Received: 14 February 2018 | Revised and accepted: 24 July 2018

DOI: 10.1111/gbb.12507

Genes, Brain and Behavior

ORIGINAL ARTICLE

Impaired GAD1 expression in schizophrenia-related WISKET rat model with sex-dependent aggressive behavior and motivational deficit

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After peri-adolescence isolation rearing (IS) and subchronic ketamine (KET) treatment, adult, selectively bred Wistar rats (named WISKET) mimic abnormal behaviors reminiscent of human schizophrenia, including reduced prepulse-inhibition of startle reflex, disturbances in cognition, locomotor activity and thermoregulation, decreased pain sensitivity and electrophysiological alterations. To further validate our WISKET rat line, regarding its translational utility in schizophrenia research, we examined their social behavior and introduced a short and simple hole-board (HB)-like test to investigate their motivational deficit that predicts the cognitive disturbance. Sex-dependent alterations in schizophrenia may yield important insights into its etiology; thus, male and female WISKET rats were also investigated and compared with their naive Wistar counterparts. Considering the contribution of the hippocampal and cortical GABAergic inhibitory circuitry in these behavioral alterations, molecular-biology studies were also performed regarding the GAD1 gene products. Impaired social activity with increased aggression, stress-related behavior, active social withdrawal, motivation deficit and decreased exploration were observed, especially in male WISKET rats, compared with Wistar ones and their corresponding females. These alterations were accompanied by sex-dependent alterations regarding GAD67 mRNA and protein expression in the prefrontal cortex and hippocampus. In conclusion, the WISKET animals are valuable tools for animal-based preclinical drug discovery studies for predictive screening of novel compounds improving negative symptoms with potential antipsychotic efficacy.

KEYWORDS

aggression, behavior, GAD1, isolation rearing, motivation, schizophrenia, selective breeding, sex, social, two-hit rat model

1 | INTRODUCTION

Schizophrenia has a relatively high prevalence and it has an early onset in young adulthood, frequently with disabling symptoms.[1] The heterogeneity of the symptoms and also the typical human nature of them (eg, hallucinations, delusions and poverty of speech) are significant challenge to the development of appropriate animal models.[2] Schizophrenia is often associated with a certain profile of behavioral impairments, which are thought to represent endophenotypes and can be used to study the etiology and elucidate the pathophysiology.[3–7] Its clinical symptoms are based on brain dysfunctions attributed to gene-environment interactions.[8] Several researchers have pursued the risk genes with the greatest impact on the predisposition to the disorder.[9] Nevertheless, there are only few animal models by which the contributions of environmental and genetic factors to the pathobiology of the disorder can be investigated.[10]

The "two-hit" hypothesis proposes that neuropsychiatric illnesses may be elicited by a combination of two or more major disruptions at specific time points during their development.[11] Based on this, our

research group developed and characterized a complex, chronic rat model with features relevant to schizophrenia, based upon the well-established postweaning isolation rearing (IS) with subchronic ketamine (KET) treatment in a selected new rat line (WISKET). Our previous results proved that the combination of these insults was associated with deficits in sensorimotor gating, cognitive performance, acute heat pain sensitivity, body temperature regulation, locomotor activity and an altered electroencephalography (EEG) pattern.[12–17]

Asociability, as a negative symptom, is a pronounced behavioral feature in schizophrenia.[18–20] Nonetheless, the underlying pathophysiology is unknown, and currently available pharmacological treatments fail to reliably produce efficacious benefits regarding them. Utilizing rodent paradigms, such as social withdrawal and social cognition, to show the neurobiological substrates underlying social dysfunction and to identify novel therapeutic targets may be highly useful to understand more about the negative symptoms of schizophrenia.

Our goal was to investigate whether our WISKET rat model has face validity for the negative symptoms on social interaction (SI) test. In order to assess whether the behavioral effects on selected animals extend to or are different in female rats, we also investigated both sexes. Reductions in social activity are already present in the premorbid stages of the illness, and it has been shown that during early childhood, pre-schizophrenic children show significantly reduced play behavior. Social withdrawal usually worsens during exacerbations of the illness and generally persists throughout the entire course of the disease.[21] To investigate the age-dependence of social behavior, we tested the animals in different ages, before and after the complex treatment.

Cognitive disturbances also represent some of the most debilitating symptoms of neuropsychiatric disorders with a strong predictor of outcome, which are currently the most poorly treated features.[22–26] WISKET rats show cognitive impairments on novel object recognition (NOR), holeboard (HB) and Ambitus tests.[12,13,17] As the three-phase (habituation, learning and trial phase) version of the apetitively motivated HB task requires a long-time food restriction for the animals to maintain their motivation, it cannot effectively be used for easy and fast testing of high amount of animals, which is required in preclinical studies. Thus, in the present study, we used a one-phase task (simplified HB-like test). However, it only gives information on the presence of motivational deficit, it higly predicts global cognitive performance. Our previous data suggested that the 10-minute learning session by themselves could be suited to predict the cognitive performance increasing its adaptability.[12] The regression analysis indicated significant correlations between the learning capacity and the working memory ratio, reference memory ratio and cognitive performance, respectively.

Glutamic acid decarboxylase (GAD), which exists in two isoforms (GAD67 and GAD65), is the key enzyme for the synthesis of GABA, and it is critical for the developmental, homeostatic and activity-dependent regulation of GABA.[27] Genetic variation[28–30] and substantial dysregulation of GAD mRNA expression, as well as reductions in the release and reuptake of GABA have been observed in schizophrenia,[31–37] which appear at the level of the prefrontal cortex (PFC), hippocampus and cerebellum as well.[38,39] We hypothesized the abnormalities in the inhibitory circuitry that may contribute to the behavioral alterations in our WISKET rats. GAD1 (encoding GAD67 enzyme) mRNA and protein expression levels were used as the indicators of the GABAergic activity in the PFC and the hippocampal region.

2 | MATERIALS AND METHODS

All experiments involving animals were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: XIV/03285/2011). Animals were kept in a 12 hours light/dark cycle under conditions of controlled temperature (22°C ± 1°C) with ad libitum water and food access; except during the HB test, when they were food deprived for 2 days before the experiment. All efforts were made to minimize animal suffering and to reduce the number of animals used in the experiment.

2.1 | The WISKET rat model

The paradigm for selective breeding through several generations has already been described.[13] Briefly, weaning animals (postnatal day 21) were tested with the tail-flick (TF) test to assess their basal acute heat pain sensitivity, and then, they underwent IS between 4 and 7 weeks of age (Table 1). The animals in each generation were treated with KET (Calypsol, Gedeon Richter Plc., Hungary; 30 mg/kg/d intraperitoneally) from 5 to 7 weeks of age. Then, the animals were returned to standard social rearing; 1 week of recovery with no treatment was given to them before behavioral tests. Animals with the lowest pain sensitivity, highest sensory gating disturbance (prepulse inhibition [PPI]), impaired recognition and working memory were used for selective breeding throughout several generations.

2.2 | Experimental paradigm

Naive, socially reared male (n = 16) and female (n = 8) Wistar rats without KET treatment and the 21st generation of selectively bred WISKET male (n = 21) and female (n = 22) rats with complex treatment (IS and KET treatment) were involved in the experiments (Table 1). The body weight of rats was measured throughout the whole investigation period. The time-points for behavioral testing were determined based on Sengupta's study, who correlated human

TABLE 1 The experimental paradigm

Postnatal age	Interventions and behavioral tests
PD 21	Weaning, TF test 1
PD 23-24	SI test 1
Weeks 4-7	Isolation rearing (WISKET rats) or social rearing (Wistar rats)
Weeks 5-7	Subchronic ketamine treatment (30 mg/kg i.p. daily)
Week 8	Resocialization of WISKET rats
Week 9	TF test 2, PPI test
Week 10	Simplified HB-like test
Week 11	SI test 2
Week 12	Termination, brain dissection for molecular-biology studies

Abbreviation: PD, postnatal day.

year with rat days in different phases of life.[40] Thus, a rat in its 4 weeks of age is in the prepubertal period, whereas the period between 9 and 11 weeks of age refers to the adolescent and young adult periods.

2.3 | A simplified HB-like task

An appetitively motivated one-phase simplified HB-like task was used to predict the cognitive performance of the animal groups.[12] Food reward (puffed rice) was used as a positive motivation after 2 days of total food-deprivation. The task was to collect all the food rewards (16) within 600 seconds. The animals were placed into the center of the arena, and the behavior was recorded with an infrared video device (WCM-21VF, CNB, China). The task was applied only once. The durations of the basic activities, such as rearing (vertical) and locomotor (horizontal) activities, the time spent in the central area and self-grooming were evaluated (Table 3). The time spent with sniffing the holes was defined as general exploratory activity, supplemented by the latency of the first hole visit and the first reward eating. The motivation of the animals was determined as:

$$\text{Motivation index} = \left[\frac{\text{number of collected food rewards} \times \text{cut-off time of the task (600 s)}}{\text{number of food rewards (16)} \times \text{time required to complete the task (s)}}\right] \times 100$$

2.4 | Social interaction test

Weight- and sex-matched, unfamiliar pairs of rats with identical treatment were simultaneously placed in opposite corners of the unfamiliar testing chamber (15 × 34 × 33 and 60 × 34 × 33 cm for postweaning and adult rats). The animals' behavior was recorded for 600 seconds with an overhead infrared video camera and analyzed offline by trained observers, who were blind to the treatment groups. The test was repeated, which allowed us to evaluate trajectories across the neurodevelopmental stages of postweaning and young adults (Table 1). The parameters evaluated for social investigation included the time spent with sniffing each other, which was defined as social interest; the number of initiating attack, fights, pushing past and crawling over each other with physical contact were defined as aggression; and running away was defined as avoidance. For nonsocial exploratory behavior, the duration of rearing was quantified, and the duration of self-grooming was related to stress-behavior/anxiety.

2.5 | RT-PCR studies

2.5.1 | Tissue isolation

After behavioral testing, 6-6 male and female rats out of the Wistar and WISKET groups were randomly selected and terminated for molecular-biology studies. Their brain was removed and dissected immediately on dry ice and placed into RNAlater-ICE Frozen Tissue Transition Solution (ThermoFisher Scientific, Hungary). The whole hippocampus was dissected without separating its subregions. For the dissection of the PFC, a rat brain atlas was used for guidance: after the discharge of the olfactory bulbs, an approximately 1.5 mm coronal slice was hand-dissected to prevent the white matter from dissection.[41] The tissues were frozen in liquid nitrogen and stored at −75°C until the extraction of total RNA.

2.5.2 | Total RNA preparation

Total cellular RNA was isolated by extraction with guanidinium thiocyanate-acid-phenol-chloroform according to the procedure of Chomczynski and Sacchi.[42] After precipitation with isopropanol, the RNA was washed with 75% ethanol, and then re-suspended in diethyl pyrocarbonate-treated water. RNA purity was controlled at an optical density of 260/280 nm with BioSpec Nano (Shimadzu, Japan); all samples exhibited an absorbance ratio in the range of 1.6 to 2.0. RNA quality, and integrity was assessed by agarose gel electrophoresis.

2.5.3 | Real-time quantitative reverse-transcriptase PCR

Reverse transcription and amplification of the polymerase chain reaction (PCR) products were performed by using the TaqMan RNA-to-C_T-Step One Kit (ThermoFisher Scientific, Hungary) and an ABI StepOne Real-Time cycler. Reverse-transcriptase PCR amplifications were performed as follows: 48°C for 15 minutes and 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The generation of specific PCR products was confirmed by melting curve analysis. Table 2 presents the assay IDs for the primers used and the reaction parameters. All samples were run in triplicate. The fluorescence intensities of the probes were plotted against PCR cycle number. The amplification cycle displaying the first significant increase of the fluorescence signal was defined as the threshold cycle (C_T).

2.6 | Western blot analysis

The brain tissues were homogenized using a Micro-Dismembrator (Sartorius AG, Germany) and centrifuged at 11.000*g* for 30 minutes at 4°C in RIPA Lysis Buffer System, which contained phenylmethylsulfonyl fluoride, sodium orthovanadate and a protease inhibitor cocktail. The total protein amounts from the supernatant were determined by spectrophotometry (BioSpec-nano, Shimadzu, Japan). Twenty-five micrograms of sample protein per well were subjected to electrophoresis on 4% to 12% NuPAGEBis-Tris Gel in XCellSureLock Mini-Cell Units (ThermoFisher Scientific). Proteins were transferred from gels to nitrocellulose membranes using the iBlot Gel Transfer System (ThermoFisher Scientific). The Ponceau S (Sigma-Aldrich, Hungary) was used to check the standard running and transfer conditions. The blots were incubated overnight on a shaker with GAD1 (67 kDa) and β-actin (42 kDa) monoclonal antibodies (Santa Cruz Biotechnology, diluted 1:200, host: mouse, specificity: mouse, rat and human) in blocking buffer. Antibody binding was detected with the Western Breeze

TABLE 2 Parameters of the applied primers and PCR reactions

TaqMan assays	Assay ID (ThermoFisher scientific)	Accession number	Assay location	Amplicon length	Annealing temperature (°C)	Reaction volume (μL)
GAD1	Rn00690300_m1	NM_017007.1	480	63	60	20
β-Actin	Rn00667869_m1	NM_031144.3	881	91	60	20

Real-time reverse transcription polymerase chain reactions were used to determine the changes in mRNA expression. In our studies, the parameters of inventoried TaqMan assays were defined by Life Technologies (ThermoFisher Scientific, Budapest, Hungary).

Chromogenic immunodetection kit (ThermoFisher Scientific). Images were captured with the EDAS290 imaging system (Csertex Ltd., Hungary), and the optical density of each immunoreactive band was determined with Kodak 1D Images analysis software (New Haven, CT, USA). Optical densities were calculated as arbitrary units after local area background subtraction.

2.7 | Statistical analysis

Behavioral data were analyzed by using factorial ANOVA with group (Wistar and WISKET) and sex as factors. The data are expressed as means ± SEM. Post hoc comparisons were performed by using the Newman-Keuls test. Repeated measurement ANOVA was used to

TABLE 3 Significance of different tests and parameters by ANOVA

Behavioral test	Investigated parameters	Factors	*F* value	Significance
	Body weight	Age	$F_{14,882} = 5985.00$	$P < 0.0001$
		Group	$F_{1, 63} = 257.24$	$P < 0.0001$
		Sex	$F_{1, 63} = 103.10$	$P < 0.0001$
		Age × group	$F_{14,882} = 65.00$	$P < 0.0001$
		Age × sex	$F_{14,882} = 332.95$	$P < 0.0001$
		Group × sex	$F_{1, 63} = 9.30$	$P < 0.0001$
		Age × group × sex	$F_{14,882} = 26.08$	$P < 0.0001$
TF test	TF latency	Age	$F_{1, 63} = 174.16$	$P < 0.0001$
		Group	$F_{1, 63} = 73.16$	$P < 0.0001$
		Sex	$F_{1, 63} = 4.83$	$P < 0.05$
		Age × group	$F_{1, 63} = 20.75$	$P < 0.0001$
		Age × group × sex	$F_{1, 63} = 4.36$	$P < 0.05$
PPI test	% PPI	Group	$F_{1, 63} = 9.97$	$P < 0.005$
SI test	Rearing activity	Age	$F_{1, 63} = 206.20$	$P < 0.0001$
		Group	$F_{1, 63} = 19.4$	$P < 0.001$
		Sex	$F_{1, 63} = 10.79$	$P < 0.005$
	Grooming activity	Age	$F_{1, 63} = 21.46$	$P < 0.0001$
		Age × group	$F_{1, 63} = 5.25$	$P < 0.05$
		Age × sex	$F_{1, 63} = 13.91$	$P < 0.001$
	Social interest	Age	$F_{1, 63} = 43.76$	$P < 0.0001$
		Sex	$F_{1, 63} = 4.03$	$P < 0.05$
	Aggression	Group	$F_{1, 63} = 6.94$	$P < 0.05$
		Age × sex	$F_{1, 63} = 4.75$	$P < 0.05$
	Avoidance	Age	$F_{1, 62} = 4.63$	$P < 0.05$
		Group	$F_{1, 62} = 7.75$	$P < 0.01$
		Age × group × sex	$F_{1, 62} = 4.29$	$P < 0.05$
Simplified HB-like test	Rearing activity	Group	$F_{1, 63} = 5.95$	$P < 0.05$
		Group × sex	$F_{1, 63} = 7.66$	$P < 0.01$
	Grooming activity	Group	$F_{1, 63} = 14.23$	$P < 0.001$
		Sex	$F_{1, 63} = 5.74$	$P < 0.05$
		Group × sex	$F_{1, 63} = 4.95$	$P < 0.05$
	Locomotor activity	Group	$F_{1, 63} = 6.84$	$P < 0.05$
		Sex	$F_{1, 63} = 6.85$	$P < 0.05$
	Sniffing time	Group	$F_{1, 63} = 15.31$	$P < 0.001$
	Time in central area	Group	$F_{1, 63} = 13.58$	$P < 0.001$
	Learning capacity	Group	$F_{1, 63} = 12.63$	$P < 0.001$
	First hole visit	Group	$F_{1, 63} = 5.44$	$P < 0.05$
	First reward eating	Group	$F_{1, 63} = 6.66$	$P < 0.05$

evaluate the age-dependent effects. The correlation of behavioral parameters was assessed by linear regression analysis and calculation of Pearson correlation coefficients (Spearman R statistic). $P < 0.05$ was considered significant (Statistica 13.1, Dell Statistica, Round Rock, Texas, USA).

The molecular biology studies were carried out on six animals per group, and they were repeated three times. The unpaired *t* test was used for statistical analysis (Prism 5.0, Graph Pad Software, La Jolla, CA, USA).

3 | RESULTS

The body weight was significantly influenced by age, group and sex, and their interactions; thus, WISKET females had the lowest body weight compared with other groups during the whole experiment (Table 3).

Similar to the results of previous generations,[12,13] the WISKET rats showed sex- and age-dependent disturbances in pain sensitivity and sensory gating (Figure 1A,B, Table 3).

3.1 | SI test

3.1.1 | Basic activities

ANOVA showed the lowest exploratory activity in WISKET males (Figure 2A, Table 3) with the longest grooming activity at the age of 11 weeks (Figure 2B, Table 3).

3.1.2 | Social interaction

The social interest was decreased in each group by age (Figure 3A, Table 3). The tendency could also be observed to decrease in WISKET males compared with their naive counterparts on week 11; however, it did not reach a significant level.

WISKET males showed the highest aggression with escape behavior during the second SI test (Figure 3B,C, Table 3), but it was not characteristic for the other groups (Figure 3C). At the age of 11 weeks, higher individual differences were found in the WISKET groups regarding the aggressive behavior, especially in males (Figure 3D).

In order to determine whether the same animals showed heightened aggression and avoidance, or they formed distinct subpopulations of the WISKET males, we correlated these parameters. A significant correlation was found ($r = 0.44$; $P < 0.05$); thus, the animals with increased aggression also showed heightened avoidance behavior. However, we should mention that we may distinguish between three subpopulations based on the ratio of aggression and avoidance counts: one with similar behavior to the control animals, without aggression and avoidance behavior ($n = 11$); another with increased aggression ($n = 4$); a third one with both increased aggression and avoidance ($n = 6$). The avoidance and aggressive behaviors were also correlated by pairs. The linear regression analysis resulted no correlation between these behavioral parameters ($r = 0.101$). Thus, the increased aggression in one rat did not necessarily result in avoidance behavior in the other one.

Regarding the sex differences, the female WISKET rats showed similar social behavior to their naive counterparts (Figure 3).

3.2 | Simplified HB-like test

3.2.1 | Basic activities

Similar to the findings on SI test, ANOVA showed significantly the lowest rearing and the longest grooming activities in WISKET males (Figure 2C,D, Table 3) indicating their higher stress and anxiety level.[43]

Furthermore, WISKET rats of both sexes spent shorter time with locomotion; however, post hoc comparison showed a significant difference between Wistar and WISKET male rats (Table 3). During the offline analysis of video recordings, there were no visible signs for the disruption of the animals' physical ability to execute movements. WISKET groups independently of sex showed decreased exploratory

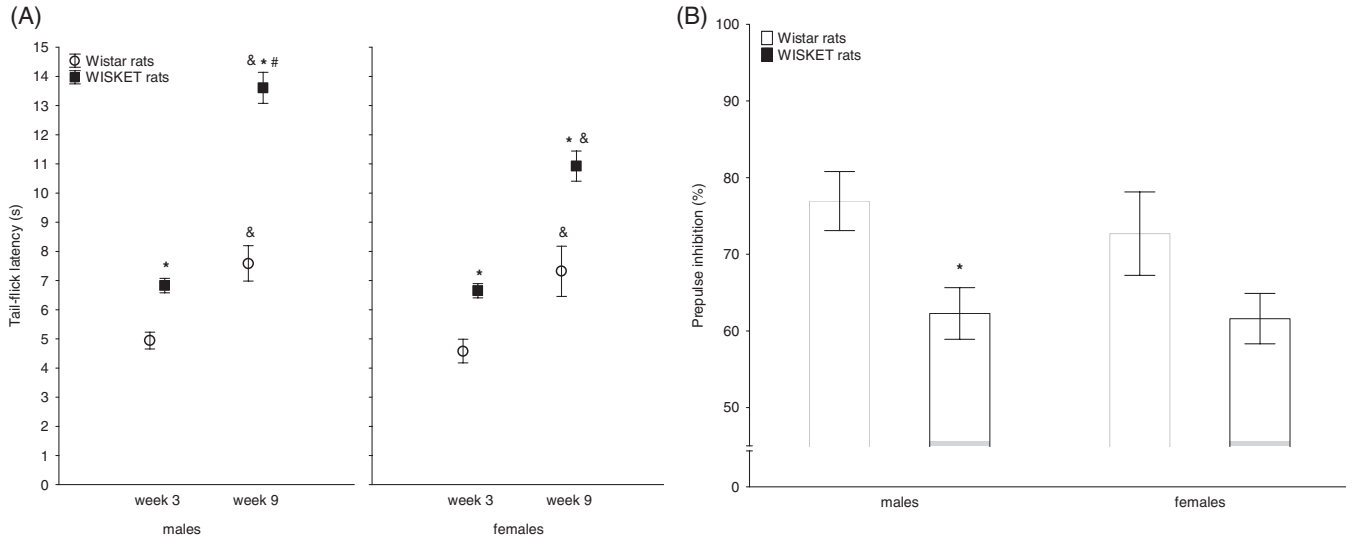

FIGURE 1 Pain sensitivity (A) and sensory gating process (B) indicated by the tail-flick latency and prepulse inhibition (%PPI) values in Wistar and WISKET rats by age and sex. Trials were performed at the age of 3 and 9 weeks, respectively. The symbols indicate significant differences ($P < 0.05$) by Newman-Keuls post hoc test between: The corresponding Wistar and WISKET groups (*); the sexes (#); and also between the trials (&). Data are presented as means ± SEM

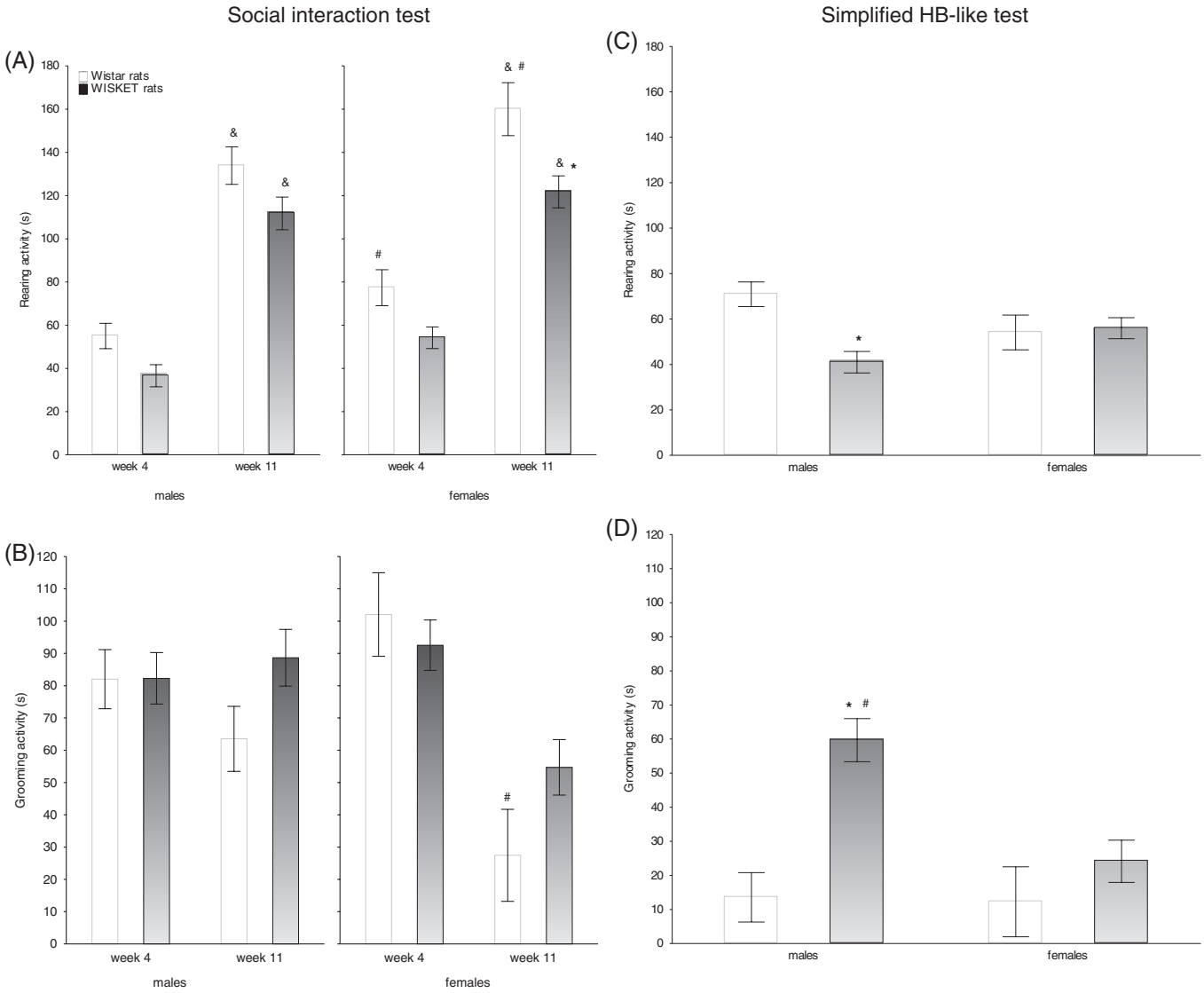

FIGURE 2 Basic activities (rearing and grooming) during the social interaction (A and B) and simplified holeboard tests (C and D) indicating the exploratory activity and stress-related behavior. The symbols indicate significant differences ($P < 0.05$) by Newman-Keuls post hoc test between: the corresponding Wistar and WISKET groups (*); sexes (#); and also between the trials (&). Data are presented as means ± SEM

behavior (sniffing time) and spent shorter time within the central part of the apparatus (Table 3).

3.2.2 | Motivation

Regarding the motivation of the animals, ANOVA showed the significant effect of the group with a lower motivation in WISKET animals, and an almost significant effect of sex ($F_{1,63} = 3.33$, $P = 0.072$, Figure 4A, Table 3). The post hoc analysis of data showed a significantly higher deficit in WISKET males compared with their female counterparts (Figure 4A), because they could collect significantly fewer food rewards (9.2 ± 1.17 vs 13.2 ± 0.66). Furthermore, WISKET males could collect fewer rewards (9.2 ± 1.17 vs 13.4 ± 1.09) and also required longer time (592.0 ± 7.95 vs 516.9 ± 26.78 seconds) to perform the task than their Wistar conspecifics. Both the latency of the first hole visit, and the first reward eating were increased in WISKET animals (Figure 4B,C, Table 3).

As there was no significant correlation between the locomotor activity and the motivation index by groups, it suggests that the decreased locomotor activity by itself could not result in the motivation deficit.

3.3 | RT-PCR and western blot studies

In male rats, the selective breeding of animals after complex treatment did not result in any significant change in the GAD1 mRNA expression in the hippocampal region, but it decreased significantly in the PFC compared with the naive samples (Figure 5A). In female WISKET rats, significantly increased mRNA expression was measured both in the hippocampal region, and in the PFC compared with the Wistar brain samples (Figure 5B). The same pattern of alterations could be observed in protein expression as well (Figure 5A,B).

4 | DISCUSSION

So far, accumulating evidence has suggested that our WISKET rat model has validity at several levels to mimic abnormal behavioral

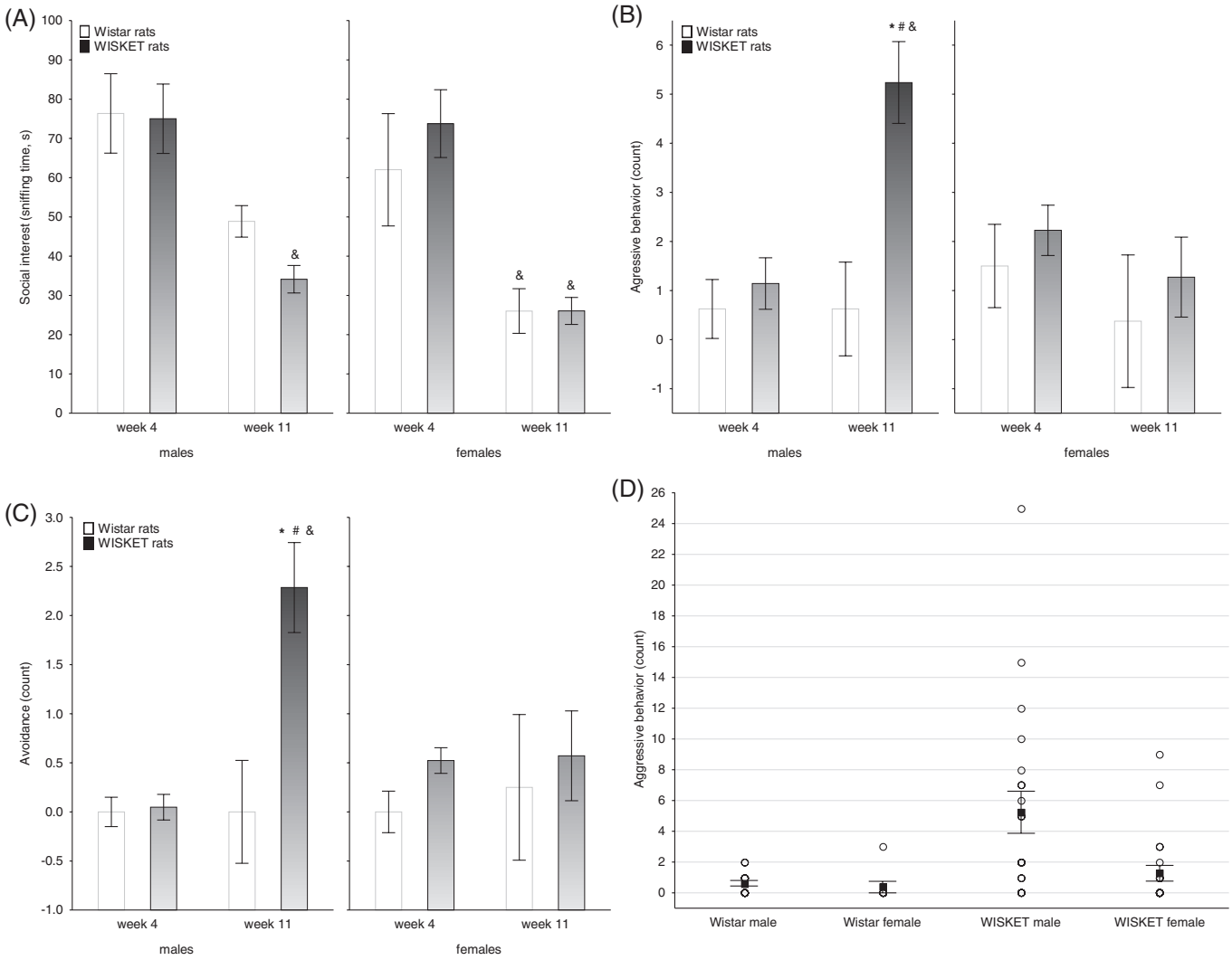

FIGURE 3 The social interest (A), aggressive behavior (B), and avoidance (C) of the animals during the social interaction test. Scatterplot with means ± SEM showing aggression behavior from individual animals at 11 weeks of age by groups (D). The symbols indicate significant differences ($P < 0.05$) by Newman-Keuls post hoc test between: the corresponding Wistar and WISKET groups (*); sexes (#); and also between the trials (&). Data are presented as means ± SEM

disturbances and neuropathology related to schizophrenia, which might be appropriate for preclinical screening of putative antipsychotic agents. We have found significant sensory gating disturbance to acoustic stimulation as well as decreased acute heat pain sensitivity, exploratory behavior, cognitive performance and social interest accompanied by increased aggression and avoidance, motivational deficit, especially in males.[12–16]

Similar to human patients, a decrease in SI has been described both in pharmacological animal models of schizophrenia, and in rats neonatally lesioned in the ventral hippocampus.[44,45] Social deprivation of rat pups from weaning also causes behavioral deficits in adulthood, which are unaltered by social re-integration in later life.[46–48]

Although it has been showed that the interaction between susceptibility genes and environmental factors after birth induces more apparent phenotypes of schizophrenia, only a few studies have investigated the social behavior in double-hit models.[49–55] KET with maternal deprivation or with sepsis has resulted in increased latency to start social behavior in adult rats without significant differences for the number of contacts and the time spent in social engagement.[52] On the contrary, rat offsprings exposed to both prenatal dietary iron deficiency and immune activation have displayed shorter latency to the first contact in the SI test compared with the groups having undergone single insults; thus, the second hit has improved the deficit.[51] No changes in social behavior have been published after concomitant IS and poly(I:C) treatment.[53] Whereas Schwabe et al.[54] have found no interaction between the effects of neonatal excitotoxic lesions of the rat medial PFC and subchronic pubertal phencyclidine (PCP) treatment on adult rat behavior, another approach has indicated that neonatal medial PFC surgery renders the brain vulnerable to the adverse effects of pubertal cannabinoid treatment, which then leads to disturbed social behavior.[55] Our present results showed that the complex periadolescent treatment was required for the observed behavioral alterations, because genetic vulnerability by itself was insufficient to elicit the same effects at the age of 3 weeks.

Sex differences in schizophrenia have long been recognized.[56] There are slightly higher rates, more severe clinical course with more negative symptoms, and earlier onset in males than in females. As sex hormones play an important role in brain development, it is perhaps

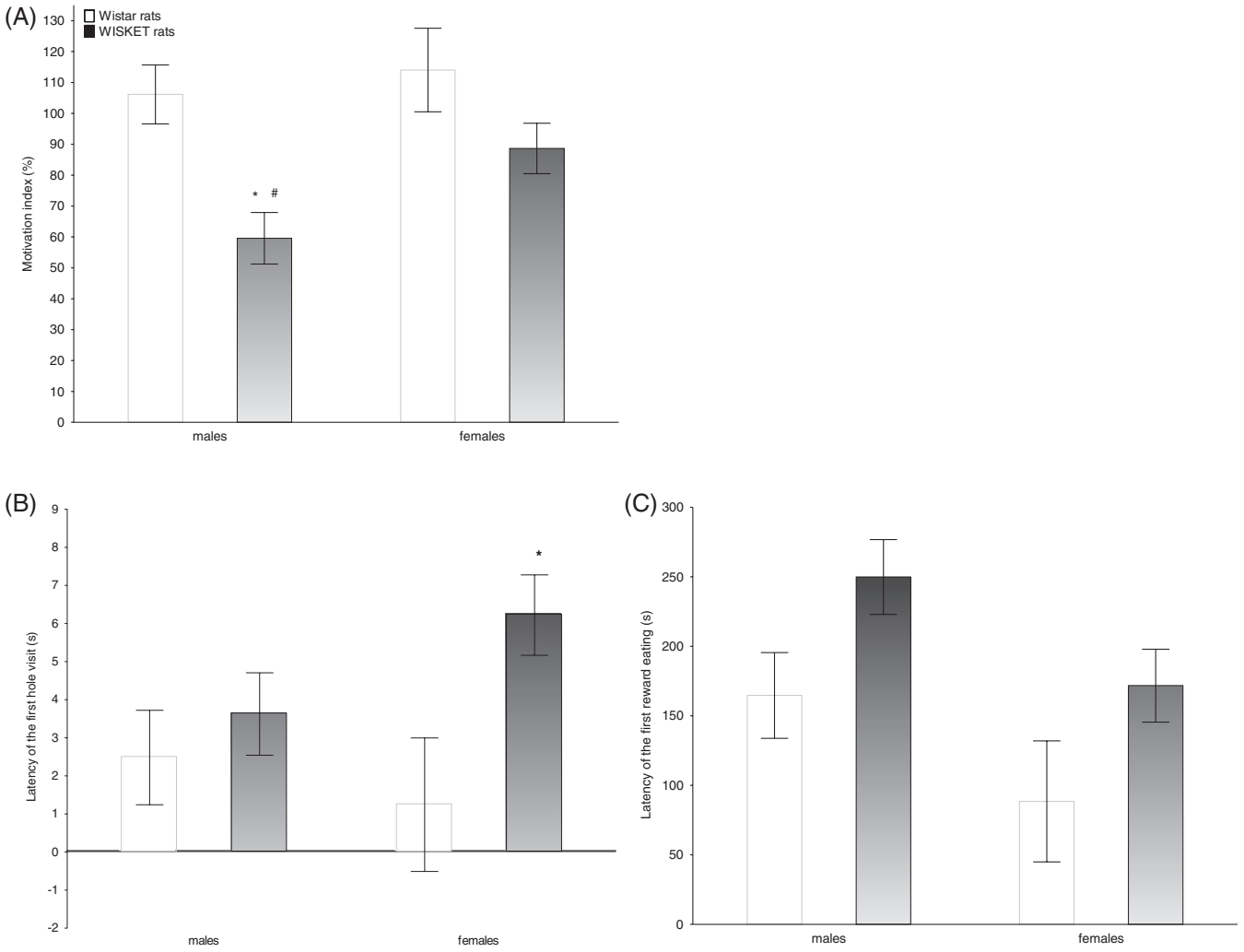

FIGURE 4 The motivation of the animals in the simplified holeboard test represented by the motivation index (A), the latency of the first hole visit (B), and eating (C). The symbols indicate significant differences ($p<0.05$) by Newman–Keuls post hoc test between: the corresponding Wistar and WISKET groups (*) and sexes (#). Data are presented as means ± SEM

unsurprising that rodents have also been shown to display sex-dependent susceptibility to the applied interventions. The majority of preclinical studies have been performed with males, each of them reporting a significantly reduced duration of active SIs and longer time spent in avoidance.[57–59]

So far, only one study has investigated sex-dependent alterations regarding social behavior in a double hit model: neonatal exposure to poly(I:C) with peripubertal unpredictable stress has resulted in more deficits in social preference in males.[49] Our present findings indicated a tendency to a decreased social interest in male WISKET rats compared with their naive counterparts at the age of 11 weeks accompanied by significant aggression and avoidance. In females, social interest decreased by age, but there were no differences regarding the groups.

Paranoia and increased anxiety in schizophrenia patients have been related to social impairments leading to misinterpretation of social stimuli and an increased sensitivity to social threat resulting in increased aggression and greater avoidance, too.[60,61] These data are consistent with those of schizophrenia-related animal studies, such as the neonatal ventral hippocampal lesion and treatment with PCP, KET and MK-801, reporting active social withdrawal.[44,45,58] A 2- to 10-fold increased incidence of aggression could be observed in schizophrenia patients.[62,63] Typically, aggressive behavior in rodents is assessed via paired interaction, where the test animal is confronted with an age-matched unfamiliar conspecific or younger intruder.[64] Similar to the findings in humans who suffered early social maltreatment, isolation reared rats show markedly more aggression than socially reared ones, which could be resilient to re-socialization procedures. It shows good correlation with our present findings, where the animals were tested 4 weeks after social rearing.[65–70] However, some studies using another strain and/or different time-intervals have found that aggression can be restored by resocialization.[71,72] Subchronic PCP treatment of male rats also reduces the initiation of affiliative contacts and increases aggressive responses, in the absence of drug outcomes on time spent in SI.[73] Although most of these studies have been performed in male rats, exposure to periadolescence social isolation can also result in increased aggression in females.[74] Our present experiment was a first attempt to shed light on sex-dependent affiliative and aggressive behavior in a "two-hit" rat model of schizophrenia with significantly higher abnormalities in males than females.

When aggression is present in schizophrenia, its relationship to psychopathology and cognitive dysfunction is unclear.[75]

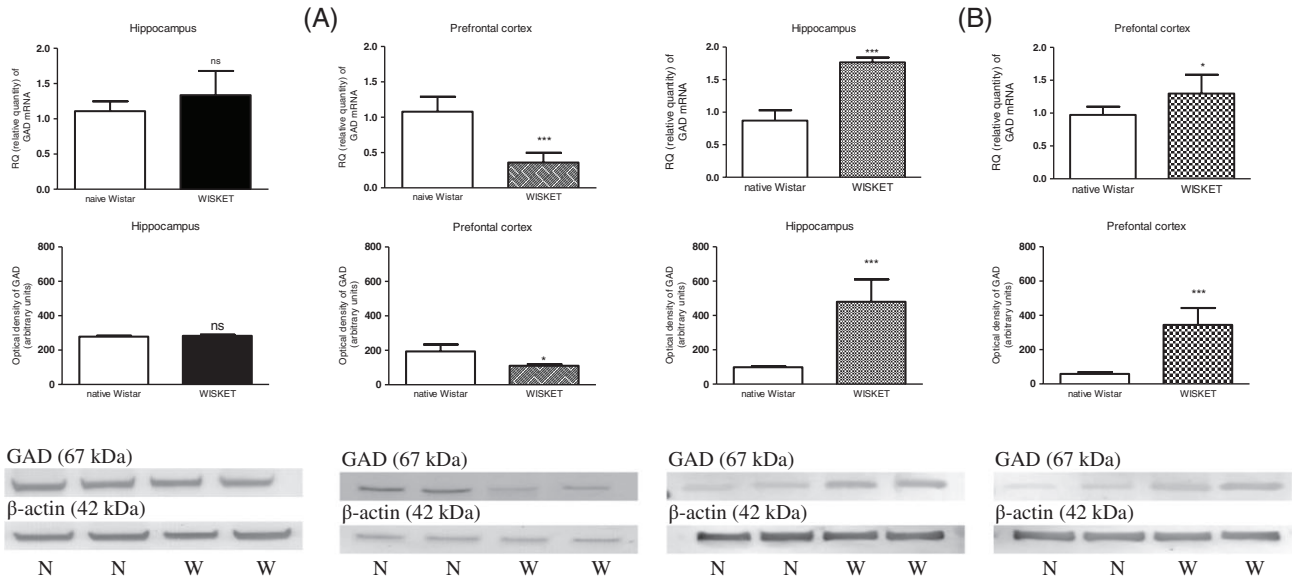

FIGURE 5 Results of RT-PCR and western immunoblotting experiments. The changes of mRNA and protein expressions of GAD1 in the hippocampal region and prefrontal cortex samples of male (A) and female (B) naive Wistar (N) and WISKET (W) rat brain. * $P < 0.05$, ***$P < 0.001$ as compared with the Wistar rat brain samples. Data are presented as means ± SEM; n = 6-6/group

Schizophrenia is associated with amotivation and avolition that interfere with a wide range of goal-directed activities.[76,77] It is difficult to distinguish what functional impairments result in decreased performance on a reward-based behavioral test. It may be due to motivational, reward valuation, or higher-order cognitive dysfunction, obscuring interpretations of specific deficits. However, the motivational deficit highly predicts global cognitive performance, and it is linked to poor cognition.[78] Additionally, besides the motivational deficit, all of the stress-related behavior that we observed, for example, decreased vertical rearing, increased total self-grooming time, negatively affect cognitive performance as well. Several studies have investigated the cognitive disturbances in "two-hit" schizophrenia rat models, especially in males. The combination of postweaning IS and postnatal MK-801 or PCP treatments has resulted in more severe impairments in recognition and contextual memory in adulthood than the single interventions.[79–81] The cognitive differences are most likely to be supported by sexual dimorphism in brain morphology and neurochemistry found in schizophrenia.[56,82] The sex-dependent cognitive deficits in the "two-hit" rat models of schizophrenia are less investigated.[83–85] Wistar rats exposed to neonatal maternal separation stress and chronic adolescent corticosterone treatment show sex-specific behavior in adulthood: male rats show marked disruptions in short-term spatial memory (Y-maze), which is absent in females, and they also show a learning delay in the Morris water maze test; however, little change in the T-maze test, and unchanged NOR and anxiety (elevated plus maze) have been detected.[84,86] These behavioral alterations were accompanied by region- and sex-specific long-term effects on brain-derived neurotrophic factor (BDNF) expression and signaling. Inescapable footshock exposure and corticosterone administration have also led to the inability to ignore irrelevant stimuli in male but not in female offsprings.[87] Shionogi mutant male developing rats treated with both a glutathione synthesis inhibitor or a dopamine uptake inhibitor have resulted in impaired recognition memory in NOR test in adulthood, whereas females have not been affected.[85] Neonatal domoic acid treatment with social IS also impairs attentional processing on latent inhibition in young adult male but not in female rats.[88] Our present results also indicated that a very simple and fast behavioral test was also appropriate to detect the presence of sex-dependent motivational deficit instead of using really complex and time-consuming tests predicting specific cognitive disturbances.[78] Thus, the performance of females was found to be less affected by the complex treatment, which could be related to the findings that women are less vulnerable to schizophrenia than men.[83] Despite the large number of studies showing sexual dimorphism in cognitive function in schizophrenia, other studies have shown no difference by gender.

Aberrant mesolimbic and mesocortical dopamine, glutamate, and serotonin neurotransmission along with inflammatory processes and altered BDNF signaling have been widely reported in parallel to the above mentioned behavioral changes.[68] The alteration in GABA concentration is also related to aggression both in humans and rodents.[89] Only some studies have investigated GABAergic system in "two-hit" schizophrenia rat models beyond phenotypic alterations in males. Combined neonatal injection of PCP or MK801 and post-weaning social isolation have produced impaired recognition memory, accompanied by significant downregulation of the hippocampal genes involved in GABA receptor signaling, decreased GAD67 expression in the medial PFC, and increased GAT-1 activity in the frontal cortex.[90] Marriott et al have also found some tendencies toward differences in GAD65 or GAD67 protein expression, in either the PFC or hippocampus, after postweaning social isolation and neonatal domoic acid treatment.[91] Similar to the recent findings of Tzanoulinou et al[92], who have found decreased level of GAD67 mRNA in the central nucleus of amygdala in addition to sociability deficits and increased aggression, we also observed suppressed GAD1 gene expression in PFC accompanied by the same behavioral phenotype in males.[93] The reduction of GAD67 expression might lead to reduced GABAergic control over the glutamatergic cells; therefore, pharmacological interventions that raise

GAD67 expression could represent novel targets for antipsychotic therapy. Interestingly, in female WISKET rats, we observed increased mRNA and protein expression both in the PFC and hippocampus, which may partially explain the behavioral gender-differences, suggesting that estradiol and/or luteinizing hormone can mitigate social deficit through the GABAergic pathway.[92] Similarly, in a trimethyltin-induced hippocampal neurodegeneration model increased expression level of GAD67 gene in CA1 stratum oriens, CA3 pyramidal layer, hilus and dentate gyrus was founded.[94] However, clearly sexual steroids can affect the activity of GAD enzyme, the results of activity assays are contradictory and inconclusive. After estrogen treatment brain region- and isoform (GAD 65 or GAD67) specific increase, decrease or no change in GAD mRNA levels were also reported in ovarectomized rats.[94–96] Furthermore, Ortiz et al have found sex- and region-specific corrrelation between the radial arm water maze acquisition of rats and GAD65 mRNA expression after chronic unpredictable stress administration.[97] While in male rats, GAD65 expression in the medial amygdala was negatively correlated with total errors on day 1 of training; in female rats a positive correlation was found in the hippocampal CA1 region. Neither complex schizophrenia-related animal models, nor post mortem human studies investigated sex-dependent alterations of GAD1 gene expression.

The lack of additional groups with single-hit (only isolation reared or KET treated) to determine the contribution of the different interventions to the face validity of the model might be seen as a major limitation of the study. However, from an animal welfare point of view, it would be difficult to argue the necessity of extra experiments for this purpose, once it has been proven that the model is valid. In our previous paper, we provided ample evidence that the WISKET rat line, after the complex treatment, has the highest validity as a schizophrenia model compared with the appropriate control groups, suggesting that the combination of genetic and environmental factors lead to the best model in this paradigm.[13] From that time, we concentrated on the characterization of this complex model in several aspects.[12,14–17] Furthermore, based on the two-hit hypothesis, some aspects of the functional impairment in schizophrenia and other neurodevelopmental diseases may be better modeled by using multiple "hit" models of disease risk.

In conclusion, our present findings may further increase the face and constructive validity of our WISKET rat model, which might reinforce its translational utility for animal-based preclinical drug discovery studies for predictive screening of novel compounds by improving negative symptoms and motivational deficits with potential antipsychotic efficacy.

ACKNOWLEDGMENTS

This work was supported by a Research Grant of the University of Szeged, Faculty of Medicine. The authors wish to thank Agnes Tandari for her excellent technical assistance and are grateful to Csilla Keresztes for the linguistic review of the manuscript.

Conflict of interest

We declare that the experiments comply with the current laws of Hungary. We certify that there is no actual or potential conflict of interest (including any financial, personal, or other relationships) in relation to this article.

Author contributions

A.B. did the animal experiments and took part in the study design. E.D. performed in vitro PCR and Western blot studies. G.H. and G.K. designed the study, wrote the protocol and the first draft of the manuscript. G.B. undertook the statistical analysis and managed literature searches. All authors contributed to and have approved the final manuscript.

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How to cite this article: Büki A, Horvath G, Benedek G, Ducza E, Kekesi G. Impaired GAD1 expression in schizophrenia-related WISKET rat model with sex-dependent aggressive behavior and motivational deficit. *Genes, Brain and Behavior*. 2018;e12507. https://doi.org/10.1111/gbb.12507