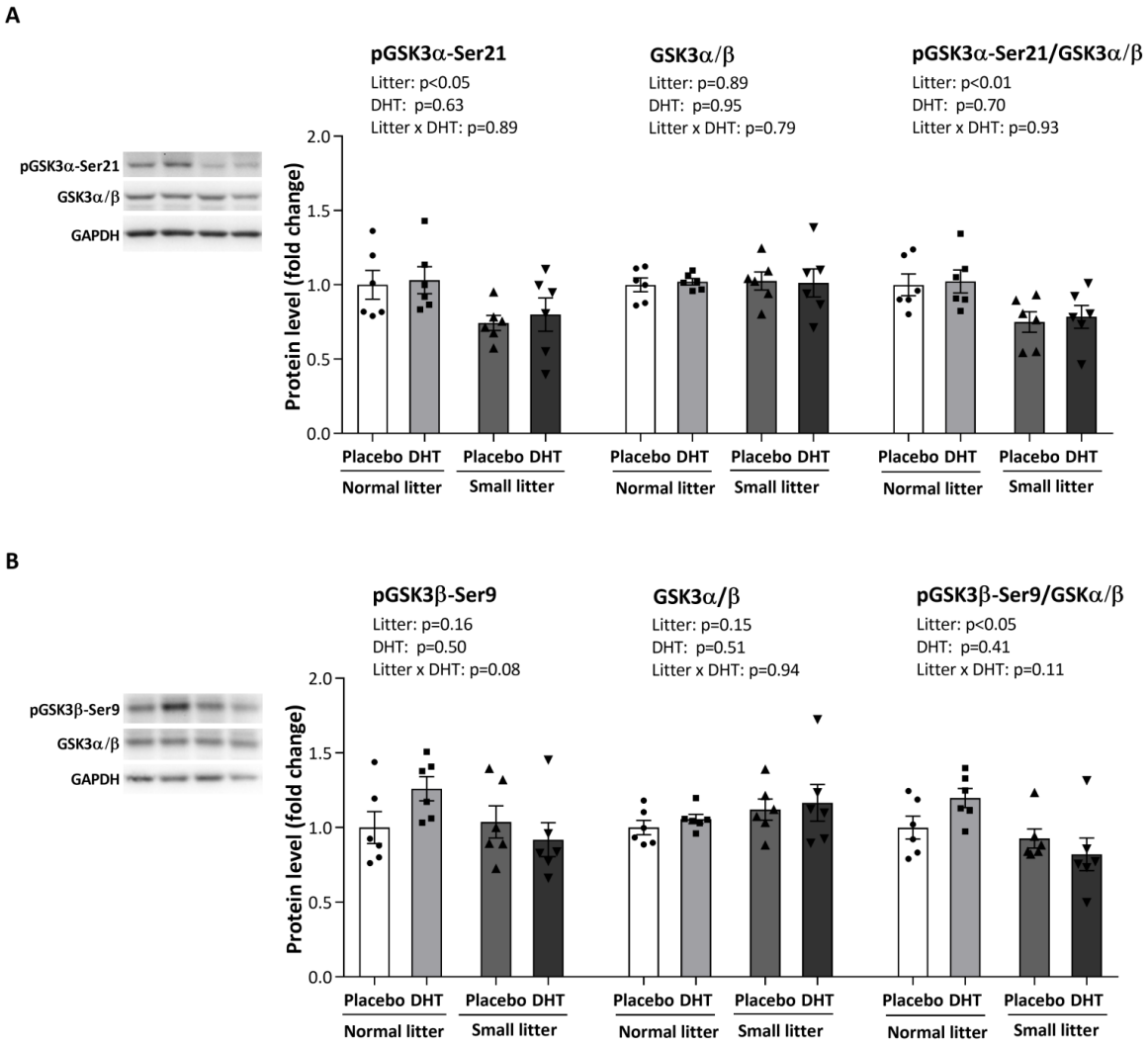
A
pGSK3α-Ser21
GSK3α/β
GAPDH
pGSK3α-Ser21
Litter: p<0.05
DHT: p=0.63
Litter x DHT: p=0.89
GSK3α/β
Litter: p=0.89
DHT: p=0.95
Litter x DHT: p=0.79
pGSK3α-Ser21/GSK3α/β
Litter: p<0.01
DHT: p=0.70
Litter x DHT: p=0.93
Protein level (fold change)
2.0
1.5
1.0
0.5
0.0
Placebo DHT
Normal litter
Small litter
B
pGSK3β-Ser9
GSK3α/β
GAPDH
pGSK3β-Ser9
Litter: p=0.16
DHT: p=0.50
Litter x DHT: p=0.08
GSK3α/β
Litter: p=0.15
DHT: p=0.51
Litter x DHT: p=0.94
pGSK3β-Ser9/GSKα/β
Litter: p<0.05
DHT: p=0.41
Litter x DHT: p=0.11

Fig. 3

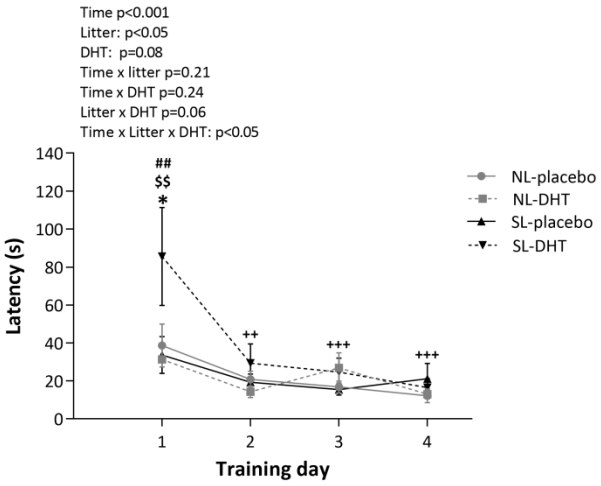
Time p<0.001
Litter: p<0.05
DHT: p=0.08
Time x litter p=0.21
Time x DHT p=0.24
Litter x DHT p=0.06
Time x Litter x DHT: p<0.05
##
$$
*
++
+++
+++
NL-placebo
NL-DHT
SL-placebo
SL-DHT
Latency (s)
Training day

Fig. 4

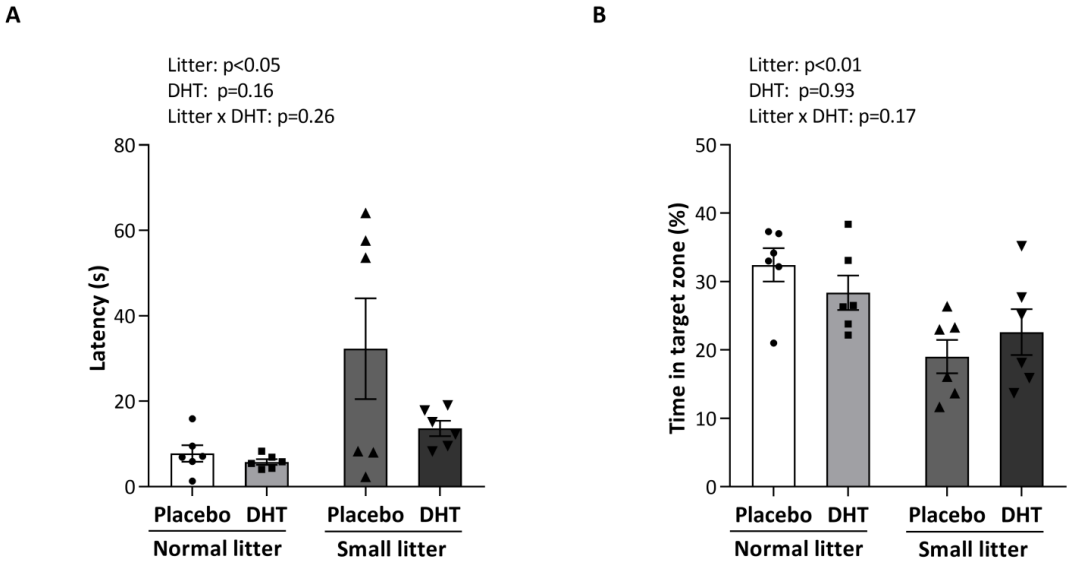
A
Litter: p<0.05
DHT: p=0.16
Litter x DHT: p=0.26
Latency (s)
Placebo DHT Placebo DHT
Normal litter Small litter
B
Litter: p<0.01
DHT: p=0.93
Litter x DHT: p=0.17
Time in target zone (%)
Placebo DHT Placebo DHT
Normal litter Small litter

Fig. 5

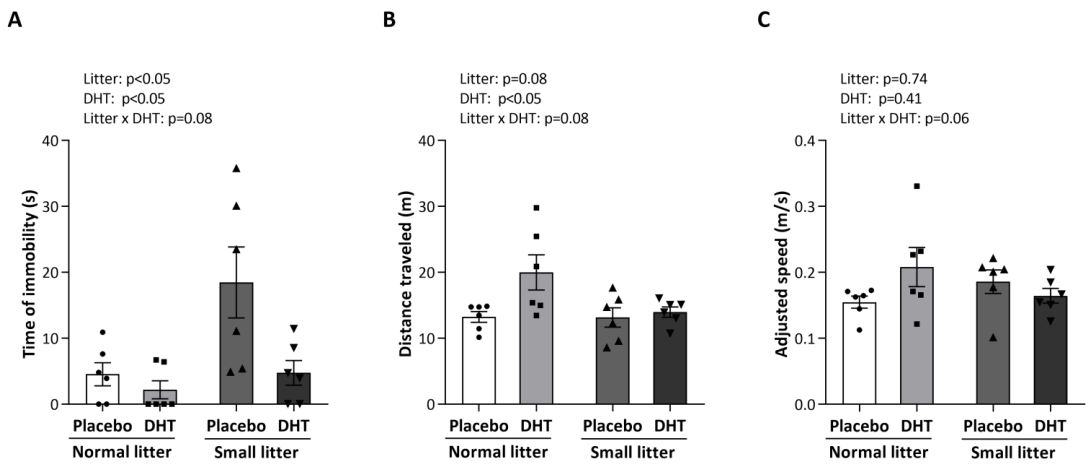
A
Litter: p<0.05
DHT: p<0.05
Litter x DHT: p=0.08
Time of immobility (s)
Placebo DHT Placebo DHT
Normal litter Small litter
B
Litter: p=0.08
DHT: p<0.05
Litter x DHT: p=0.08
Distance traveled (m)
Placebo DHT Placebo DHT
Normal litter Small litter
C
Litter: p=0.74
DHT: p=0.41
Litter x DHT: p=0.06
Adjusted speed (m/s)
Placebo DHT Placebo DHT
Normal litter Small litter

Fig. 6

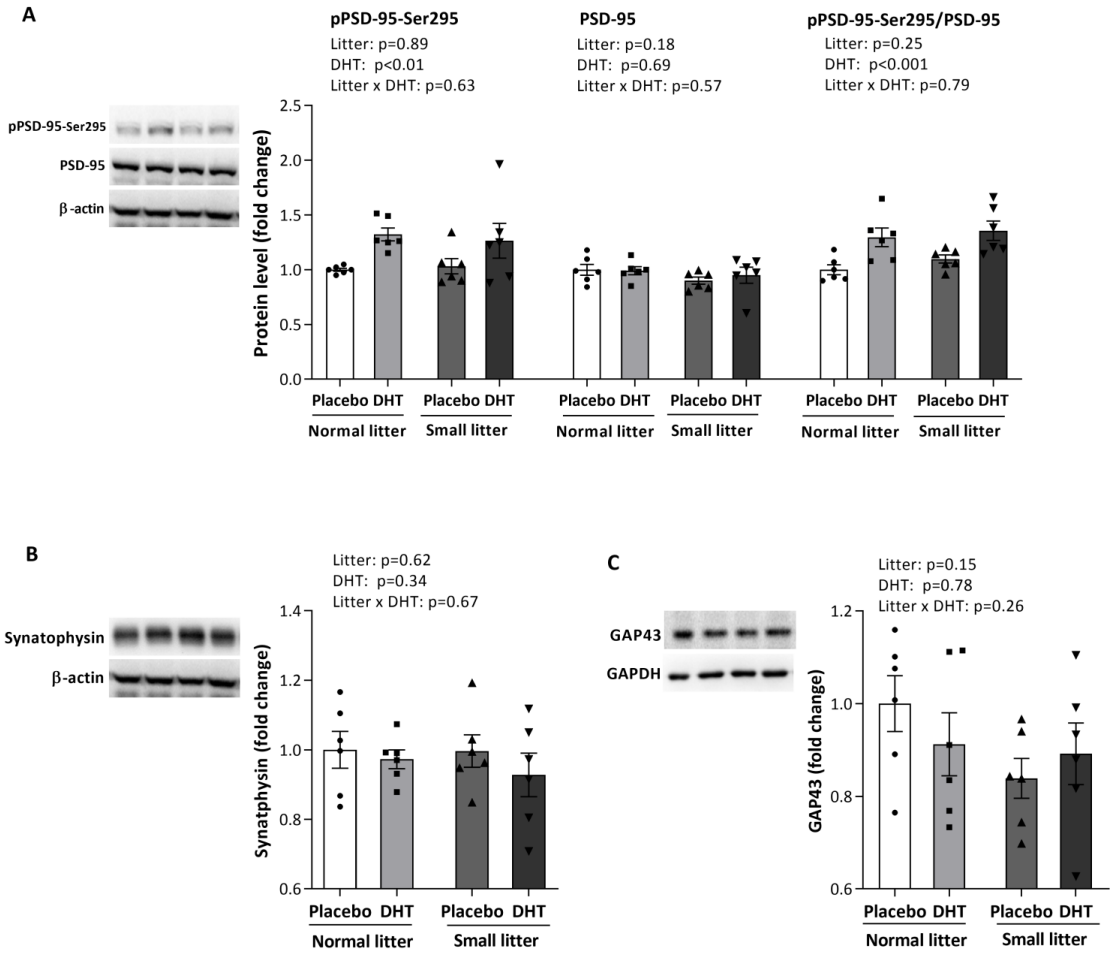
A
pPSD-95-Ser295
PSD-95
β-actin
pPSD-95-Ser295
Litter: p=0.89
DHT: p<0.01
Litter x DHT: p=0.63
PSD-95
Litter: p=0.18
DHT: p=0.69
Litter x DHT: p=0.57
pPSD-95-Ser295/PSD-95
Litter: p=0.25
DHT: p<0.001
Litter x DHT: p=0.79
Protein level (fold change)
Placebo DHT Placebo DHT
Normal litter Small litter
B
Synatophysin
β-actin
Litter: p=0.62
DHT: p=0.34
Litter x DHT: p=0.67
Synatphysin (fold change)
C
GAP43
GAPDH
Litter: p=0.15
DHT: p=0.78
Litter x DHT: p=0.26
GAP43 (fold change)

Fig. 7