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Research report

Sex-specific alterations in behavioral and cognitive functions in a "three hit" animal model of schizophrenia

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HIGHLIGHTS

- A three hit animal model of schizophrenia induced sex-specific alterations.
- Males were more severely affected than their female counterparts.
- Decreased pain sensitivity and increased motor dexterity were observed.
- Decreased working and reference memory ratios indicated cognitive disturbances.
- The results highlight the importance of taking into account sex-specific differences.

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ABSTRACT

Whereas schizophrenia affects both human sexes, there are known sex-dependent disparities. We developed a chronic animal model that shows some schizophrenia-related deficits in rats by applying selective breeding after subchronic ketamine administration connected with postweaning social isolation (complex treatment).

Our aim was to determine the sex-specific effects of these interventions on several processes. Sensory gating to acoustic stimulation, pain sensitivity, motor behavior, spatial learning and memory deficits on the hole-board test were assessed in the 17th generation of selectively bred Wistar rats compared to their naive counterparts with or without complex treatment.

We found differences between the sexes: selectively bred males with complex treatment showed the lowest pain sensitivity; however, the results of the prepulse inhibition test indicated that female rats showed enhanced impairment of sensory gating and increased acoustic startle reaction. The cognitive performance, working and reference memory ratios were significantly decreased by selective breeding and showed sex-specific alterations, with the smallest value in male rats of the new substrain.

Based on these results, the animals of the new substrain could be classified into the high-risk for schizophreniform phenotype with the highest sensitivity of males with complex treatment. Decreased cognitive performance highlighted spatial learning deficits in the selectively bred and treated rats that escalate the validity of our new and complex rat model of schizophrenia. The results indicate the same sex selectivity as observed in humans, with increased incidence of risk ratios for men to develop schizophrenia relative to women.

Abbreviations: TF, tail-flick; PPI, prepulse inhibition; HB, hole-board; NaNo, naïve non-treated; NaTr, naïve treated; SelNo, selectively bred non-treated; SelTr, selectively bred treated; PA, pulse alone; PP, prepulse-pulse pair; PPA, prepulse alone; relASR, relative acoustic startle response; WM ratio, working memory ratio; RM ratio, reference memory ratio.

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1. Introduction

Currently it is well accepted that besides the negative and positive symptoms, sensory and cognitive deficits (attention and memory dysfunctions) are common in schizophrenia. Cognitive disturbances receive increasing attention, as they are a core feature of the disease, and perhaps the strongest predictor of outcome [1]. Despite widely recognized developmental nature of schizophrenia, these impairments do not emerge until late adolescence and reflect abnormalities in specific cortical circuits arising at that time [2]. Patients present with extremely heterogeneous symptom combinations, making diagnosis and treatment problematic, however, the last revision of Diagnostic and Statistical Manual of Mental Disorders (DSM-5) provides a more precise delineation of schizophrenia [3]. The changes also facilitate measurement-based treatment and concurrently provide a more useful platform for research that will elucidate the nature of schizophrenia [3]. However, many of the symptoms used to establish psychiatric diagnoses in humans, e.g. hallucinations, delusions and thought disorders, cannot be convincingly ascertained in animals [4], certain aspects of psychiatric disorders can indeed be modeled and studied in animals.

Animal models of schizophrenia based on pharmacological or genetic manipulations induce behavioral changes indicative of specific features of this illness but are often limited to perturbations in a single neurotransmitter system or specific genetic loci [5]. It is unlikely that a single gene mutation or a single adverse life event is sufficient to increase the incidence of multifactorial disorders, such as schizophrenia, diabetes mellitus, asthma or ischemic heart disease [6]. Thus, we developed a complex, chronic, "three hit" animal model. The first hit is pharmacological, namely subchronic ketamine treatment; the second one is postweaning social isolation as an environmental stress with appropriate timing for exposure. The third hit is selective breeding (instead of using knockout animals) as a predisposing genetic factor causing anomalous neuronal development and subtle changes in behavior. This model mimicked simultaneously several aspects of schizophrenia, i.e., decreased pain sensitivity, sensory gating disturbances, decreased rearing activity and recognition memory dysfunction were observed [7]. Our previous studies were restricted to male rats.

Nevertheless, schizophrenia affects both human sexes, and there are known sex-dependent disparities in prevalence, age of onset, clinical characteristics, and the course and prognosis of the disease [8]. Thus, we proposed to investigate the sexually dimorphic effects of the applied animal model on responses to acoustic stimulation and pain sensitivity, impaired in this neuropsychiatric disorder [9].

In order to increase the face validity (derived from phenomenological similarity between the behavior in the animal model and the specific symptoms of the human condition) of our animal model, we conducted further behavioral phenotyping of these selectively bred animals to characterize their cognitive processes, i.e., the spatial learning ability and memory function with the modified hole-board (HB) test. It is a widely used spatial memory task that is adequate for simultaneously assessing exploration, anxiety-like behavior and spatial reference, and working memories in rats [10]. An additional aim of the current study was to investigate the influence of sex on cognitive and behavioral processes, and motor functions using the HB task.

2. Materials and methods

All experiments involving animal subjects were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: XIV/03285/2011). Animals were kept with a 12 h light/dark cycle under conditions of controlled temperature ($22 \pm 1\,^{\circ}C$) with ad libitum water and food access (except during the HB test, when they were food deprived).

2.1. Selective breeding process

The paradigm for selective breeding through several generations was described previously [7]. Briefly, after weaning at 3 weeks of age (21–23 days), rats were tested with the tail-flick (TF) test to assess their basal pain sensitivity and then housed individually for 28 days (between 4–7 weeks of age). The animals in each generation were treated with ketamine (Calypsol, Richter Gedeon Nyrt., Budapest, Hungary; 30 mg/kg intraperitoneally, 4 mL/1000 g body weight, daily, 5 times/week, 15 injections in total) from 5 to 7 weeks of age. At the end of the treatment, animals were re-housed in a group setting (4–5 rats per cage) and 1 week of recovery, with no treatment followed. Starting from a population of outbred Wistar rats, animals with the lowest pain sensitivity, impaired recognition memory and the highest sensory gating disturbance were used for selective breeding throughout several generations. Male and female rats of the 17th generation were involved in the present experiment.

2.2. Experimental paradigm

Eight experimental groups of Wistar rats were compared: naive socialized male ($n = 6$) and female ($n = 7$) rats without any treatment (NaNo), or with isolation and ketamine treatment (NaTr, $n = 7$ and $n = 8$), and the 17th generation of selectively bred male ($n = 6$) and female ($n = 6$) rats with no treatment (SelNo) or with complex treatment (SelTr, $n = 12$ and $n = 8$). The body weight of rats in all experimental groups was measured throughout the whole investigation period.

Behavioral assessment started at the age of 9 weeks with the tail-flick (TF) test. At the age of 10 weeks, we conducted the prepulse inhibition (PPI) test to investigate sensory gating. Spatial memory functions were examined by the HB test at the age of 13–18 weeks.

2.3. Procedures

2.3.1. Nociceptive testing

Acute nociceptive threshold was assessed by TF test. The reaction time was determined by immersing the distal 5 cm portion of the tail in hot water (48 °C) until a tail-withdrawal response was observed (cut-off time: 40 s). TF latencies were obtained four times at 0, 30, 60, and 90 min and, since they did not differ significantly, were averaged to establish the pain threshold for each group.

2.3.2. Sensory gating testing

PPI of the acoustic startle response was measured in four startle chambers as described previously [7]. Briefly, the Plexiglas startle chamber was in a sound-attenuated room and was divided into four identical compartments ($12 \times 17 \times 15.3$ cm each). Noise bursts were applied through a speaker mounted close to the backside of the chamber. Under the cage, a piezoelectric accelerometer (i.e., force transducer) sensitive to rat startle-like movements produced an electrical signal that was amplified by a signal conditioner and visualized on a computer screen. Rats were allowed to habituate to the background noise (65 dB) for 10 min, immediately thereafter, they were exposed to three different trial types: the pulse alone (PA), in which a 40 ms 95 dB white noise burst was presented; the prepulse alone (PPA; 20 ms 76 dB); and the prepulse–pulse pair (PP) in which prepulse stimuli were followed by the acoustic startle stimulus with a latency of 150 ms. All types were performed 15 times in PA-PPA-PP order. The interstimulus intervals ranged from 7 to 13 s, and there was a 10 min resting period between each

trial types. The %PPI values were calculated as percentages using the following equation: %PPI = [1 − (startle response for PP)/(startle response for PA)] × 100.

As in case of startle reflex the force of the muscle contraction is measured, the body weight can confound its value, thus we adjusted the response to body weight accordingly: relative acoustic startle response (rel ASR) = (startle response × 100)/(body weight (g)).

2.3.3. Appetitively motivated cognitive HB-type task

The HB test has been shown to be a differential and non-stressful cognitive-behavioral test in rodents suitable for the detection of long-term neurocognitive deficits [11]. This task is adequate for simultaneously assessing exploration, anxiety-like behavior and spatial reference and working memories in rodents [12–14]. Spatial learning ability was studied in a HB apparatus using food reward as a positive motivation after two days of total food-deprivation. Additional food restriction process remained throughout the experiment with decreased amount of food (approximately the 50% of the daily nutritional requirement [15]). The floor of the arena (an 80 × 80 cm square arena with 40 cm high black walls) contained 16 cylinders (5 × 5 cm diameter) in a 4 × 4 array. The animals were placed into the center of the arena in every case. The apparatus was cleaned with 70% alcohol solution after each animal.

Behavioral testing on the HB was performed between 8:00 a.m. and 3:00 p.m. over a 5-day period, and consisted of three distinct and consecutive phases. During the *habituation phase* the animals were allowed to acclimatize to the test arena for 20 minutes. Each of the 16 holes was white and empty. In the *learning phases*, puffed rice was put into each hole that was also marked with blue tapes (to learn the key to the test procedure). One session was performed in the afternoon of the habituation phase, and three additional learning sessions were performed on the next day (with an inter-session interval of 2 h). Each learning session lasted until all 16 rewards were obtained (cut-off time: 600 s). After learning the key, spatial memory was conducted in the *trial phase*: four of 16 cylinders were marked blue and contained puffed rice reward in a fixed pattern that remained constant, the others were white without rewards. Each trial lasted for 5 × 60 s (cut-off time: 300 s) or until all four rewards were obtained. All animals were tested on three consecutive days with 3, 3 and 2 sessions of trials each day with an intersession interval of 2 h.

Behavior was recorded with an infrared video device (WCM-21VF, CNB, China). The scoring of the different behaviors was carried out by investigators blind to the group. Durations of stereotypic behaviors were evaluated in both the habituation and learning phases, such as rearing (vertical) and locomotor (horizontal) activities and self-grooming. The exploratory activity was evaluated in all three phases: during the habituation phase, an average time spent with sniffing of the cylinders (as exploratory activity) was calculated for a 5 min interval; during the learning phase, the sniffing time during the first 5 min session of the first learning session was used. To make the results comparable among the eight sessions of the trial phase with different durations, the exploratory activity was expressed as an average frequency.

The level of anxiety was determined by the immobility time (total time minus the summarized activity that involves rearing, sniffing, walking and grooming activity), and the place preference (% time spent in the center – within the outer line of the holes – during the observation period). Cognitive processes were characterized during the learning and trial phases by different parameters as indicated in Table 1.

2.3.4. Statistical analyses

Data are expressed as means ± SEM. Behavioral data were analyzed using factorial ANOVA with treatment (complex or no treatment), bred (naive or selectively bred) and sex as factors. Post hoc comparisons were performed using the Fisher LSD test. Repeated measurement ANOVA was used to evaluate the time course effects on HB test. The relationships of learning capacity with other cognitive parameters were assessed by linear regression analysis and calculation of Pearson correlation coefficients (Spearman *R* statistic). Only probabilities lower than 0.05 were considered significant. For the analyses, STATISTICA Version 12 (Statsoft Inc., Tulsa, OK) was used.

To classify animals as either being low- or high-risk for schizophreniform phenotype, the median split, quartile-based scoring method was used for transforming continuous variables into categorical ones. After sorting the data by their values, the animals in the first quartile received 0 point (lowest risk), values in the last quartile received a score of 2 (highest risk), and the values between them received 1 point respectively. Parameters (Table 1) showing significant differences in any aspects were rated separately from 0 to 2; then these points were averaged on three dimensions (negative, cognitive and other symptoms), and finally the points of the three dimensions were summarized to generate the total schizophrenia score, which ranged from 0 to 6. The three categories of the symptoms are represented in total score with the same proportions. The male and female rats were scored separately, since certain observed parameters are highly influenced by sex.

3. Results

Body weight observed during the whole experiment significantly differed between the sexes from postnatal day 31, with significantly lower values in females than males. While in females

Table 1
Parameters used to generate the total schizophrenia score (ranged from 0–6) classifying the animals to low- or high-risk for schizophreniform phenotype.

	Rated aspects	Parameters	Calculations
Negative (behavioral) symptoms	General exploration	Directed exploratory activity	total number of holes visited (rewarded, repeated, wrong and unsuccessful) divided by the duration of the individual trial sessions
	Fine motor parameters	Motor dexterity	the frequency of unsuccessful hole visits
Cognitive symptoms	Learning process	Learning capacity (%)	$\left[\frac{\text{number of collected food rewards} \times \text{cut-off time of the learning phase(600 s)}}{\text{number of food rewards(16)} \times \text{time required to complete the task(s)}}\right] \times 100$
	Cognitive performance	Cognitive performance (%)	$\left[\frac{\text{number of collected food rewards} \times \text{cut-off time of the trial phase(300 s)}}{\text{number of food rewards(20)} \times \text{time required to complete the task(s)}}\right] \times 100$
	Visuo-spatial short-term memory	Working memory (WM) ratio	the number of food-rewarded visits divided by the number of visits and revisits to the baited holes during the trial phase
	Visuo-spatial long-term memory	Reference memory (RM) ratio	the number of visits and revisits to the baited holes divided by the total number of hole visits during the trial phase
Other symptoms	Pain sensitivity	Tail-flick latency (s)	the reaction time (s) until a tail-withdrawal response (48 °C)
	Sensory gating of the acoustic startle response	Prepulse inhibition (% PPI)	[1 − (startle response for prepulse + pulse trial)/(startle response for pulse alone trial)] × 100

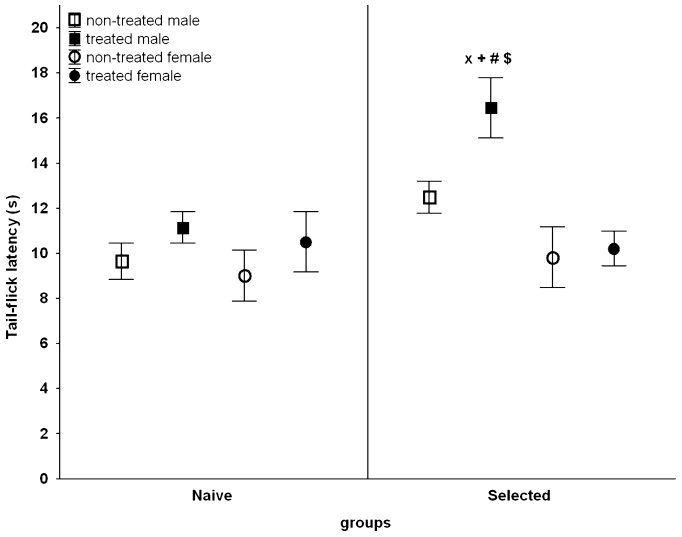

Fig. 1. Pain sensitivity indicated by the tail-flick latency at the age of 9 weeks. The symbols indicate significant differences compared to: x naive non-treated (NaNo); + naive treated (NaTr); # selectively bred non-treated (SelNo) groups. The symbol $ denotes significant difference between the sexes of the corresponding groups. Data are presented as means ± SEM.

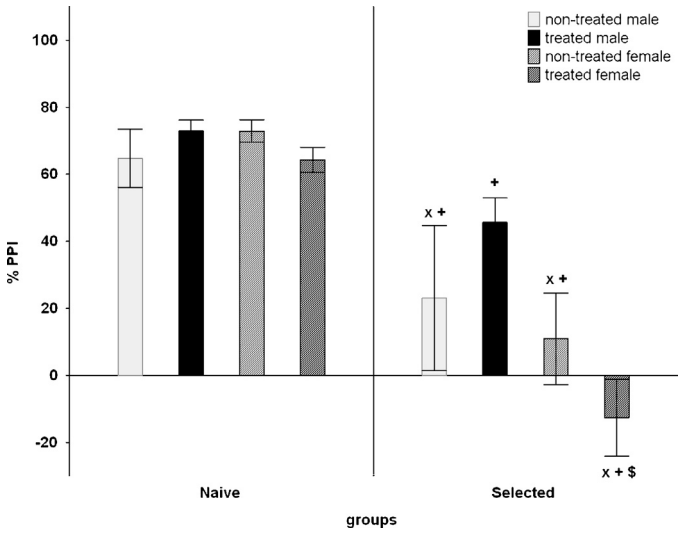

Fig. 2. Sensory gating process indicated by %PPI values in the different groups. The symbols indicate significant differences compared to: x naive non-treated (NaNo) and + naive treated (NaTr) groups. The symbol ($) denotes significant differences between the sexes of the corresponding groups. Data are presented as means ± SEM.

none of the treatments influenced the body weight, in males both NaTr and SelTr groups showed significantly decreased body weight compared to the NaNo group from day 41 and 34, respectively (data are not shown).

3.1. Somatosensory processes

3.1.1. Pain sensitivity assessed by the TF test

Factorial ANOVA revealed a significant effect of bred ($F_{(1,52)} = 6.56$, $p = 0.01$), treatment ($F_{(1,52)} = 4.70$, $p < 0.05$), sex ($F_{(1,52)} = 8.99$, $p < 0.005$) and bred and sex interaction ($F_{(1,52)} = 5.07$, $p < 0.05$) on the TF latencies measured at 9 weeks of age with the lowest pain sensitivity in SelTr male rats, while the pain threshold was similar in all 4 female groups. Thus we may consider that the males showed the highest sensitivity for selective breeding and treatment procedures in this respect (Fig. 1).

3.1.2. Sensory gating assessed by PPI of acoustic startle response

Regarding the relative startle response after PA, we observed that in selectively bred animals the female rats showed higher degree of startle reflex (SelNo: 95.69 ± 13.115; SelTr: 98.96 ± 14.944) compared to their male counterparts (SelNo: 42.27 ± 5.924; SelTr: 72.02 ± 9.566).

ANOVA of %PPI revealed a significant effect of bred ($F_{(1,52)} = 53.23$, $p < 0.0001$) and sex ($F_{(1,52)} = 6.19$, $p = 0.016$), furthermore of sex and treatment ($F_{(1,52)} = 4.86$, $p = 0.032$), and sex and bred ($F_{(1,52)} = 6.03$, $p = 0.018$) interactions. While treatment alone did not influence this parameter in naive rats, selective breeding significantly decreased it in both sexes (males: from 64.76 ± 8.673% to 23.06 ± 21.656%; females: from 72.88 ± 3.366% to 10.91 ± 13.636%, respectively). The treatment caused a significantly increased deficit in selectively bred females having the smallest %PPI (Fig. 2).

3.2. Motor and cognitive processes assessed by the HB test

3.2.1. Weight gain

The physiological effect of food restriction was monitored by weighing the animals before the beginning and on the days of hole-board test (6 times).

The body weight was significantly influenced by time ($F_{(5,260)} = 190.53$, $p < 0.0001$), bred ($F_{(1,52)} = 6.23$, $p < 0.05$) and sex ($F_{(1,52)} = 210.50$, $p < 0.0001$), and the interaction between time, bred, treatment and sex ($F_{(5,260)} = 3.63$, $p < 0.005$), that is the weight decreased with time and was the lowest in naive untreated female rats.

3.2.2. Behavioral processes

ANOVA of the summarized activity using the rearing, exploratory and locomotor activities during the habituation phase revealed that it was significantly influenced by selective breeding ($F_{(1,52)} = 4.66$, $p < 0.05$) and sex ($F_{(1,52)} = 8.79$, $p < 0.015$), with a lower activity in males and/or selectively bred animals (Fig. 3). The separate analysis of exploratory activity during the habituation phase showed decrease by time. Selective breeding resulted in a significantly decreased exploratory activity in all

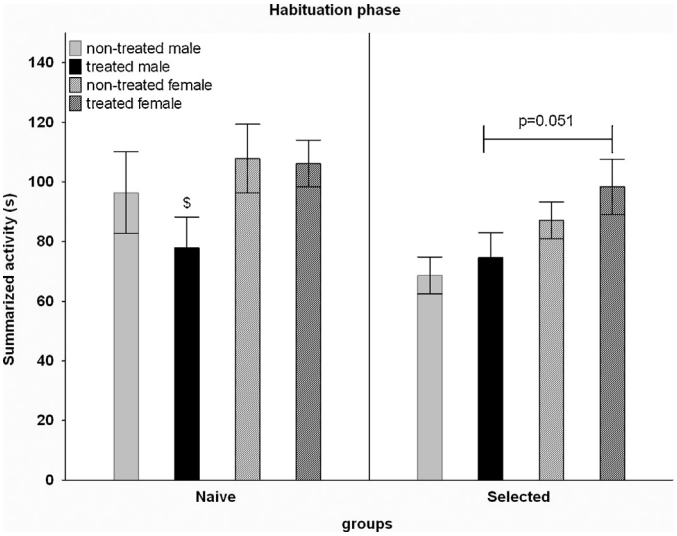

Fig. 3. Summarized activity (rearing, exploratory and locomotor activities) during the habituation phase in the hole-board task. It was calculated as an average of 5 min intervals. The symbol ($) denotes significant differences between the sexes of the corresponding groups. Data are presented as means ± SEM.

phases: habituation ($F_{(1,52)} = 7.85$, $p < 0.01$), learning ($F_{(1,52)} = 4.23$, $p < 0.05$) and trial phase ($F_{(1,52)} = 24.94$, $p < 0.00001$).

The incidence (frequency) of unsuccessful hole visits in trial phase correlates with the impairment in motor dexterity as a fine motor parameter [11]. Factorial ANOVA revealed that it significantly decreased by time ($F_{(7,364)} = 13.18$, $p < 0.00001$) and was effected by bred ($F_{(1,52)} = 31.84$, $p < 0.00001$), with significantly lower incidence in naive than in new substrain animals. Additionally the interaction of bred, treatment and sex was also significant ($F_{(1,52)} = 6.59$, $p < 0.05$) with the highest frequency in treated male rats of the new substrain.

The immobility time and the place preference were used to describe the anxiety-related behavior during the habituation and learning phases. These parameters were not influenced significantly either by the selective breeding, complex treatment or sex, but there was a tendency in the females to spend shorter time within the central part of the apparatus during the habituation (female: 12.34 ± 1.081 vs. male: 15.26 ± 1.771%) and learning phases (female: 8.27 ± 1.462 vs. male: 12.00 ± 1.962%).

Grooming activity, which may describe the level of physiological arousal increased by time and was not affected by selective breeding or treatment alone during the habituation phase; however, a tendency to an increased grooming behavior could be registered in treated male animals.

3.2.3. Cognitive processes

Both the naive and selectively bred animals were able to successfully learn the navigation task as indicated by the significant time and bred interaction of the learning capacity during the subsequent 10-minute learning sessions ($F_{(3,156)} = 3.41$, $p < 0.05$). However, selectively bred rats had significantly lower learning capacity than their naive counterparts, as reflected by the significant effect of bred ($F_{(1,52)} = 25.37$, $p < 0.0001$). It also revealed significant differences between the sexes ($F_{(1,52)} = 10.85$, $p < 0.005$), with lower values in males.

The cognitive parameters were significantly lower in selectively bred animals during both learning ($F_{(1,52)} = 25.53$, $p < 0.0001$) and trial ($F_{(1,52)} = 29.48$, $p < 0.0001$) phases (Fig. 4A and B). In addition, a significant effect of sex was also found during the learning phase ($F_{(1,52)} = 13.71$, $p < 0.001$), with the lowest learning capacity in male SelTr group animals. During the trial phase the interaction of bred, treatment and sex was also significant ($F_{(1,52)} = 4.56$, $p < 0.05$), with the poorest cognitive performance of the selected males.

The characterization of the visuo-spatial short term and long term memories during the trial phase further highlighted the cognitive deficits in the selectively bred and treated rats as indicated by the working memory (WM) and reference memory (RM) ratios calculated for each trial day (days 1–3).

The WM significantly improved by time ($F_{(2,104)} = 39.09$, $p < 0.00001$), decreased by selective breeding ($F_{(1,52)} = 19.98$, $p < 0.0001$) and revealed a significant effect by bred and sex ($F_{(1,52)} = 5.97$, $p < 0.05$) and bred, treatment and sex interactions ($F_{(1,52)} = 8.71$, $p < 0.005$) (Fig. 5A and B). Furthermore, the bred, treatment and sex interaction was also significant in time ($F_{(2,104)} = 4.61$, $p < 0.05$), with the smallest WM ratio in treated and selected males on the first trial day.

The RM ratio improved by time ($F_{(2,104)} = 35.19$, $p < 0.00001$) and revealed a significant decrease by selective breeding ($F_{(1,52)} = 15.80$, $p < 0.001$). The bred, treatment and sex interaction was significant ($F_{(1,52)} = 4.68$, $p < 0.05$), with the lowest RM ratio values in selected and treated males (Fig. 5C and D).

3.2.4. Correlation between the cognitive parameters

The regression analysis indicated significant correlations between the learning capacity and the other cognitive parameters determined during the trial phase: average WM ratio ($r = 0.74$), average RM ratio ($r = 0.50$) and cognitive performance ($r = 0.69$).

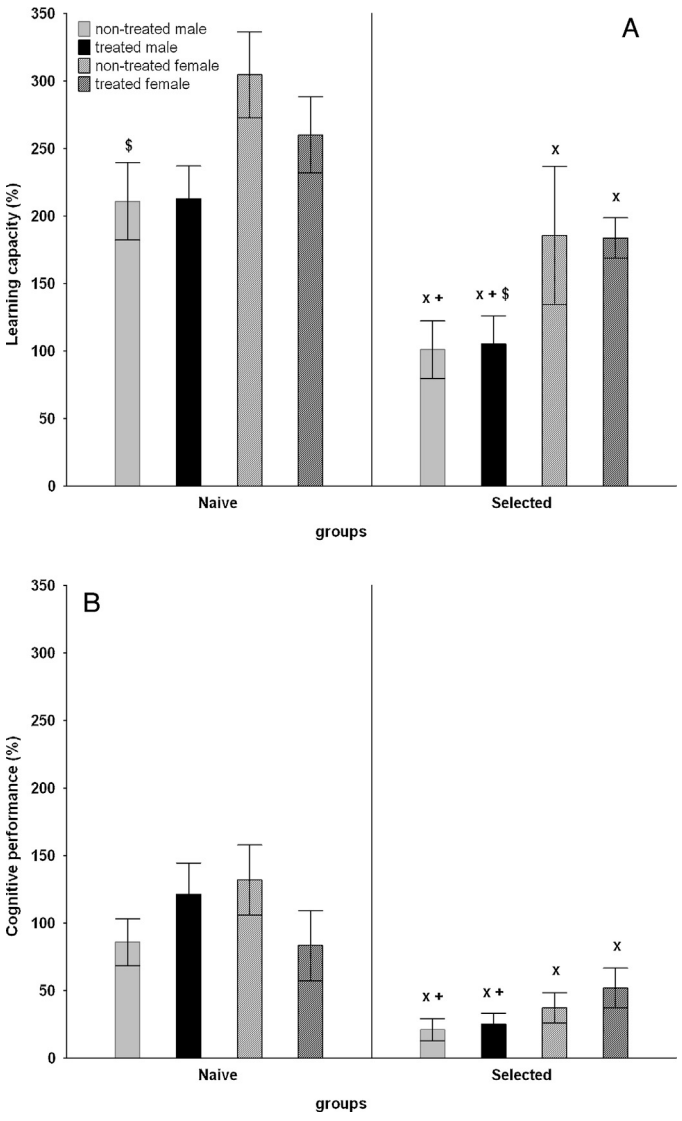

Fig. 4. Cognitive processes of the animals in the hole-board task. The cognitive processes are represented by learning capacity (A) and cognitive performance (B). The symbols indicate significant differences compared to: x naive non-treated (NaNo); + naive treated (NaTr) groups. Data are presented as means ± SEM.

3.2.5. Total schizophrenia score

Applying the median split, quartile-based scoring method for several parameters (indicated in Table 1), we classified the animals as either low- or high-risk for schizophrenia. Factorial ANOVA revealed a significant effect of bred ($F_{(1,52)} = 73.70$, $p < 0.00001$), bred, treatment and sex interactions ($F_{(1,52)} = 7.25$, $p < 0.01$). Thus the selectively bred animals received a significantly higher total score than their naive counterparts; furthermore, the selected treated male rats compared to female ones (4.42 ± 0.233 vs. 3.50 ± 0.250) (Fig. 6). Regarding phenotyping, it was not influenced significantly by complex treatment; thus, there was no significant difference between the SelNo and SelTr groups.

4. Discussion

An ongoing challenge of schizophrenia research is the development of chronic animal models with high validity. Here we applied

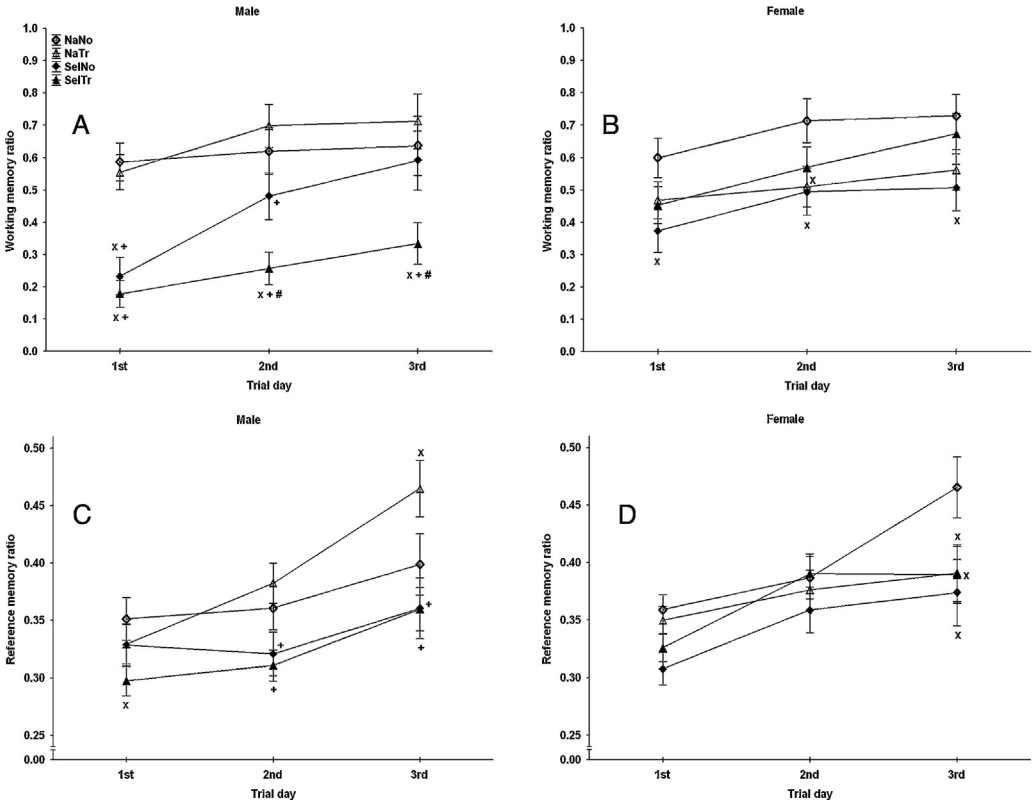

Fig. 5. Visuo-spatial long-term and short-term memory functions of the animals in the hole-board task. The memory functions are represented by working (A,B) and reference (C,D) memory ratios of males (A,C) and females (B,D) calculated by the trial phase results. The symbols indicate significant differences compared to the: x naive non-treated (NaNo) and + naive treated (NaTr) and # selectively bred non-treated (SelNo) groups. Data are presented as means ± SEM.

a "three hit" rat model with selective breeding after postweaning social isolation and subchronic ketamine treatment to develop a rat line with schizophrenic phenotype, furthermore, to assess resultant changes in adult animals with deficits commonly observed in schizophrenia. Regarding the pain sensitivity and sensory gating, present results reinforced our previous findings [7] on male rats; furthermore, also proved the sex specificity of these parameters:

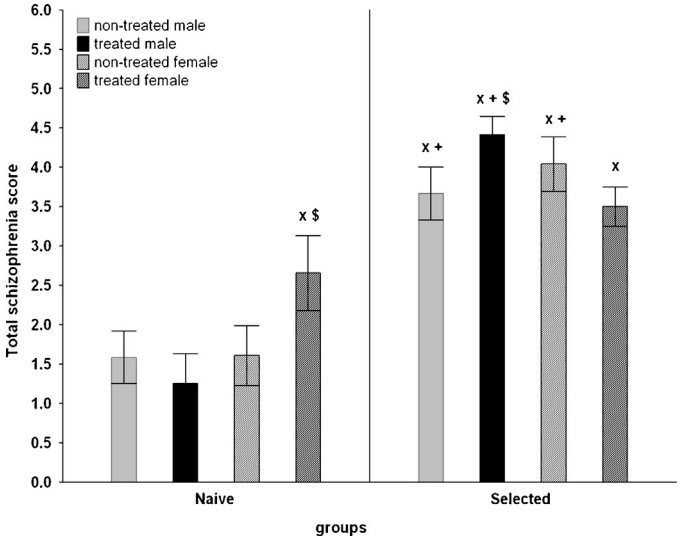

Fig. 6. Total schizophrenia score in the different groups. The symbols indicate significant differences compared to: x naive non-treated (NaNo) and + naive treated (NaTr) groups. The symbol ($) denotes significant differences between the sexes of the corresponding groups. Data are presented as means ± SEM.

females showed no alterations in pain sensitivity but produced higher level of diminished sensory gating and increased acoustic startle response compared to their male counterparts. However, treatment alone had no main effect on the observed parameters (except pain sensitivity), factorial ANOVA revealed significantly altered motor behavior, deficit in spatial learning capacity and memory functions by bred, treatment and sex interaction, which verify the significance of all three hits. Thus selectively bred and treated male rats showed impairment in motor dexterity and worse cognitive performance than their female counterparts indicated by significantly decreased WM and RM ratios. However, further experiments are required to exclude that not only the motor impairments of the selected animals reduced the ability of the rats to perform the task. The three-phase (habituation, learning and trial phase) version of the appetitively motivated cognitive type HB task is time consuming, thus, it cannot effectively be used for easy and fast testing of high amount of animals. The significant correlations between the learning capacity and the cognitive parameters of the trial phase suggest that the 10-minute learning session results by themselves could be suited to predict the cognitive performance making the task faster and increasing its adaptability.

However, schizophrenia affects both human sexes; known sex-dependent disparities exist in age of onset, clinical characteristics, treatment response, the course and prognosis of the disease [8,16–18]. The prefrontal cortex undergoes dramatic, sex-specific maturation during adolescence that shows significant sexual dimorphisms [19]. A study by Satterthwaite et al. has established for the first time that during this vulnerable period the patterns of development of cerebral perfusion (using arterial spin labeled MRI) are markedly different in males and females with significant implications for neuropsychiatric disorders with adolescent onset and strong sex disparities, such as schizophrenia

[20]. These findings may be relevant to both the development of normal sex differences in cognition and differential male–female vulnerability to psychiatric conditions. Other studies have suggested that the sex differences may instead be attributed to the male sex steroid hormone, testosterone, playing a detrimental role in schizophrenia [21]. It is suggested that sex hormones modulate and support neurotransmitter systems implicated in schizophrenia, such as serotonergic, dopaminergic and glutamatergic pathways [18]. A recent study of Locklear et al. suggests commonalities and fundamental differences in the intracortical amino acid transmitter mechanisms that regulate DA homeostasis in the male and female rat PFCs [22]. Several studies have shown that the behavioral and neurochemical effects of NMDA receptor antagonists can be modulated by sexual steroids [17,23–26], furthermore, the NMDA-receptor function appears to be modulated by brain-derived neurotrophic factor (BDNF) in a sex-specific and/or estrogen-dependent manner as well [16,27]. Chronic treatment with estradiol modulates NMDA receptor density in the rat brain [28], and it is protective against MK-801-induced PPI disruptions [17].

Both clinical data and experimental studies pointed out that many patients with schizophrenia are less sensitive to pain [29,30], have a deficit of the identification and categorization of pain both in themselves and in others [31] not only related to their empathic capacities, suggesting that these patients have a specific deficit of pain processing. Newson et al. have found that neonatal capsaicin treatment of rats as an intrinsic sensory deprivation produces brain changes (i.e., significant decrease in brain weight and corpus callosum thickness), especially in males but not in females, that are similar to those found in brains of subjects with schizophrenia [32], suggesting that the reduced pain sensitivity in schizophrenia might be the result of abnormal capsaicin-sensitive primary sensory afferent neurons and reduced connectivity in the somatosensory cortex. This could be a possible interpretation of the sex-differences in pain sensitivity found in our present study with significant decrease only in the treated males of the new rat line.

Prepulse inhibition of the acoustic startle response is a phenomenon that has been widely used as an operational measure of sensorimotor gating or information processing [33]. It is regulated by a cortico-limbic-striato-pallidal circuit and is impaired by a variety of experimental conditions, including brain lesions, drug treatment (i.e., NMDA receptor antagonist), social isolation, which makes it a viable animal model with relevance to schizophrenia as patients with schizophrenia have reduced PPI; however, the PPI impairment is non-specific [17]. Regarding the results of Schwabe' laboratory, the stable phenotype of breeding-induced PPI-deficits and reduced startle habituation in Wistar rats indicates that PPI has strong genetic determinants, and that selectively bred rats can be used for neuropsychological analysis [33]. Similarly to our present findings, Marriott et al. have found a significant effect on sensory gating for sex with males showing greater %PPI than females after postnatal (PD8-14) subchronic domoic acid treatment of SD rats as a putative animal model with relevance to schizophrenia; however, it was phase sensitive, detected only during the dark phase [34].

Motor abnormalities, both slowing of psychomotor activity associated with negative and depressive symptom clusters [35], and excessive motor activity more often accompanied by positive symptoms [36] are common in schizophrenia [9]. Although dopaminergic abnormalities have been implicated, the precise neurobiological basis of motor impairments in schizophrenia remains to be clarified [9]. Increased locomotor activity in response to a novel environment (HB test) and in forced swim test has also been registered in several rat models of schizophrenia [37–42]. Although, similarly to our findings, other studies have found decrease [43–45] or no change [46,47] in motor activity. It is important to know that there is basic difference between sexes regarding the locomotor activity and place preference, that is the activity both in the peripheries and in the central area is higher in females than in males [48]. Some schizophrenia-related animal studies show further sex-specific alterations in locomotor activity using open-field test with males being most affected, i.e., decreased total horizontal movement, low mobility time and center entries over the course of development [44,45,48].

A recent review [2] discusses evidence supporting the ideas that impairments in certain cognitive processes are the core features of schizophrenia, which reflect abnormalities in specific cortical circuits, i.e., prefrontal cortex, hippocampus, anterior cingulate, ventral tegmentum and parts of the basal ganglia [2,19,49–52], and may be present for years prior to the diagnosis of the illness [53]. Thus, to increase the face validity of schizophrenia animal models, the cognitive performance should also be assessed. The novel object recognition might be interpreted as a memory deficit, and the underlying process is a possible analog of declarative memory in humans [54–56]. Our previous results showed that selective breeding or complex treatment by itself or in combination resulted in impairment in the novel object recognition test, i.e., the ability to discriminate between novel and familiar objects was disturbed in male rats [7]. A recent study of Wiscchof et al. reported that prenatal lipopolysaccharide administration induced impaired recognition memory in both sexes of the offsprings with males being more affected [57]. To characterize the memory deficits in detail requires the application of other memory tests, i.e., for spatial memory. In animal experiments, working and reference memory performances are reflected by an animal's choice behavior, i.e., by visits and revisits of baited and unbaited locations [50]. Spatial working memory, characterized by a transitory limited capacity to hold information relevant only within a specific trial current, is considered a form of short-term memory; and spatial reference memory, which holds trial independent information, for example about the locations where food is hidden, is a form of long-term memory [50,58,59]. For optimal foraging, animals must learn to visit the baited locations only, and avoid revisiting locations from which they collected the bait within a trial. Pronounced deficits in spatial navigation have been described in some schizophrenia rat models using water maze task, indicated by a significantly longer latency to reach the target platform across training sessions and by making more working memory errors [60–62]. Furthermore, spatial learning on "Hebb-Williams maze", cognitive flexibility and working memory (spontaneous alternation and set-shifting procedures) was found to be negatively affected in young male Wistar rats after chronic neonatal MK801 treatment without affecting recognition memory [43,46]. In contrast, Li et al. in 2003 found that repeated PCP administration did not induce impairments in arm re-entry errors using the eight-arm radial maze task [47].

Though less widely used than variants of mazes, a number of HB-type tasks have proved suitable for investigating the effects of a large number of naturally occurring differences (i.e., strain, sex and age differences) and experimental manipulations on spatial learning and memory functions [49,50,58]. Most of the studies used it only as a non-cognitive version to determine the locomotor, anxiety-related, exploratory activity and stereotypes of the animals [37,40,44]. The task is sensitive to several interventions that are used to induce schizophrenia-related alterations. These interventions produced cognitive deficiencies and impaired acquisition in male rats indicated by the deficits of working and reference memories [38,52,63–66], increased trial duration and more errors [67] using HB-type tasks. The present results reinforced our previous findings regarding the recognition memory in males, that selective breeding produced impaired memory functions, since both the working and reference memories were disturbed [7]. Moreover, the defective working memory on the first testing day suggested a delayed acquisition of the task's working memory rule for selected

animals. The WM ratio of the treated, selectively bred animals also failed to reach the performance of the controls till the end of the task.

Sex differences also exist in cognitive functions; however, despite this fact, the majority of studies are still not including female animals. In animal research, sex differences in spatial tasks are not displayed consistently. The results of Faraji et al. have indicated task-specific sex-differences in spatial performance of naive Long-Evans rats in the wet-land Morris water versus the dry-land ziggurat task [49]. We also found a tendency in naive rats with a better cognitive performance in females. Lebowitz and Brown (1999) using a pole box for testing the animals show that, although female rats appear to have required more choices than the males to complete the task, there is no reliable difference between males and females [68].

The influence of sexually dimorphic behaviors in cognitive processes in schizophrenic patients [69] and schizophrenia rat models [24,49,68] have not yet been widely evaluated. A recent study has shown that sex differences in PCP-induced cognitive deficits may be attributed to BDNF expression [24]. In this study, female rats were shown to be more sensitive to PCP-induced deficits in attentional set shifting tasks and showed significant reduction in BDNF mRNA levels in several forebrain regions compared to males. Both long-lasting and single injections of estradiol were also protective against cognitive impairments induced by PCP treatment in the novel object recognition task [70,71]. Our present study was aimed at expanding previous studies on sexual dimorphism in spatial function using the cognitive-type HB task in selected animals with schizophreniform phenotype. We found a significantly higher sensitivity of SelTr males.

The concept of the endophenotype of psychiatric diseases [72] assumes that the biological basis of complex neuropsychiatric diseases can eventually be understood by studying phenotypes that are related both to the clinical symptoms but also to a fundamental biological mechanism [33]. It gives strong rationale for the investigation of the genetic determinants of behavioral traits in animals that can be quantified. In light of the putative association between our complex animal model and schizophrenia, the discrete behavioral differences observed may help elucidate the neurobiological correlates of schizophrenia. Our model appears to be informative for schizophrenia research from a behavioral perspective: selective breeding with subchronic juvenile ketamine treatment and social isolation is associated with (a) increased pain sensitivity, (b) disrupted sensory gating, (c) decreased directed exploratory motivation, (d) increased spatial learning time with decreased learning capacity and (e) deficiencies of cognitive performance, reference and working memory. Furthermore, most of the observed parameters showed sex-specific alterations with a higher sensitivity of male rats to schizophrenia-related symptoms (except sensory gating disturbance) that highlights the implication of sex as a factor in assessing schizophrenic animal models. The present results support the utility of our putative "three hit" animal model for studying the cognitive changes occurring in psychiatric disorders; furthermore, they reinforce its constructive and face validity to model schizophrenia in rats; however, it needs yet to be validated pharmacologically and the molecular biological background of these alterations should also be verified.

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