Behavioral and autonomic characterization of chronic schizophrenia rat model (WISKET)

Phd. Thesis

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ABBREVIATIONS

ANS – autonomic nervous system

DA – dopamine

GABA – gamma-aminobutyric acid

GAD – glutamic acid decarboxylase

GLU – glutamate

HB – holeboard

i.p. – intraperitonally

MAM – methylazoxymethanol acetate

NMDA – N-Methyl-D-aspartatic acid

NOR – novel object recognition

PCP – phencyclidine

PLR – pupillary light reflex

PolyI: C – polyriboinosinic-polyribocytidilic acid

PPI – pre-pulse inhibition

TF – tail-flick

1 INTRODUCTION

1.1 Definition of schizophrenia

Schizophrenia has been described as the "worst disease affecting mankind" [1]. It is an extraordinarily complex syndrome with multiple psychological domains including, impaired mental and social functions. While written documents **that** identify schizophrenia can be traced for more than two thousand years; schizophrenia was classified as a distinct mental disorder only in 1887 by Kraepelin. He was the first who did a distinction in the psychotic disorders between what he called dementia praecox and manic depression. In 1911 Eugen Bleuler developed Krapelin's ideas on the diagnosis of this condition and introduced the word "schizophrenia". The concept of schizophrenia subsequently was revised several times over the past century and the current clinical criteria is described by two major classification systems, Diagnostic Statistical Manual V and International Classification of Disease ICD-11 [2].

1.2 Symptoms

Schizophrenic disorders are characterized by various signs, which are generally classified into positive (hallucinations, delusions and thought disorder), negative (deficits in social interaction, emotional expression, motivation and speech difficulties) symptoms and cognitive dysfunctions (impaired attention, information processing, problem-solving, verbal and visual learning and memory) [2]. Several other, non-specific sings can also be observed in schizophrenia, such as sensory gating disturbance, decreased pain sensitivity and alterations of the autonomic nervous system (ANS) [3-10]. Most of these abnormalities are differentially expressed across patients and through the course of the illness [2]. Sensory gating, as measured by prepulse inhibition (PPI) of the acoustic startle reflex, includes neurological processes of filtering out redundant or unnecessary stimuli in the brain, and it prevents an overload of irrelevant information in the higher cortical centers of the brain [11]. It is reduced in a variety of neuropsychiatric disorders, including Huntington's disease, Tourette's syndrome, autism spectrum disorder, bipolar or panic disorder, and schizophrenia as well [3, 4]. Various studies have described that persons with schizophrenia appear to be less sensitive to pain compared to healthy controls [7, 12-15]. Thus, they have enhanced threshold and tolerance to different nociceptive stimulus.

1.2.1 Cognitive dysfunction

Cognitive impairment in schizophrenia have been recognized since the description of dementia praecox by Kraepelin [2]. It is highly prevalent in patients, precedes the onset of substantial symptoms and generally persists throughout the entire course of the disease. A rather unique feature of the cognitive deficits is that they remain relatively stable in severity and character within the same patient over time [16].

The range of cognitive deficits in schizophrenia is broad and multiple domains are affected: attention, executive functions, verbal and spatial working memory [17-23], which means the subject cannot maintain the spatial location of visual information. Schizophrenia patients show inflexible thinking, they have trouble adapting to changes in environment that require different behavioral responses. They also have reduced ability to learn new information, but the ability remains intact to retain information once it has been learned [24-26].

In the development of cognitive impairments some of the discovered genes (e.g. disrupted in schizophrenia 1, reelin, dysbindin and neuregulin 1) associated with schizophrenia are responsible for disrupted regulation of neuritic growth and migration in the developing brain, which can lead to an altered neuroplasticity [16]. The imbalance in the major neurotransmitter systems (e.g. dopamine, glutamate and GABA) (see section 1.3) leads to abnormal synaptic connections and plasticity playing a major role in impaired learning and information processing [27] .

1.2.2 “Social brain”

The social cognitive skills develop in infants over time, the individual consciousness arises from the mother and then from the social experience [28, 29]. The “social brain” concept has been described by Brothers as the higher cognitive and affective system in the brain that evolved as a result of increasingly complex social selective pressures [30]. It provides the substrate for intact social cognition, social behavior and affective responsiveness. It identifies anatomical and functional neural basis, which consists of complex neural interconnections that links the lobes of the brain and connects the superficial cortex to the limbic system [28, 31-33]. This network includes the dorsolateral prefrontal cortex, the orbitofrontal cortex, the anterior cingulate cortex, the amygdala, the superior temporal gyrus and the parietal association cortex [31-34].

There is strong evidence for “social brain” dysfunction in a variety of mental disorders; schizophrenia particularly represents the “ultimate” social brain disorder [28]. In schizophrenic patients functional and structural connectivities of this network are damaged.

The basic problem relates to the sense of detachment and disembodiment from “social self” and “social world” in them [28], which manifest in deterioration of patients’ interpersonal abilities, such as reduced play behavior in the premorbid stages of the illness (childhood), nonpurposeful aggression and avoidance [26, 28]. These disturbances contribute mainly to poor functional outcomes, including the lack of motivaton and inability to engage in meaningful work [27].

1.2.3 Autonomic impairments

Several studies suggest that schizophrenic patients have an elevated risk of stress sensitivity and ANS imbalance with the dominance of the sympathetic nervous system [35, 36]. This leads to reduced heart rate variability, disturbed thermoregulation and diminished pupillomotor control. All these impairments may develop to serious cardiac events and metabolic complications leading to increased mortality and shortened life expectancy [36-40].

A body of evidence has been amassed over time that demonstrates impaired thermoregulation in people with schizophrenia. Disturbed adaptation to heat or cold stress, altered baseline temperature with abnormal daily range/fluctuation of temperature has been reported [41-44]. Biochemical and physiological explanations of the above are inconclusive, with researchers postulating a dichotomy of theories: a “peripheral” abnormality is related to impaired heat loss through peripheral vasodilatation via abnormalities in responses to niacin and prostaglandin E1, and a “central” abnormality due to disruption of the mesolimbic dopamine system might be responsible for temperature regulation and psychosis, too [45]. The reality is that the underlying mechanism(s) may well involve a combination of both mechanisms, either with equal importance or with one playing a predominating role [45].

Clinical studies obtained in schizophrenic patients revealed diminished pupillomotor control including increased resting pupil diameter, abnormal latencies and decreased reactions to light stimuli [36, 38-40, 46-48]. These results also suggest an increased sympathetic modulation and/or decreased parasympathetic activity in these patients [48-51]. The pupillary function is determined by a balance between the sympathetic and parasympathetic ANS depending on a number of factors, such as genetic influences, age, wakefulness, accommodative state and ambient lighting conditions [52].

1.2.4 Epidemiology and etiology

Schizophrenia affects approximately 1 % of the population worldwide [53, 54]. Despite the intense study over the past century, its etiology and pathophysiology remain relatively obscure.

The incidence (the number of new cases annually) is about 1.5 per 10,000 people [55]. The age of onset is typically during adolescence, childhood and late-life onsets (over 45 years) are rare [55, 56].

It is well known that this disorder aggregates in families, thus the heritability of schizophrenia are now well established. Epidemiologically, schizophrenia results from the cumulative effects of genetic susceptibility and the environmental factors contribute about 80 % of the liability for schizophrenia demonstrated also by twin studies [56-59]. Thus, it is unlikely that a single gene mutation or a single adverse life event is sufficient to increase the incidence of schizophrenia, and it is thought that it is the interactions between or within such factors [60]. Studies to discover the genes associated with schizophrenia have been carried out globally by metaanalyses: N-methyl-D-aspartatatic acid (NMDA) receptor subunit 1 and 2A, disrupted in schizophrenia 1, neuregulin 1, dysbindin, and reelin have been proposed to date as risk factors [61]. Various prenatal and postnatal environmental factors, (e.g. infectious diseases during pregnancy, malnutrition, or hypoxia in the fetus, environmental toxins, vitamin D deficiency, disruption of social environment, chronic stress accompanied with urbanicity, migration and poverty, cannabis and other substance use) all together may lead to disturbed maturation of the brain and contribute to the development of schizophrenia and subsequently result in manifestation of schizophrenia-like phenomena in young adulthood [56, 61-66].

1.2.4.1 Sex differences

The sex differences in clinical appearance and outcome of schizophrenia have long been recognized. Slightly more men than women are diagnosed with schizophrenia (on the order of 1.4:1) and the onset is between 18 and 25 years of age for men and between 25 and 35 for women [67-69]. Women have shorter hospitalizations and relapses and superior functioning, whereas they tend to have more affective symptoms and fluctuation of their psychopathology [67]. Possible explanation for these differences may include the protective role of oestrogens [70, 71].

Sex differences have been observed in the cognitive deficits, i.e. males consistently express more severe cognitive deficits from the premorbid phase of the illness, thus poorer performance in men were detected in attention, language, verbal learning, memory and

executive function compared to women [68]. Sex-specific neuroanatomical differences and their relation to cognitive deficits have been addressed: the prefrontal cortex, the anterior cingular cortex and the hippocampal region tend to be preserved in women relative to men [72].

The social functioning in schizophrenia also shows considerable sex differences from puberty on [69]: social deterioration is less severe in premenopausal women than in man [68]. Male patients with schizophrenia show socially adverse illness behavior, a higher degree of social dysfunctioning especially in adolescence and early adulthood. Moreover dissocial behavior and aggressiveness are more likely developing in men than in women. Female patiens tend to come to terms with the illness better and show a higher tendency to social conformity and therapy compliance; however, they show a greater frequency of neurotic disorders [68, 69].

Regarding the autonomic regulation no substantial sex differences have been observed in patients with schizophrenia [73].

1.3 Neurochemical theories

Abnormalities of the major neurotransmitter systems such as the dopaminergic, glutamatergic and gamma-aminobutyric acid (GABAergic) ones have been reported in the brains of individuals with schizophrenia [74, 75]. It is supposed, that one of the following theories, or more likely the interactions of them, could explain the variety of symptoms seen in schizophrenia.

1.3.1 Dopaminergic system

The “dopamine (DA) hypothesis of schizophrenia” attributes the symptoms to abnormalities in DA transmission. The baseline mesolimbic and mesocortical dopamine output yield normal psychiatric functioning. Hyperactivity of mesolimbic dopaminergic pathway is primarily the cause of positive symptoms of schizophrenia [76]. The dopaminergic hyperactivity in the striatum may be the result of a greater number of DA D2 receptors that have a higher affinity for DA and/or an enhanced presynaptic accumulation of DA in the striatum (Fig. 1A) [77-79] Thus, dopaminergic agonist agents may trigger the positive signs, DA D2 receptor antagonists relieve them (Fig. 1A) [80, 81].

The negative symptoms are mainly associated with hypodopaminergic mesocortical transmission, that might be a direct result of little amount of DA neuronal firing originating in the midbrain and allowing poor DA release and activity of the frontal cortex (Fig. 1B) [82]. The association of negative symptoms with hypodopaminergic input in the prefrontal cortex is

suggested to involve DA D1 receptors. In schizophrenic patients, reductions in density of DA D1 receptor and dopaminergic innervation have been described in the prefrontal cortex [83].

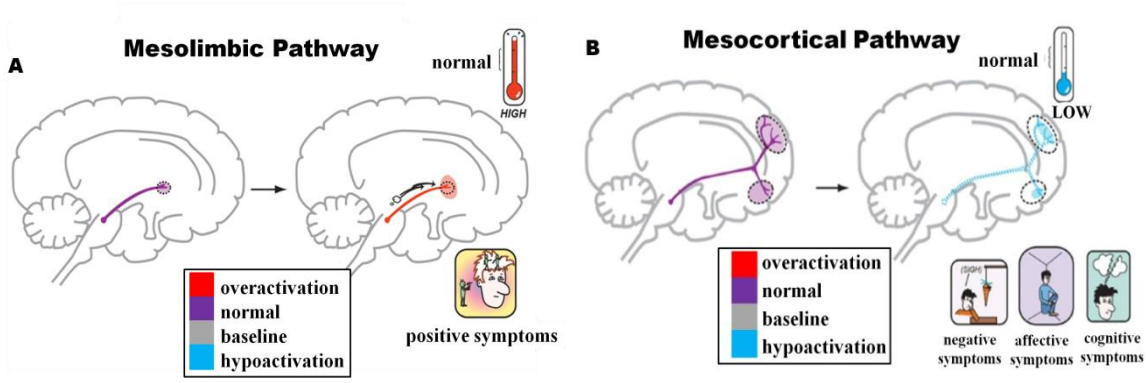

Figure 1. Hyperactivity of the mesolimbic pathway leads to the development of positive symptoms (A), while hypoactivity of mesocortical pathway is responsible for the negative symptoms of schizophrenia (B) [82].

1.3.2 Glutamatergic system

The "GLU hypothesis of schizophrenia" as an alternative hypothesis to the DA model was first proposed in the early 1990s [84]. Normal GLU-GABA-GLU-DA and GLU-GABA-GLU-*GABA*-DA circuits are balanced and with normal psychiatric functioning right amount DA is projected to the frontal cortex and allows the correct DA activity to occur (Fig. 2A) [82]. Positive symptoms are generated, when GLU-GABA-GLU-DA circuit is abnormal: GABA interneurons with poorly functioning NMDA receptors no longer fire adequately. The loss of GABA activity will cause the secondary GLU neurons to abnormally increase their firing rates, which stimulates the DA mesolimbic pathway directly and causes excessive DA neuronal activity (Fig. 2A) [82]. Thus, NMDA receptor hypofunction due to the reduced expression or structural alterations of NMDA receptors in cortical neurons can lead to an impaired GABAergic transmission, therefore the disinhibition of mesolimbic dopaminergic system generates positive symptoms [90].

This excessive phasic DA activity during psychotic episodes increases the glutamate release, resulting in oxidative stress, neuronal damage and neurotoxicity, which can induce morphological changes and a decrease in mesocortical dopamine producing negative symptoms as well as cognitive deficits (Fig. 2B) [95-98]. These disturbances and the hypofrontality can also occur by an abnormal GLU-GABA-GLU-*GABA*-DA circuitry [87]. The secondary GLU neurons stimulate another GABA interneurons trough α-amino-3-hydroxy-5-methyl-4-

isoxazolepropionic acid also known as AMPA receptors leading to enhanced GABA release. The increase in GABA causes DA neurons to be inhibited and fire less, thus the mesocortical DA pathway becomes hypoactive [87].

Consistent with these theories, compounds blocking neurotransmission at NMDA-type ionotropic glutamate receptors, such as phencyclidine (PCP) and ketamine, induce psychotic symptoms and neurocognitive disturbances, including thought disorder, social withdrawal and catatonia in healthy volunteers [89, 99-101].

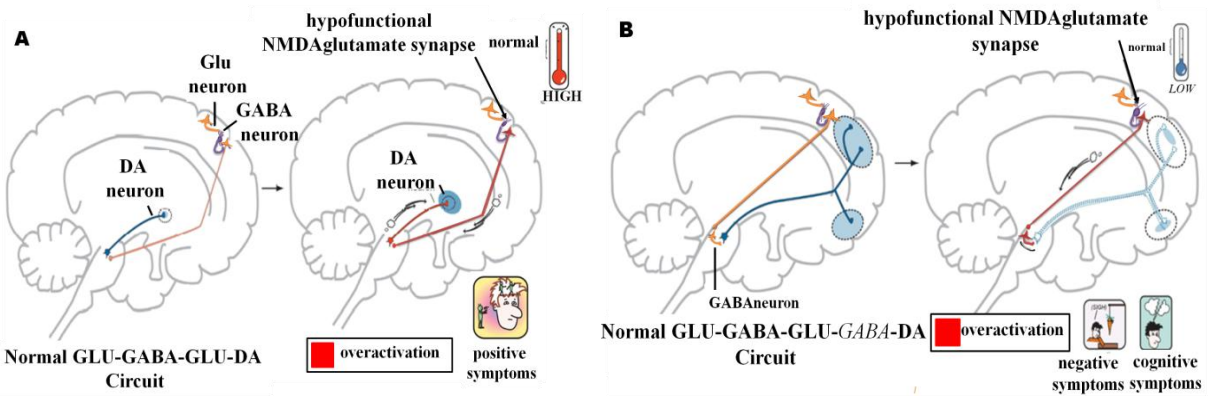

Figure 2. Overactivation of a normal GLU-GABA-GLU-DA circuit generates the positive symptoms (A). Hypofrontality and negative symptoms occur by abnormal GLU-GABA-GLU-*GABA*-DA circuitry (B) [87].

1.3.3 GABAergic system

As it was mentioned abovement (see section 1.3.2), deficits of the GABAergic system have also been reported in patients with schizophrenia, implicating alterations in both pre- and postsynaptic components of GABAergic neurotransmission (Fig. 2B) [102].

GABA is synthesized by glutamic acid decarboxylase (GAD), which exists in two isoforms (GAD67 and GAD65). GAD67 is responsible for basal GABA levels and the majority (80–90 %) of GABA synthesis [103, 104]. One of the most consistent postmortem finding in schizophrenia is the reduction of GAD67 mRNA and protein expressions and therefore decreased extracellular GABA level in the central nervous system [104-112].

GABA exerts its activity by binding to three types of receptors, such as the ionotropic $GABA_{A/C}$ and the metabotrop $GABA_B$ receptors. $GABA_A$ receptors mediate the fast-inhibitory action of GABA and serve as the major source for inhibitory tone, while the $GABA_B$ receptors produce slow inhibitory signal and modulate the release of neurotransmitters. $GABA_A$

receptors include α, β, γ, δ, ε, π, and θ subunits, $GABA_B$ receptor consists of $GABA_B$ receptor 1 and 2 subunits. The $GABA_C$ receptor is composed of five ρ subunits and found to be expressed in the retina [113]. Upregulation of $GABA_A$ α2—receptor subunit and reduced expression of $GABA_B$ receptor 1 and 2 subunits have been demonstrated in schizophrenia [114-117].

GABA transporter 1 is responsible for the synaptic reuptake of GABA and regulates the duration and efficacy of synaptic GABAergic neurotransmission [118]. Several studies have found reduced mRNA level encoding for GABA transporter 1 protein in schizophrenia [114, 119-121].

All these alterations in GABAergic system have been found mostly in dorsolateral prefrontal cortex, but other brain regions are also affected: changes in GAD67 expression have been observed in basal ganglia, thalamus and cerebellum as well, alterations in $GABA_B$ expression have been reported in hippocampus and cerebellum [106, 111, 122-125]. The observed changes in GABAergic neurotransmission have functional significance and are related to cognitive deficits and aggression both in humans and rodents [102, 126-128].

1.4 Animal models of schizophrenia

Animal models of complex and heterogeneous psychiatric disorders are clearly valuable preclinical tools to investigate the different features and neurobiological basis of these diseases, and to identify new drug targets providing more effective treatment in the future [129]. The animal models should be validated on the basis of how well their performances in a given test reflect the key symptoms observed in human patients with schizophrenia, and how they fulfill the criteria of the appropriate triad of validites. Thus, face validity or symptom homology is defined the accuracy of an animal model reproducing the symptoms of a human condition. Constructive validity includes how well an animal model replicates the theoretical neurobiological rationale and pathology. Predictive validity assess the pharmacological response to treatment by known antipsychotics [129, 130].

It is unrealistic to expect animal models that exhibit uniquely human symptoms, like hallucinations and delusions. However, they can be expected to display some abnormalities that are found in schizophrenia patients, including sensory gating and cognitive deficits [131, 132].

In animal model of schizophrenia both DA agonists and non-competitive NMDA receptor antagonists can replicate psychosis effectively, the latter are able to produce also the cognitive deficits and negative symptoms associated with schizophrenia [133]. The different domains of

cognitive functioning affected in schizophrenia (see section 1.2.1) have been tested in rodents. However, for proper evaluation and interpretation of performances of the animals in a cognitive test, it is important to take into account the different factors, which can influence/alter the motivation: e.g. the motor function, the arousal level, the sensory capability and the innate behavior [134-137].

The enhanced social withdrawal, avoidance as negative symptoms are assessed in social interaction tests in rodent paradigms. Measurements of social interactions in animals are relatively straightforward in comparison with other negative symptoms, such as apathy [133, 138]. Typically, aggressive behavior in rodents is assessed via paired interaction where the test animal is confronted with an age-matched unfamiliar conspecific or a younger intruder [139]. A major factor for determining the usability and reliability for this test is its cross-species translatability, which means that measures in rodents are directly analogous to social interaction measures in humans [133, 138].

The autonomic disturbances in schizophrenia animal models have been investigated in few studies only, which focused on primarily the cardiovascular and thermoregulatory alterations [140-142]. Pupillometry is a simple, convenient and noninvasive technique for evaluating and assessing autonomic function, in human and in animal models as well [143, 144]. The physiology, pathophysiology and pharmacology of pupillary functions were investigated in rodents; however, no data on pupillary function are available from animal models of neuropsychiatric disorders, such as schizophrenia [145, 146].

Although some of the animal models of schizophrenia considerable overlap in the methodology/principle used, they fit into three different induction categories: neurodevelopmental, pharmacological and genetic manipulations [63, 129, 147]. Recently, it is supported that animal models with one intervention cannot recreate the diversity and complexity of schizophrenia, but the combination of these different procedures may help to produce a more reliable animal model, as the fourth type of schizophrenia animal model [148-152].

1.4.1 Pharmacological animal models

Pharmacological animal models of schizophrenia are based on current understanding of the alterations in various neurotransmitter systems including DA and GLU ones. The first animal model of schizophrenia has been developed in the 1950s, which was the amphetamine model and based on the DA hypothesis of schizophrenia [63]. Amphetamine is a synthetic psychoactive drug, which increases dose-dependently the contrentration of monoamines in the

synaptic cleft and inhibits the reuptake of them [153]. Its administration induces enhancement in locomotor activity and stereotyped movements, disrupted PPI and spatial learning [154-156]. However, the amphetamine treated model is not able to induce negative symptoms, thus, it is not suitable as a complete model of schizophrenia [90]. Based on the glutamate hypothesis of schizophrenia, NMDA–receptor antagonists (e.g. PCP, MK-801, and ketamine) are also applied to evoke some signs of the positive and negative symptoms and cognitive deficits, including disrupted PPI, spatial learning, altered social interaction with decreased locomotion and exploratory behavior [63, 157-161].

The disadvantage of pharmacological animal models is that the schizophrenia-like symptoms can not be observed after the wash-out period of a given drugs, thus the model does not have construct validity to the proposed neurodevelopmental origin of schizophrenia (see below) [162, 163].

1.4. 2. Neurodevelopmental/environmental animal models

Neurodevelopmental animal models of schizophrenia are generated by interventions applied during the prenatal or early postnatal periods.

Maternal immune activation with the injection of lipopolisacharides (bacterial endotoxin) or polyriboinosinic-polyribocytidilic acid (polyI:C; synthetic analogue of double-stranded ribonucleic acid as viral mimetic) as prenatal interventions have been reported [63]. Both of them stimulate the production of pro-inflammatory cytokines that are involved in the regulation of neurite growth and synaptic plasticity, thus their increased level can disturb the early brain development [164-170]. The prenatal injection of these substances to pregnant rodent dams causes the expression of schizophrenia-like phenomena in their adolescent offsprings, such as reduced PPI, spatial working and recognition memory and enhanced behavioral response to amphetamine [171, 172].

Prenatal neurotoxin methylazoxymethanol acetate (MAM) injection given at a specific gestational phase to rat dams, disrupts neurogenesis and inhibits dezoxyribonucleic acid synthesis [63]. The administration of MAM results in disturbed motor activity, social interaction, cognitive function and reduced PPI [63, 173, 174]. However, Gomes and his collegues have revealed that MAM treatment did not provide an appropriate model of schizophrenia, but instead it caused animals having increased responses to stress [175, 176].

Neonatal hippocampus lesion belongs to the early postnatal interventions that results schizophrenia-like abnormalities, like reduced social interaction, learning capacity and PPI in

rats [177, 178]. One caveat with the induced lesion model is that it reflects far greater damage than what is seen in the brains of individuals with schizophrenia [130].

Postweaning social isolation is an alternative, early postnatal intervention that produces a number of behavioral consequences in adulthood that are similar to schizophrenia symptoms, including deficits in PPI, motor activity, pain sensitivity and enhanced response to psychoactive drugs [179-181]. This intervention also causes alterations in the prefrontal cortex, significant neurotransmission abnormalities including enhanced DA and serotonin functions in the basal ganglia [182-184].

1.4.2 Genetic animal models

The recent advances in genetic technology have led to great progress in modeling of schizophrenia. The high level of homology between human and mouse genomics allows testing the alteration of schizophrenia-susceptibility candidate genes [162]. The effects of genes associated with schizophrenia (see section 1.2.4) have been assessed in genetically modified mice (knockdown: NDMA receptor subunit 1 and 2A, knock-out: disrupted in schizophrenia 1, neuregulin, or naturally occurring mutant: dysbindin). These animals show some features related to schizophrenia, e.g. impaired cognitive function, but their constructive and predictive validities are questionable [185, 186]. Advances of genetic manipulation technology in rats have progressed more slowly than in mice, thus in rats selective breeding have been developed with the goal of studying schizophrenia-related genetic features [162].

Selective breeding (or artificial selection) is a process, in which rats are bred for a particular trait or phenotype since strong heritable variations in behaviors can be found among individuals within one strain [187]. This method produces animals that often mimic more schizophrenia-relevant symptoms (which is not so common when using knock-out mice) providing a powerful strategy to unravel the genetic basis of the disorder [162, 187].

Thus, selected rats with low-PPI show decreased flexibility in different tasks, deficits in social behavior and motivation [5, 188, 189]. Roman High-Avoidance and Roman Low-Avoidance rat strains are bidirectionally (psychogenetically) selected for their rapid versus extremely poor (respectively) acquisition of the two-way active avoidance response [190, 191]. Roman High-Avoidance rats show enhanced impulsive behavior, impaired acquisition and retention of fear conditioning in different paradigms, moreover worsened performance in spatial or non-spatial and working memory tasks [192-196]. Another genetically-based animal model of schizophrenia is constituted by the apomorphine susceptible and unsusceptible rats, based on

the bidirectional selection for extreme gnawing responses to the DA agonist apomorphine [197, 198]. Apomorphine susceptible rats have a hyperreactive hypothalamus-pituitary-adrenal axis, diminished PPI, impaired latent inhibition, and high sensitivity to the locomotor-stimulating effects of amphetamine [197-199]. Recently the "Enhanced Dopamine in Prodromal Schizophrenia" animal model has been developed, in which rate-limiting enzymes in a genetic construct coding for DA synthesis are injected into the substantia nigra pars compacta of adolescent rats [200]. The treated animals show increased amphetamine-induced hyperlocomotion and deficits in PPI and their glutamatergic response to amphetamine also altered in the striatum. However, it is not known whether enhanced dopamine in prodromal schizophrenia animals display any negative symptom phenotypes, or any cognitive deficits [200].

It is clear that susceptibility to schizophrenia is hereditary, however, none of the identified risk genes are specific to schizophrenia, but rather indicate a general vulnerability to mental health disorders [201-203]. The weakness of this type of models is that genetic vulnerability by itself is insufficient to elicit the same behavioral impariments without environmental factors (see below).

1.5 Animal models with combinations of different factors

Since the development of this human disease is due to the complex interaction of genetic and environmental factors, the "multiple hit" hypothesis based translational model of schizophrenia seems to be a more useful through combining different interventions of the above mentioned models. In these animals it is possible to control the different factors and their impacts experimentally in a way that is not possible in humans, such as susceptibility genes, environmental factors or pharmacological treatments [62, 152, 203, 204].

The combination of genetic and environmental factors, e.g. transgenic transformation of disrupted in schizophrenia 1 gene and polyI: C immune activation in mice, results in deficits of short-term memory, object recognition, increased anxiety, depression-like responses and decreased sociability [151, 185]. The pituitary adenylate cyclase-activating polypeptide knockout mice with isolation rearing display remarkable hyperactivity in the novel environment, high level impulsivity, sensorimotor gating deficits and prolonged immobility in the forced swimming test [186].

As combination of environmental–environmental factors, neonatal immune activation and adolescent PCP treatment in mice exhibit high level impulsivity, social deficits, and impairment of memory in novel object recognition (NOR) test [186]. The social isolation in MAM-E17 (pregnant dams are injected with MAM on gestational day 17) rat model results in deficit in recognition memory, sensory gating and GABA synthesis [205, 206].
As far as we know no article was found with "three hit" schizophrenia animal model, except our WISKET model developed from Wistar rats (see below).

1.5.1.1 WISKET animal model

The WISKET rat line (see section 3.3.1) originates from Wistar rat strain developed in our laboratory applying a complex periadolescent treatment [162, 202].
This chronic rat model is based upon the well-established postweaning isolation rearing with subchronic ketamine treatment in this selected new rat line (WISKET). 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, based on behavioral phyenotypes, as a genetic factor causing disturbances in neuronal development and induces changes in behavior. Animals showing the most significant disturbances related to schizophrenia are used for selective breeding throughout several generations. This model mimics simultaneously several aspects of schizophrenia. Our previous results proved that the combination of these insults was associated with impairments in acute heat pain sensitivity, sensorimotor gating, locomotor activity, cognitive performance, body temperature regulation, and electroencephalograpy pattern [142, 202, 207-209]. Furthermore, molecular-biology studies revelaed disturbed functions in opioid and cannabinoid receptor systems [210, 211].

2 AIMS OF THE STUDY

The goal of this study was to characterize WISKET model in several new aspects, including:

I. the investigation of age- and sex-dependence of social behavior of Wistar (control) and WISKET animals in social interaction test,
II. the assessment of sex differences in exploratory activity, cognitive function and anxiety-like behavior of Wistar and WISKET rats in a simplified holeboard test,
III. the examination of the exploratory activity and learning capacity of male Wistar and WISKET rats in a newly developed cognitive test (AMBITUS), and
IV. the analysis of the pupillary function in male Wistar and WISKET animals under sedation or anesthesia.

3 MATERIALS AND METHODS

3.1 Subjects

All experiments were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: XIV/03285/2011, XIV/1248/2018). Animals were kept with a 12 h light/dark cycle under controlled temperature (22 ±1 °C) with ad libitum water and food access (except for the experiments in the holeboard test and AMBITUS system, when the animals were food deprived for two days, see section 3.5.1 and 3.6.1). The body weight of rats was measured weekly throughout the whole investigation period. The experiments were performed between 8 a.m. and 3 p.m.

3.2 Drugs

All the employed drugs, including ketamine hydrochloride (Calypsol), diazepam (Seduxen) and chloral hydrate (all from Gedeon Richter Plc.) were dissolved in and diluted with saline.

3.3 Experimental paradigm

3.3.1 Interventions and basal tests

The paradigm for selective breeding (based on basal tests: tail flick [TF] and PPI) through several generations has already been described (Table 1) [202]. The complex treatment includes early-life interventions as social isolation and subchronic ketamine treatment. After weaning (postnatal day 21) all rats were tested with the TF test to assess their basal acute heat pain sensitivity and then WISKET animals were housed individually for 28 days (between 4–7 weeks of age) and treated with ketamine (30 mg/kg intraperitoneally [i.p.], 4 ml/1000 g body weight, daily, 5 times/week, 15 injections in total) from 5 to 7 weeks of age. Then the animals were re-housed in a group setting (3–4 rats per cage) and 1 week of recovery with no treatment followed (Table 1). Starting at the age of 9 weeks, TF and PPI tests were applied.
To further characterize our model in new aspects we tested the animals in two series:

3.3.2 Series 1

Besides the basal tests (see section 3.3.1) the social interaction test was performed at the age of 3 and 11 weeks and the cognitive test at the age of 10 weeks. Naive male (n=16) and female (n=8) Wistar rats and WISKET male (n=21) and female (n=22) rats were involved in the experiments (Table 1).

3.3.3 Series 2

One week after the basal tests (see section 3.3.1) the cognitive and two weeks later the pupillary light reflex tests (Table 1) were performed in male Wistar rats (n=31) and male WISKET rats (n=42).

Table 1. Schedule for the interventions and behavioral tests in series 1 and series 2.

Group		**Postnatal age**							
		PD 21	PD 23-24	week 4-7	week 5-7	week 8	week 9	week 10	week 11
Series 1	**Wistar**	weaning, TF test 1	Social interaction test 1	social rearing			TF test 2, PPI test	HB test	Social interaction test 2
	WISKET			isolation rearing	ketamine treatment (30 mg/kg i.p. daily)	resocialization			
Series 2	**Wistar**			social rearing				AMBITUS test	PLR test
	WISKET			isolation rearing	ketamine treatment (30 mg/kg i.p. daily)	resocialization			

Abbreviations: HB – holeboard, i.p. – intraperitoneally, PD – postnatal day, PLR – pupillary light reflex, PPI – pre-pulse inhibition, TF – tail-flick.

3.4 Basal tests

3.4.1 Nociceptive test

The acute heat pain sensitivity was assessed with TF test 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: 20, 40 s, on the 3^{th} and 9^{th} weeks, respectively). TF latencies were obtained four times with 30 min intervals and were averaged to establish the pain threshold for each group.

3.4.2 Sensory gating test

The degree of sensory gating of the acoustic startle response was measured as described previously [202]. Briefly, after 10 min habituation in plexiglass startle chambers (12x17x15.3 cm) rats were exposed to two different trial types: the pulse alone (PA), in which a 40 ms 95 dB white noise burst was presented; and the prepulse–pulse pair (PP) in which prepulse stimuli (20 ms, 76 dB) were followed by the startle stimulus with a latency of 150 ms. Both types of stimuli were applied 20 times in random pattern. The interstimulus intervals ranged from 7 to 13 s. Degree of prepulse inhibition was calculated as percentage using the following equation:

$$\text{PPI (\%)} = \left(1 - \left[\frac{\text{startle response for PP}}{\text{startle response for PA}}\right]\right) \times 100$$

3.5 Procedures in series 1

3.5.1 Holeboard test

This test can be applied for the assessment of locomotor activity and exploration, anxiety-like behavior and cognitive functions in rodents [212-214]. The one-phase holeboard (HB) test is an appetitively motivated test, in which food reward (puffed rice, 20 mg) was used as a positive motivation after two days of total food-deprivation.

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, and their behaviors were recorded with an overhead infrared video device (WCM-21VF, CNB, China) and analyzed offline by trained observers, who were blind to the treatment groups. The apparatus was cleaned with 70 % alcohol solution after each animal. The task was to collect all rewards (16) within 600 s. The durations of locomotor (horizontal)

and rearing (vertical) behaviors were defined basic activities. The grooming and the place preference (time ratio spent in the central area) were defined as anxiety. The latency of the first hole–visit and the first reward eating were also registered and the learning capacity of the animals was determined as:

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

3.5.2 Social interaction test

Weight- and sex-matched, unfamiliar pairs of rats from the same group were simultaneously placed in opposite corners of an unfamiliar testing chamber (15×34×33 and 60×34×33 cm for postweaning and adult rats, respectively). The animals' behavior was recorded for 600 s with an overhead infrared video camera (WCM-21VF, CNB, China) and analyzed offline by trained observers, who were blind to the treatment groups. The evaluated parameters for social behavior 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 denoted as aggression; and running away was defined as avoidance. The basic activities, thus the time spent with rearing and the times spent of self-grooming were quantified as exploratory behavior and degree of anxiety, respectively.

3.6 Procedures in series 2

3.6.1 AMBITUS test

The AMBITUS system is a newly developed appetitively motivated cognitive-behavioral test suitable for the detection of exploratory activities and learning capacity in rats [209]. This instrument is a combination of HB and corridor tests (with several food rewards but without an open field). Briefly, it is a square corridor of clear plexiglass on black floor (outer diameter of 80 cm, width of 8 cm and height of 50 cm), and all the eight walls have two equally spaced sites (side-boxes: 5x5x5 cm) with food reward in each of them (puffed rice, 20 mg) and equipped with infrared photocells for the automatic detection of the exploratory activity (visits into the side-boxes) of the animals (Fig. 3). The behavior of the animals was also recorded with an infrared video device (WCM-21VF, CNB, China) fixed above the apparatus and analyzed offline (determination of the time required to complete the task) by trained observers, who were blind to the treatment groups.

Trials commenced by placing the rats into the same starting point within the corridor (Fig. 3A); thereafter, the experimenter immediately left the room. The animals were allowed to explore the corridor and collect the 16 food rewards (Fig. 3B) within 300 s minutes (cut-off time). The number of food rewards eaten was recorded at the end of each trial by the experimenter. The apparatus was cleaned with 70 % alcohol after each animal. The trials were repeated two times with 1 min apart (trial 1 and trial 2). The number of side-box visits was used to characterize the exploratory activity, while learning capacity was calculated the same way as in the HB test [208, 209]:

$$\text{Learning capacity \%} = \left[\frac{\text{number of collected food rewards} \times \text{cut-off time of the task (300 s)}}{\text{number of food rewards (16)} \times \text{time required to complete the task (s)}}\right] \times 100$$

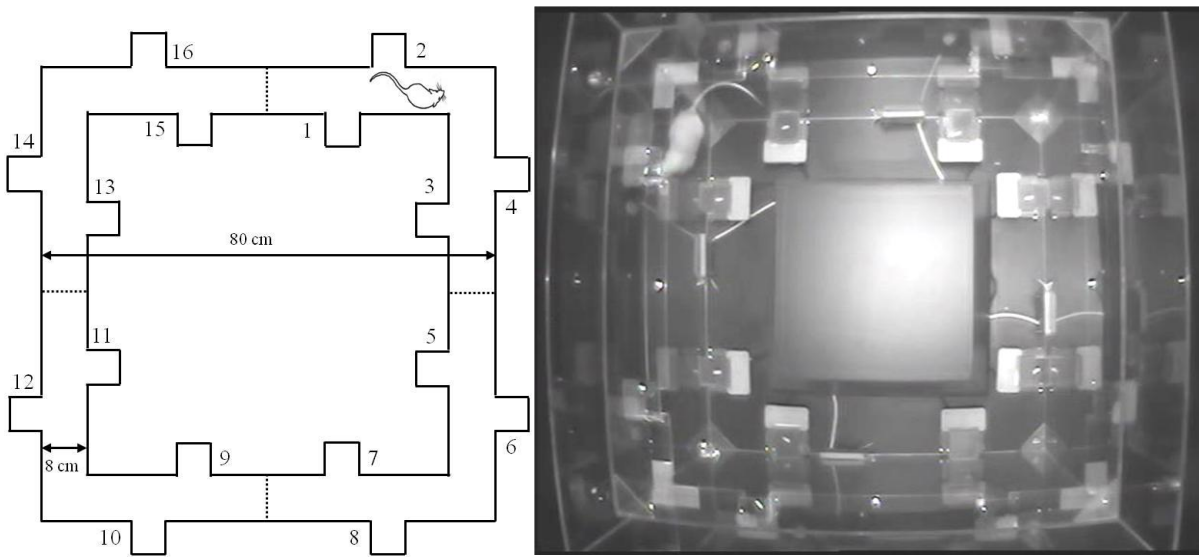

Figure 3. Ground plan of the corridor with 16 side-boxes equipped with photo beams with a rat at the starting point (A). Image of the AMBITUS system captured from an offline video. In the left upper corner, a rat is sniffing in Box 14 (B).

3.6.2 Pupillary measurements

Several psychological and physiological variables can influence the initial pupil diameter and the PLR [51, 215]. The fear responses can be decreased with sedation and/or anesthesia, thus the test was conducted under two conditions.

In the first condition, the pupils were tested 15 min after diazepam-induced sedation (2.5 mg/kg i.p., WISKET n=22, Wistar n=17). The sedated animals had slow righting reflex and they accepted the slight restrain during the test period.

Since anesthesia allows an appropriate and convenient investigation of pupillary reactions for a longer period compared to the investigation under sedation, therefore, in the second condition, the pupils were tested 15 min after chloral hydrate-induced anesthesia (200 mg/kg i.p., WISKET n=20, Wistar n=14) [216]. The anesthetized animals had no righting reflex and there was no response to mechanical stimuli.

A modified digital camera (Nikon D7000) was used to record pupillary responses at a speed of 24 frames-per-second under infrared illumination. After the induction of sedation or anesthesia, a 15-minute dark adaptation period was followed. Thereafter, the animals were positioned in front of the camera (the sedated animals were gently restrained). Then an intensive light stimulus (approximately 300 cd/m^2 for 600 ms) was applied to the left eye, along with an infrared flash and the response was recorded for up to 15 s in sedated animals and to 60 s in anaesthetized animals [217].

The custom image and signal processing methods were developed in MATLAB (Version 2015b, Mathworks) to extract automatically the required parameters before and after the light stimuli on each recorded frame (Table 2) [218]. The pupil diameter was expressed as a ratio of the diameter of iris in % (Fig. 4) [219, 220]. The automated feature extraction method produced 13 features from the pupilogram (Table 2). Many of them were basic, traditional parameters like initial diameter, which was determined 1 s before the stimulus, minimum diameter; constriction latency; maximum of the redilated diameter. Several other features were implemented to obtain information about the dynamics of the response, thus time related descriptors were introduced, such as the flatness of the curve, total constriction time and in the redilation phase: time required reaching different percentages of the initial size of the pupil (Table 2 and Fig. 5).

It has to be mentioned that in the video-recorded images, the exact definition of the pupil in the iris may be challenging due to scattering movements, significant blur, low contrast difference between the pupil and iris, noise and reflections [218]. Besides the robust handling of these, the measurement process needs to be fast, accurate and reproducible. The solution was a novel, energy attenuation model-based ray propagation method, which used mathematical, geometrical and physical relations to explore and analyze the structure of the iris and pupil regions and to estimate the latter’s diameter. The evaluation of the proposed method on 20 manually processed video recordings showed that the overall diameter measurement error was less than ± 2 % [218].

Table 2. Investigated pupillary parameters.

PARAMETERS	DEFINITION
Initial pupil diameter (%)	Pupil diameter before light stimulus
Minimum diameter (%)	Minimum diameter after light stimulus
Constriction latency (ms)	Time interval between light stimulus and constriction onset
Amplitude of constriction (%)	Initial diameter – minimum diameter
Degree of constriction (%)	(1– (minimum pupil diameter / initial pupil diameter))x100
Duration of constriction (ms)	Time interval between constriction onset and to reach the minimum diameter
Flatness of the curve (ms)	The time the pupil remains at the maximal constriction
Total constriction time (ms)	Duration of constriction + flatness of the curve
25% redilation time	Time to reach the 25% of the initial diameter
33% redilation time	Time to reach the 33% of the initial diameter
50% redilation time	Time to reach the 50% of the initial diameter
Maximum redilated pupil diameter (%)	Maximal redilation value
Redilation time (ms)	Time to reach the maximum redilation

All diameters are expressed as percentage of iris diameter. The calculated parameters are based on earlier studies: [51, 217, 221-224].

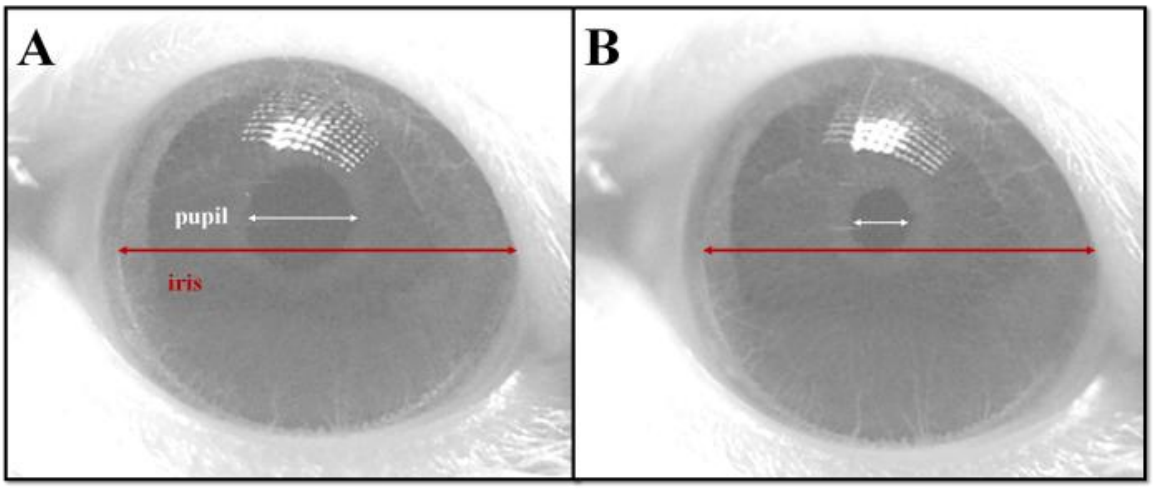

Figure 4. Two representative examples of rat eye images: (A) pupil size before light stimulus, and (B) shortly after the stimulus. Red lines represent the calibration lines: adjust to the diameter of the iris, this value is 100 %. White lines represent the measuring lines: adjust to the diameter of the pupil.

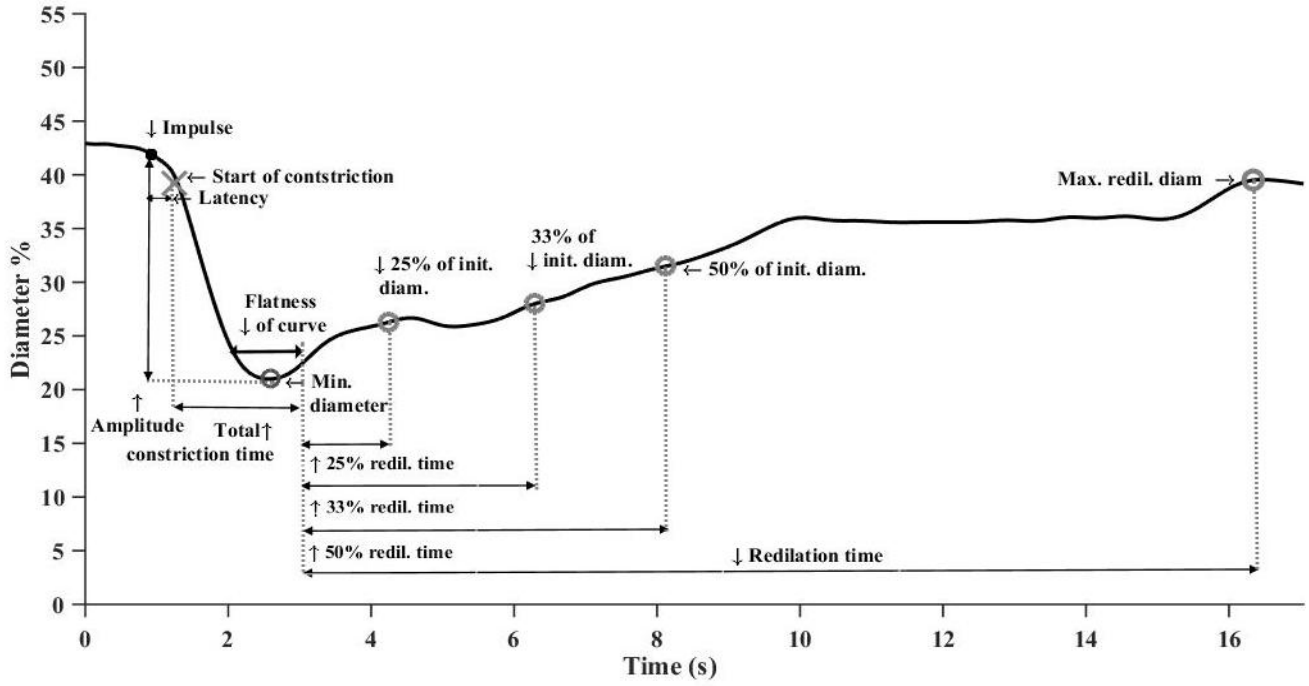

Figure 5. The evaluated pupillary light reflex parameters are defined on a representative reflex curve. *Abbreviations*: init. diam. – initial diameter, redil. diam. – redilated diameter, redil. time – redilation time.

4 STATISTICAL ANALYSIS

Data are expressed as means ± SEM. For the analyses, STATISTICA program (Version 13.4.0.14, TIBCO Software Inc., Palo Alto, USA) was used. Data were analyzed by using one-way, repeated or factorial ANOVA with group (Wistar and WISKET), age, sex and condition as factors.

For the correlation analysis linear regression and calculation of Pearson correlation coefficients were assessed. For the post-hoc comparisons, the Newman-Keuls test was used. Only probabilities lower than 0.05 were considered significant.

5 RESULTS

5.1 Basal parameters

In agreement with our recent studies [142, 202, 208-211, 225] the Wistar and WISKET rats showed significant differences in body weight, and in basal behavior parameters of the experiments (Table 3).

Table 3. Significant effects of body weight, tail flick and pre-pulse inhibition tests in both series.

		Series 1		Series 2	
Investigated parameters	**Factors**	**F value**	**Significance**	**F value**	**Significance**
Body weight (g)	age	$F_{(8,504)}$=6614.96	p<0.0001	$F_{(8,568)}$=2453.248	p<0.0001
	group	$F_{(1,63)}$=224.18	p<0.0001	$F_{(1,71)}$=34.099	p<0.0001
	sex	$F_{(1,63)}$= 214.18	p<0.0001		
	age x group	$F_{(8,504)}$=61.71	p<0.0001	$F_{(8,568)}$=27.899	p<0.0001
	age x sex	$F_{(8,504)}$=385.16	p<0.0001		
	group x sex	$F_{(1, 63)}$= 17.62	p<0.0001		
	age x group x sex	$F_{(8,504)}$=24.92	p<0.0001		
TF latency (s)	age	$F_{(1,63)}$=174.16	p<0.0001	$F_{(1,71)}$=196.055	p<0.0001
	group	$F_{(1,63)}$=73.16	p<0.0001	$F_{(1,71)}$=121.128	p<0.0001
	sex	$F_{(1,63)}$=4.83	p<0.05		
	age x group	$F_{(1,63)}$=20.75	p<0.0001	$F_{(1,71)}$=18.987	p<0.0001
	age x group x sex	$F_{(1,63)}$=4.36	p<0.05		
PPI (%)	group	$F_{(1,63)}$=9.97	p<0.005	$F_{(1,71)}$=13.029	p<0.005

Abbreviations: TF – tail flick, PPI – pre-pulse inhibition.

WISKET rats had lower body weight compared to Wistar groups in both series (Fig. 6), and the differences increased with age. The results obtained in series 1 confirmed the well-known sex-specific difference, and WISKET females had the lowest body weight compared to other groups (Table 3 and Fig. 6A).

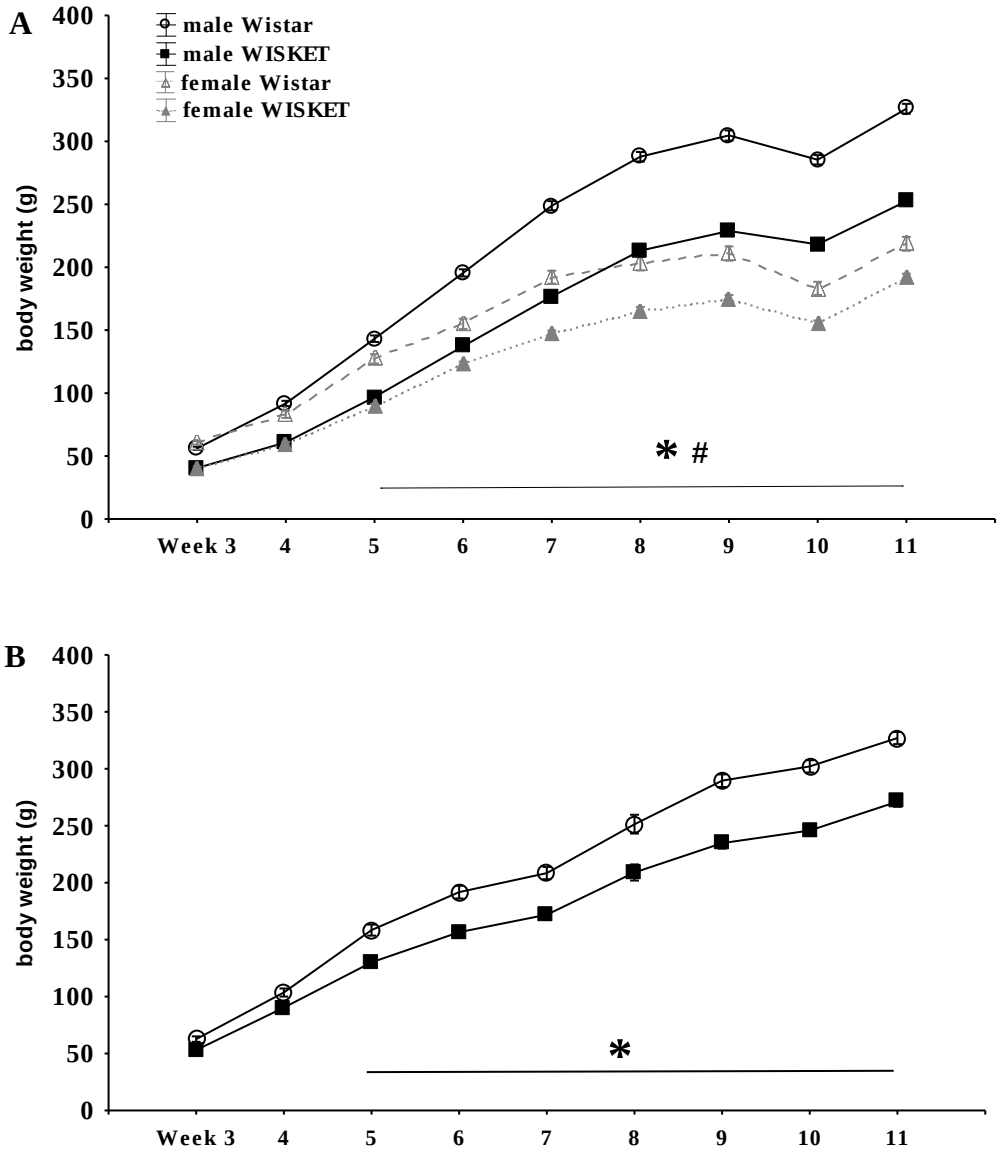

Figure 6. Body weight in series 1 (A) and in series 2 (B). The symbols indicate significant differences ($p<0.05$) by Newman-Keuls post hoc test between: the corresponding Wistar and WISKET groups (*) and sexes (#).

The pain threshold increased with the age in each group and it was significantly lower in WISKET rats compared to Wistar animals in both series and in both trials (Table 3 and Fig. 7). The adult WISKET rats showed sex dependent disturbances in pain sensitivity. The male WISKET rats at the age of 9 weeks had the longest reaction time compared to other groups in series 1 (Table 3 and Fig. 7A).

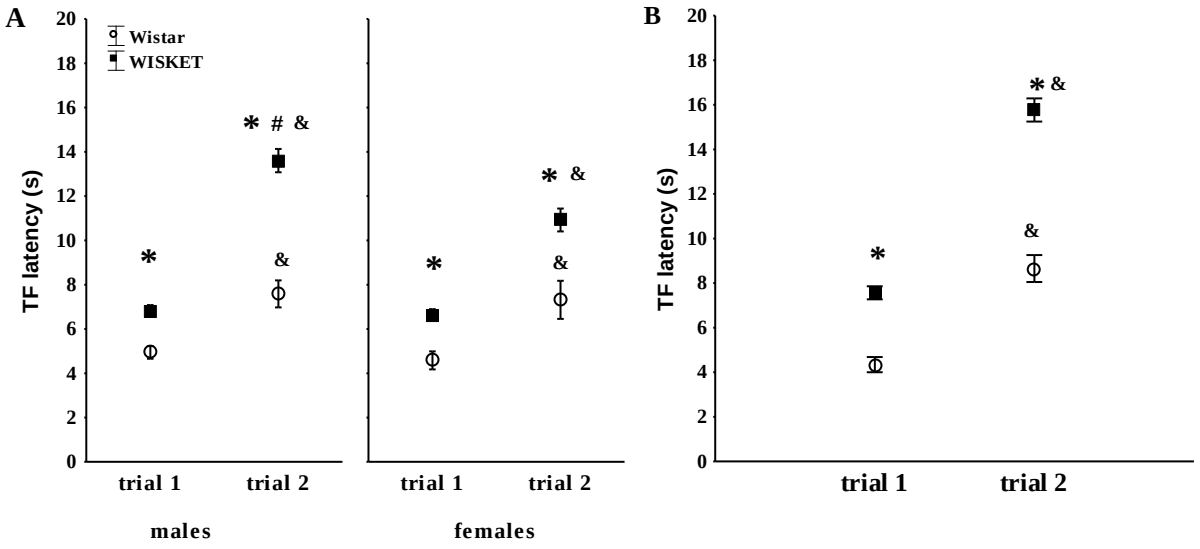

Figure 7. Pain sensitivity in series 1 (A) and in series 2 (B) indicated by the tail-flick (TF) latency values in Wistar and WISKET rats. The symbols indicate significant differences ($p<0.05$) by Newman-Keuls post hoc test between: the corresponding Wistar and WISKET groups (*); the sexes (#) and trials (&).

Regarding the sensory gating, the WISKET rats had lower PPI compared to the Wistar animals (Table 3 and Fig. 8); significant differences were observed between male WISKET and Wistar rats (Table 3 and Fig. 8).

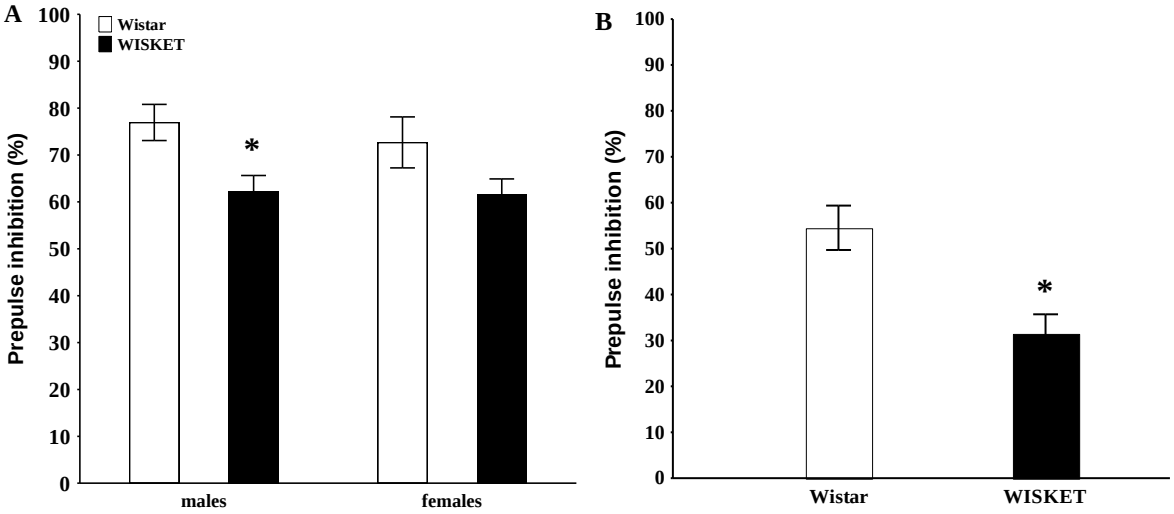

Figure 8. Degree of sensory gating in series 1 in both sexes (A) and in series 2 (B) defined by % PPI values in Wistar and WISKET rats. The symbol (*) indicates significant differences ($p<0.05$) between the corresponding Wistar and WISKET groups.

5.2 Results of series 1

5.2.1 Holeboard test

During the offline analysis of video recordings, there were no visible signs for the disruption of the WISKET animals' physical ability to execute movements.

The WISKET rats showed significant impairments in the HB test, which were sex dependent in some parameters (Table 4 and Fig. 9).

Table 4. Significant effects in basic activities and cognitive performance in HB test.

Investigated parameters	**Factors**	**F value**	**Significance**
Locomotor activity (s)	group	$F_{(1,63)}=6.84$	$p<0.05$
	sex	$F_{(1,63)}=6.85$	$p<0.05$
Rearing acivity (s)	group	$F_{(1,63)}=5.95$	$p<0.05$
	group x sex	$F_{(1,63)}=7.66$	$p<0.01$
Grooming activity (s)	group	$F_{(1,63)}=14.23$	$p<0.001$
	sex	$F_{(1,63)}=5.74$	$p<0.05$
	group x sex	$F_{(1,63)}=4.95$	$p<0.05$
Time in central area (s)	group	$F_{(1,63)}=13.58$	$p<0.001$
Learning capacity (%)	group	$F_{(1,63)}=12.63$	$p<0.001$
First hole visit (s)	group	$F_{(1,63)}=5.44$	$p<0.05$
First reward eating (s)	group	$F_{(1,63)}=6.66$	$p<0.05$

Male WISKET rats spent shorter time with locomotion and rearing compared to the Wistar ones (Fig. 9A, B), and female WISKET rats showed enhanced locomotion compared to the male ones. Regarding the anxiety related behaviors, WISKET males spent more time with grooming, compared to the control males and female WISKET rats (Fig. 9C). Furthermore, both sexes of the WISKET animals showed significant reductions in the time spent in central area compared to the controls (Fig. 9D).

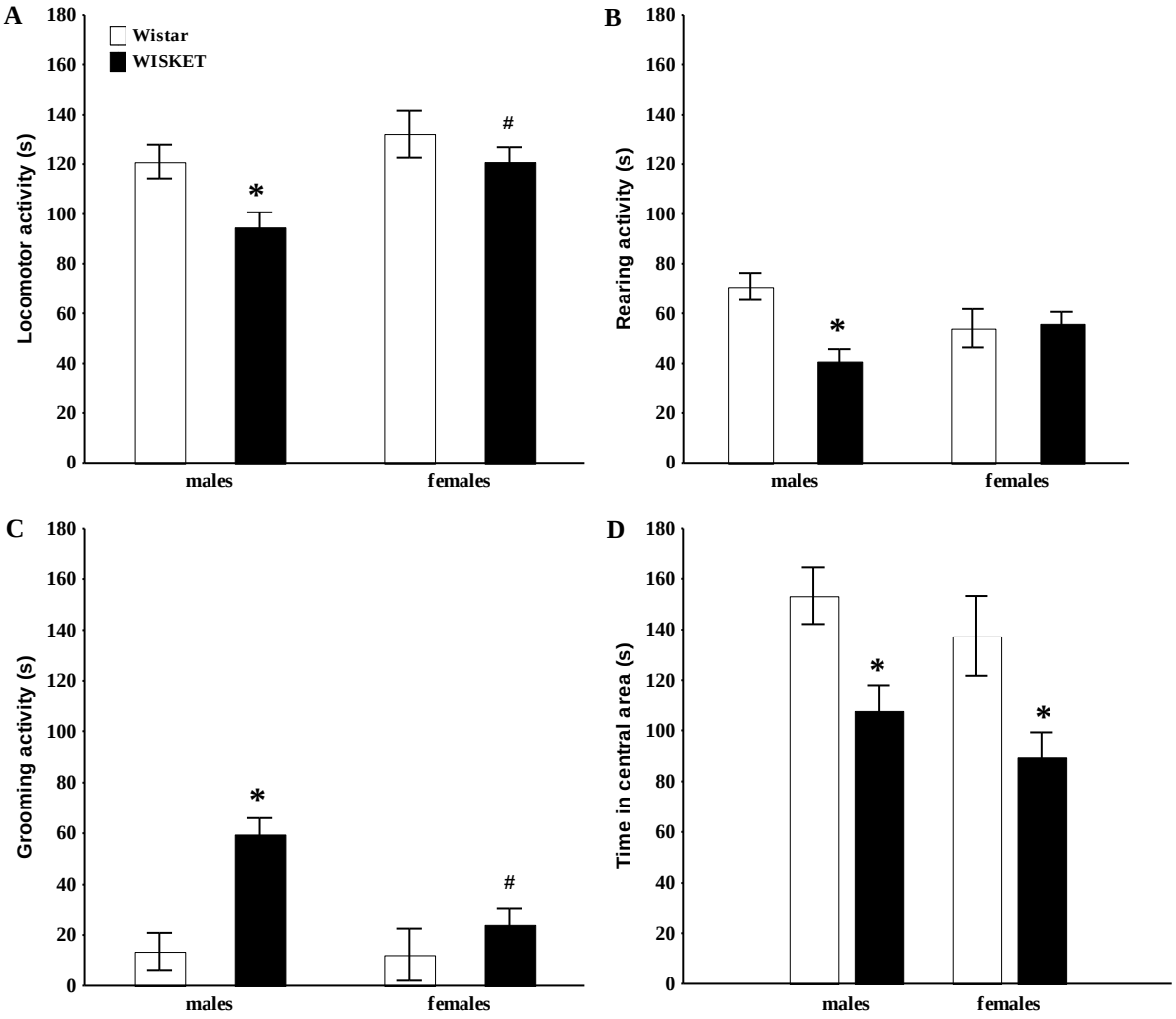

Figure 9. Locomotory activity (A), rearing (B), grooming (C), and the time spent in the central area (D) during the HB test. The symbols indicate significant differences ($p<0.05$) between the corresponding Wistar and WISKET groups (*) and sexes (#).

ANOVA revealed significant effect of the group with a lower cognitive performance in WISKET animals (Table 4 and Fig. 10A), and close to significant effect of sex ($F_{(1,63)}$=3.33, p=0.072). The post hoc comparison revealed that female WISKET rats showed higher level of learning capacity compared to the male ones. Both the latency of the first hole−visit (Fig. 10B), and the first reward eating were significantly increased in WISKET animals (Table 4 and Fig. 10C).

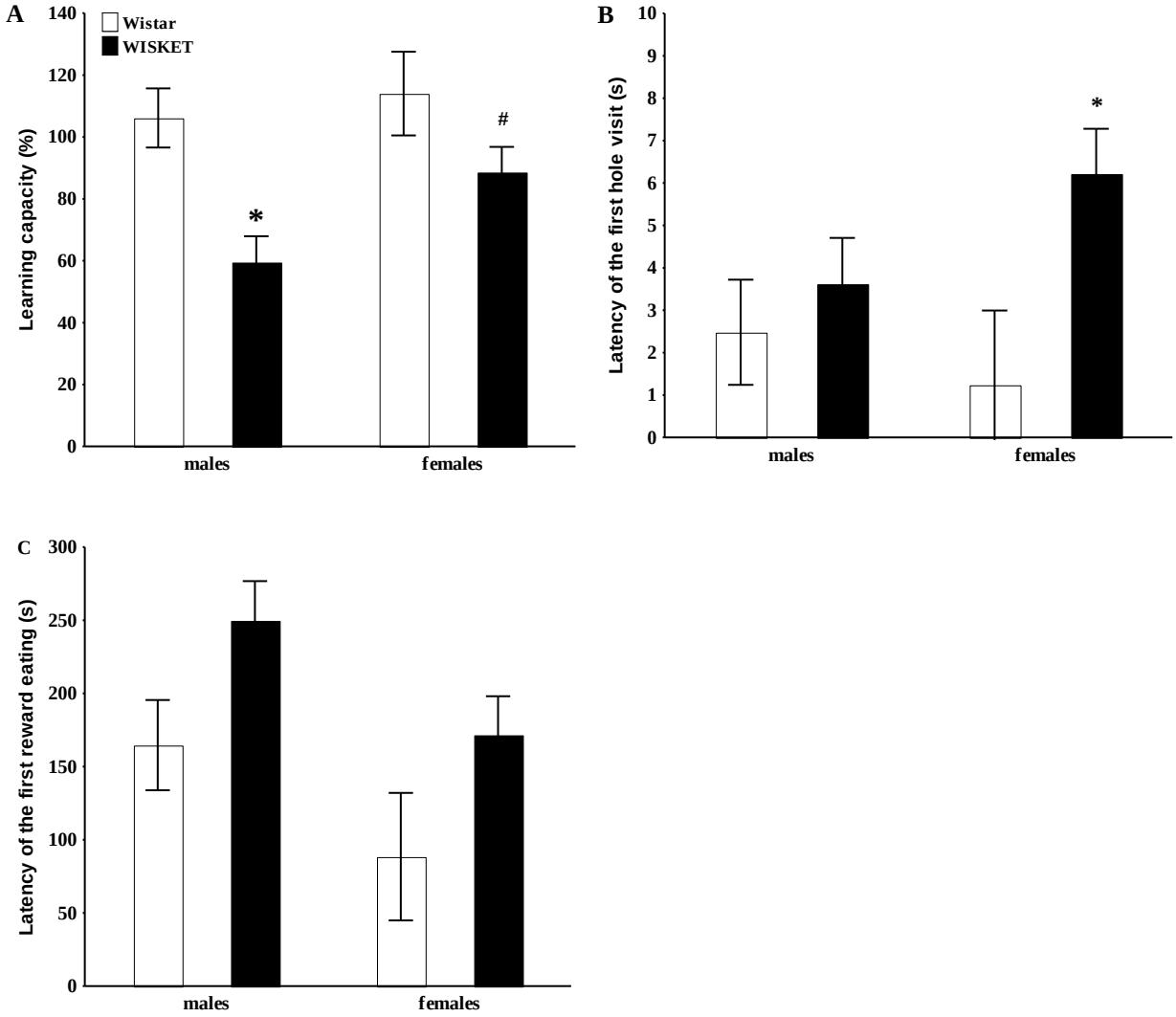

Figure 10. Cognitive performance of the animals in HB test represented by learning capacity (A), the latency of the first hole visit (B) and eating (C). The symbols indicate significant differences ($p<0.05$) between the corresponding Wistar and WISKET groups (*) and sexes (#).

5.2.2 Social interaction test

The rearing and grooming activities were influenced by the different factors and their interactions (Table 5).

Table 5. Significant effects in basic activities and social behavior in social interaction test.

Investigated parameters	**Factors**	**F value**	**Significance**
Rearing acivity (s)	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 (s)	age	$F_{(1,63)}=21.46$	$p<0.0001$
	age x group	$F_{(1,63)}=5.25$	$p<0.05$
	age x sex	$F_{(1,63)}=13.91$	$p<0.001$
Sniffing time (s)	age	$F_{(1,63)}=43.76$	$p<0.0001$
	sex	$F_{(1,63)}=4.03$	$p<0.05$
Aggressive behavior (count)	group	$F_{(1,63)}=6.94$	$p<0.05$
	age x sex	$F_{(1,63)}=4.75$	$p<0.05$
Avoidance behavior (count)	age	$F_{(1,63)}=4.74$	$p<0.05$
	group	$F_{(1,63)}=7.66$	$p<0.01$
	age x group x sex	$F_{(1,63)}=4.44$	$p<0.05$

The rearing activity increased with age in all groups, but it decreased in WISKET animals in both trials compared to control ones (Fig. 11A). The Wistar female animals had higher level of rearing activity compared to the males, but not the WISKET ones (Fig. 11A). Grooming activity decreased with age especially in females, and the post hoc analysis revealed significant decrease in adult Wistar female animals compared to males (Fig. 11B).

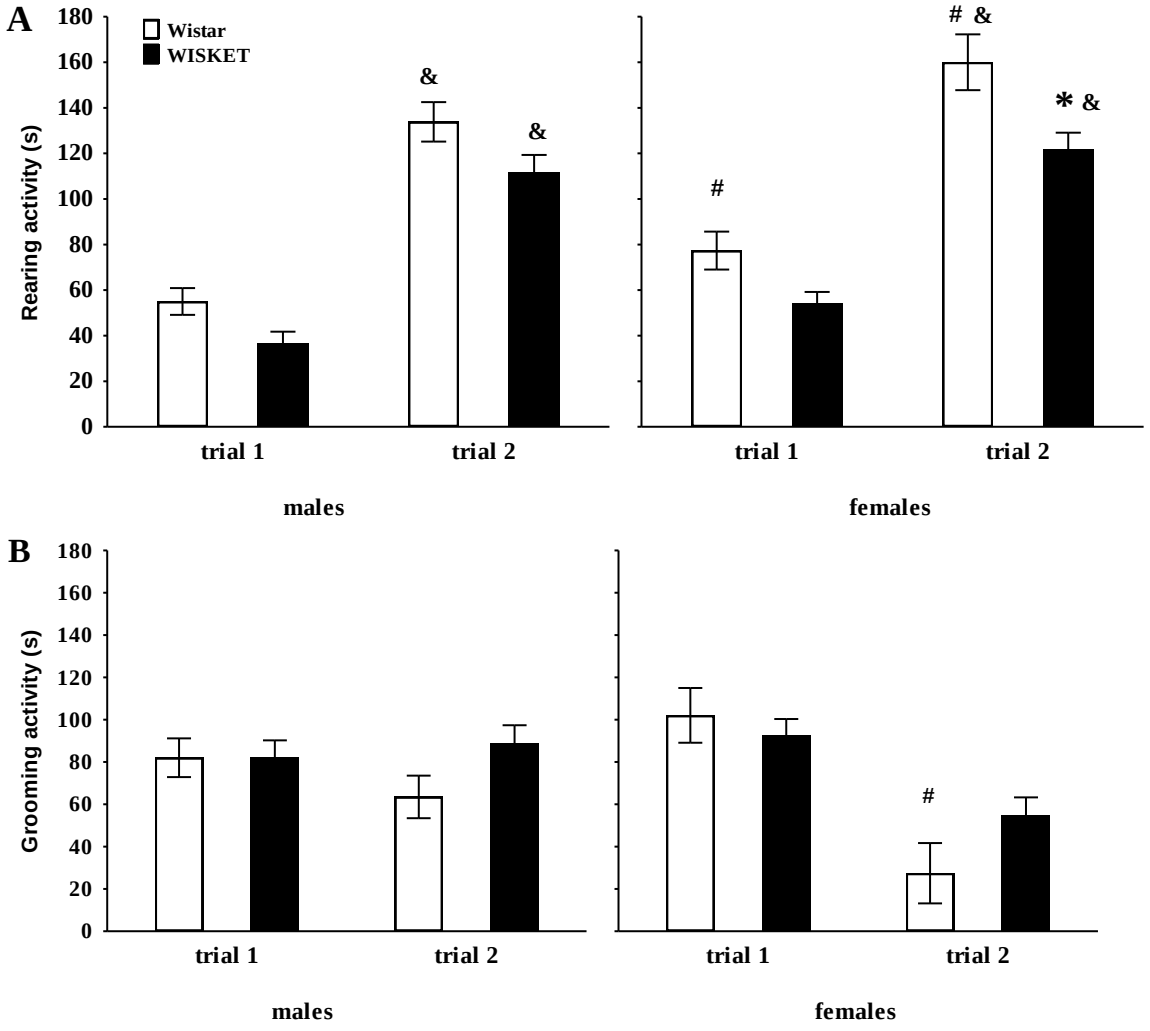

Figure 11. Time spent with rearing (A) and grooming (B) during the social interaction test. The symbols indicate significant differences ($p<0.05$) by Newman-Keuls post hoc test between: the corresponding Wistar and WISKET groups (*), sexes (#) and trials (&).

The social interest decreased in each group with the age (Table 5 and Fig. 12). In contrast, the aggressive and avoidance behaviors increased with age in male WISKET rats (Fig. 13A, B), they showed the highest aggression with escape behavior during the second social interaction test (Table 5 and Fig. 13), but it was not characteristic for the other groups (Fig. 13). Furthermore, regarding the aggressive behavior, higher degree of individual differences was found in the WISKET male groups at the age of 11 weeks (Fig. 13C).

The female WISKET rats showed similar social behavior as the Wistar ones (Fig. 12, 13).

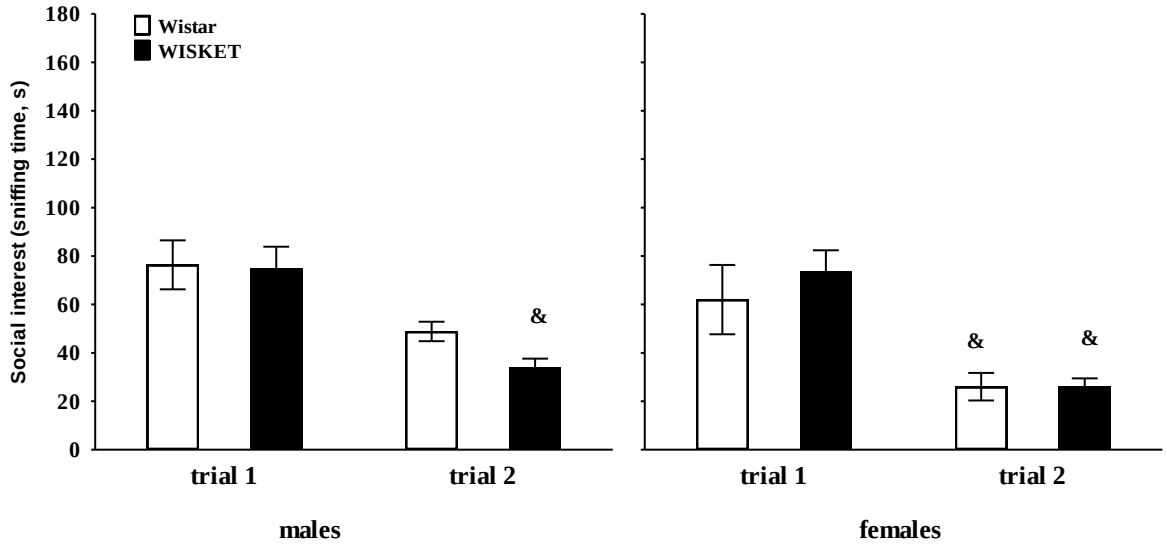

Figure 12. The social interest of the animals is shown during the social interaction test. The symbol (&) indicates significant difference ($p<0.05$) by Newman-Keuls post hoc test between the trials.

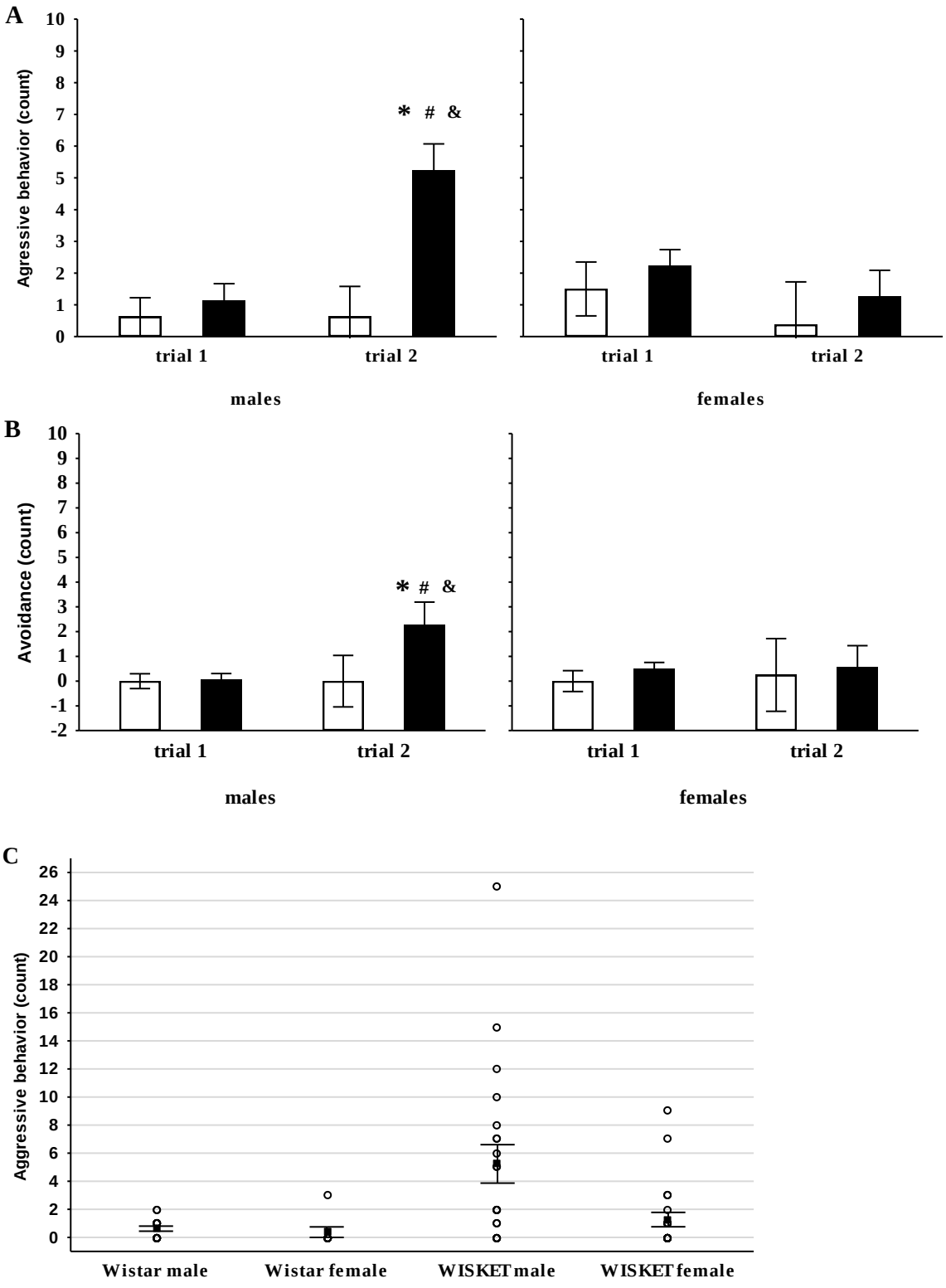

Figure 13. The aggressive behavior (A), the avoidance (B) of the animals during the social interaction test. Scatterplot with means ± SEM showing aggression behavior from individual animals at 11 weeks of age by groups (C). The symbols indicate significant differences ($p<0.05$) by Newman-Keuls post hoc test between: the corresponding Wistar and WISKET groups (*); sexes (#); and trials (&).

5.3 Results of series 2

5.3.1 AMBITUS test

The Wistar rats showed enhanced exploratory activity and learning capacity with the trials, but not the WISKET animals (Table 6 and Fig.14). They visited less boxes and collected fewer food rewards during both trials, which manifested in the decreased learning capacity (Fig. 14).

Table 6. Results of exploratory activity and learning capacity in AMBITUS test

Investigated parameters	Factors	F value	Significance
Exploratory activity (count)	group	$F_{(1,71)}$=56.450	p<0.0001
Learning capacity (%)	group	$F_{(1,71)}$=60.571	p<0.0001
	trial	$F_{(1,71)}$=7.091	p<0.01
	group x trial	$F_{(1,71)}$=9.058	p<0.01

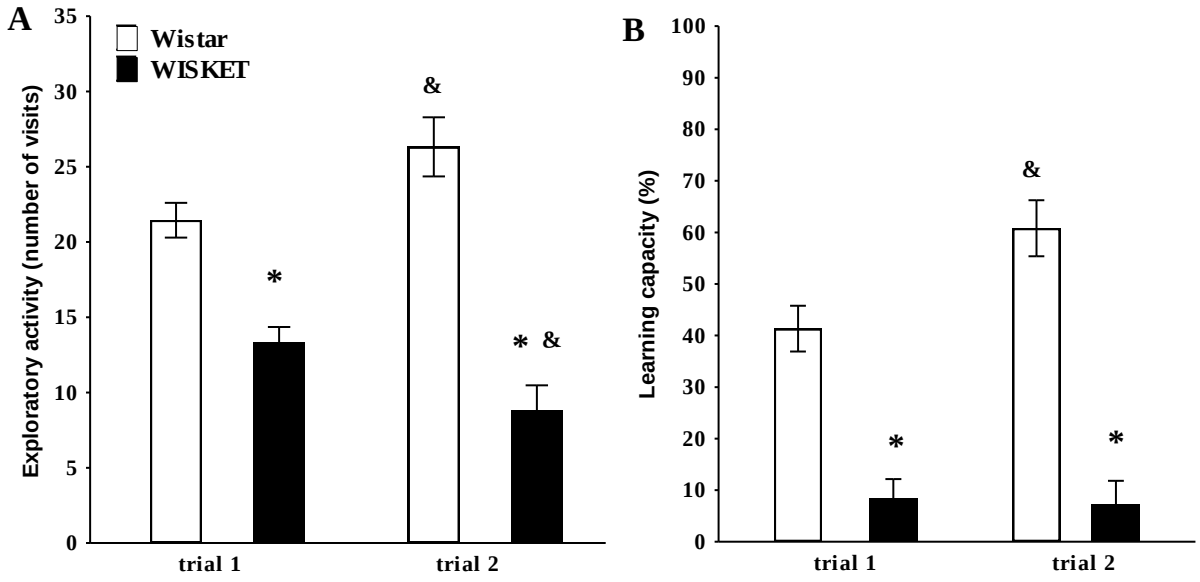

Figure 14. Exploratory activity (A) and cognitive performance represented by learning capacity (B) of Wistar and WISKET animals during the two trials in the AMBITUS test. The symbols indicate significant differences (p<0.05) between the corresponding Wistar and WISKET groups (*) and trials (&).

5.3.2 Pupillary light reflex

Regarding the pupillary parameters, the light stimulus caused significant pupillary constriction in both groups and conditions. The curves show fast constriction followed by a slow recovery (Fig. 15).

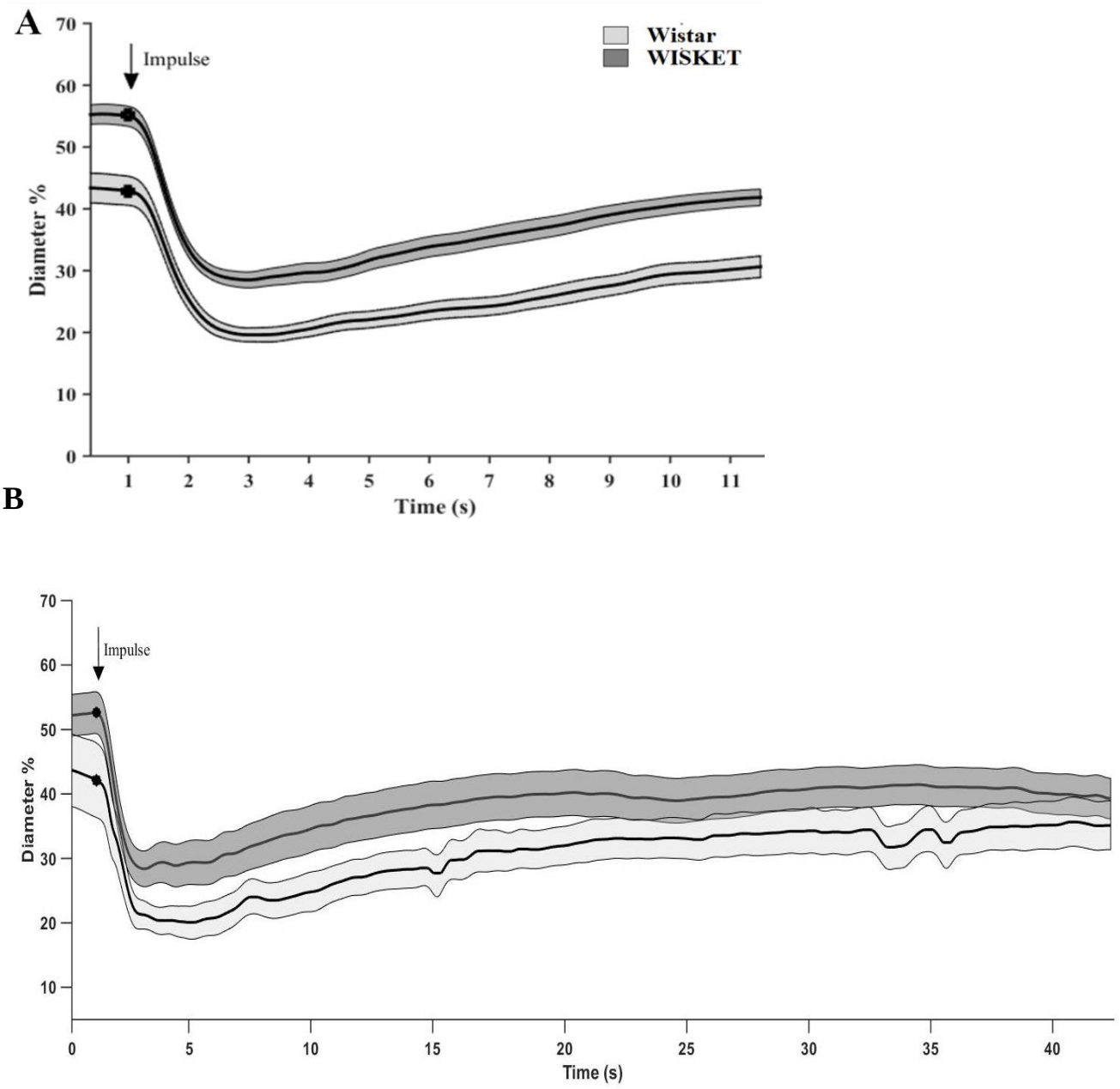

Figure 15. Illustrations of the pupilograms, responses to single light stimuli in sedated (A) and in anesthetized (B) animals.

5.3.2.1 Pupillary function in sedated animals

Five out of the analyzed parameters showed significant differences between the groups (Table 7). First of all initial and the minimum pupil diameters were larger in the WISKET animals (Table 7 and Fig. 16A), with a higher variance of the latter one in the WISKET rats compared to the Wistar ones (0.31 vs 0.16).

No significant differences were detected in the latency and the duration of constriction, while the flatness of the curve and the duration of total constriction time were significantly shorter in the WISKET group (Table 7 and Fig. 17A). The amplitude of constriction did not show significant differences between the groups, however, the degree of the constriction was significantly lower (Table 7 and Fig. 18A), and its variance was higher in the WISKET group compared to the control (49.9 vs 39.0). Regarding redilation, the time to reach the 25%, 33% and 50% of the initial diameter did not differ significantly (Fig. 19A).

Table 7. Significant effects of the pupillary light reflex test between groups or conditions.

	Sedation		**Anaesthesia**		**Condition**	
Investigated parameters	**F value**	**Significance**	**F value**	**Significance**	**F value**	**Significance**
Initial diameter (%)	$F_{(1,37)}=12.537$	$p<0.01$				
Minimum diameter (%)	$F_{(1,37)}=32.944$	$p<0.0001$	$F_{(1,32)}=5.574$	$p<0.05$		
Duration of constriction (ms)					$F_{(1,69)}=16.522$	$p<0.001$
Flatness of curve (ms)	$F_{(1,37)}=10.044$	$p<0.01$			$F_{(1,69)}=22.082$	$p<0.0001$
Total constriction time (ms)	$F_{(1,37)}=5.428$	$p<0.05$			$F_{(1,69)}=25.962$	$p<0.0001$
Degree of constriction (%)	$F_{(1,37)}=19.431$	$p<0.0001$			$F_{(1,69)}=5.707$	$p<0.05$
25% Redilation (s)					$F_{(1,69)}=17.51$	$p<0.0001$
33% Redilation (s)					$F_{(1,69)}=15.718$	$p<0.001$
50% Redilation (s)					$F_{(1,69)}=25.006$	$p<0.002$

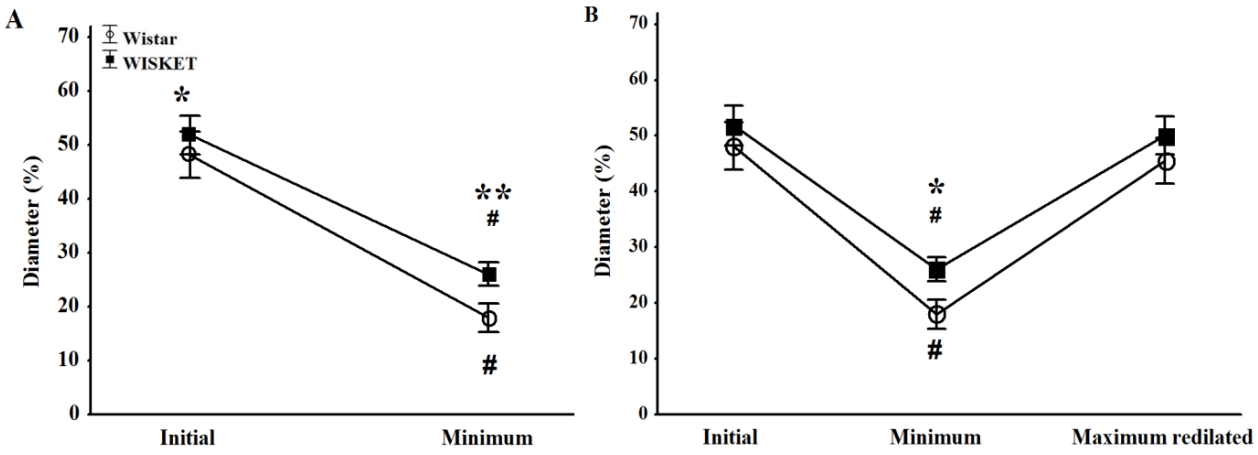

Figure 16. Initial and minimum pupil diameters in sedated (A) and initial, minimum and maximum redilated pupil diameters in anesthetized (B) animals are presented. The symbols indicate significant differences between the corresponding Wistar and WISKET groups (*p<0.05 or **p<0.01) and the initial and the minimum diameter within a group ($^{\#}$p<0.05).

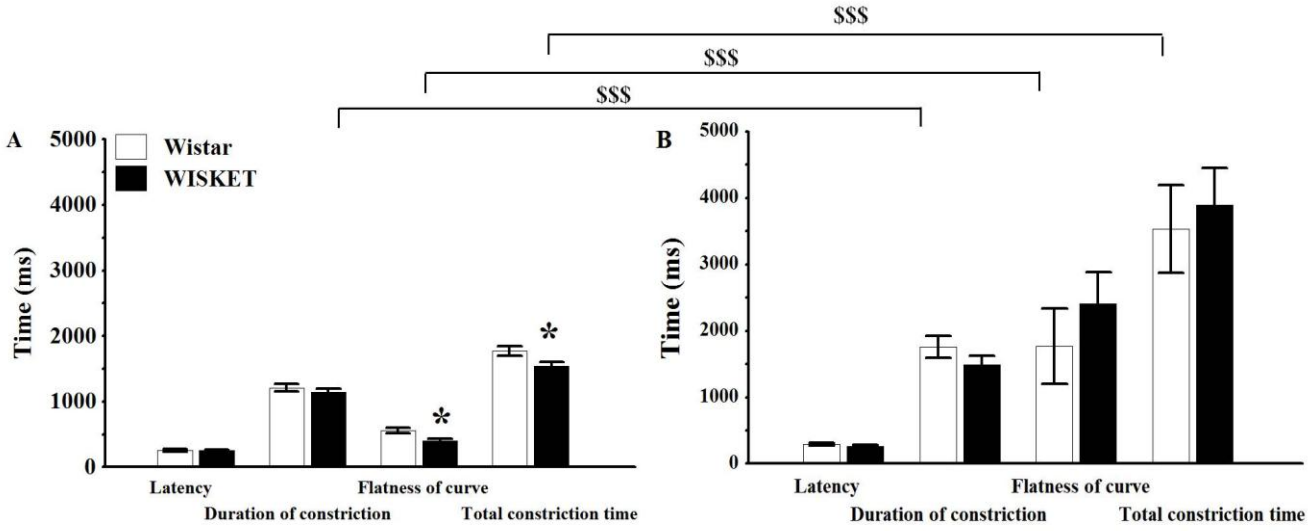

Figure 17. Analysis of the time related parameters: latency, duration of constriction, duration of flatness of the curve and the total constriction time in sedated (A) and in anesthetized (B) animals. The symbols indicate significant differences between the corresponding Wistar and WISKET groups (*p<0.05) and conditions ($^{\$\$\$}$p<0.0001).

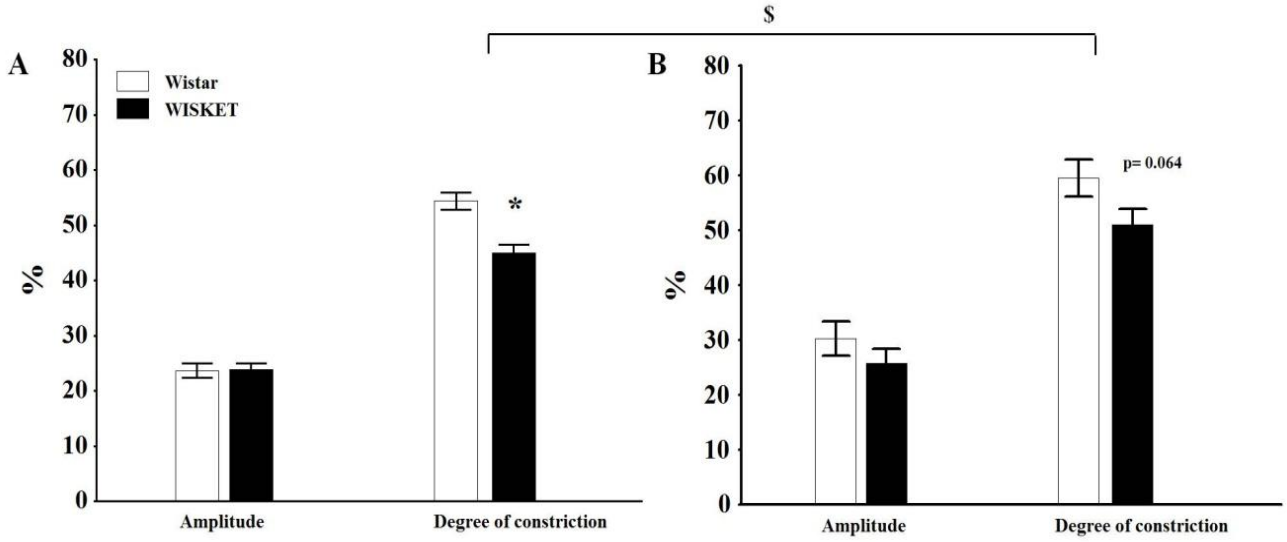

Figure 18. Amplitude and degree of constriction in sedated (A) and in anesthetized (B) animals. The symbols indicate significant differences between the corresponding Wistar and WISKET group (*$p<0.05$) and conditions ($^{\$}p<0.05$).

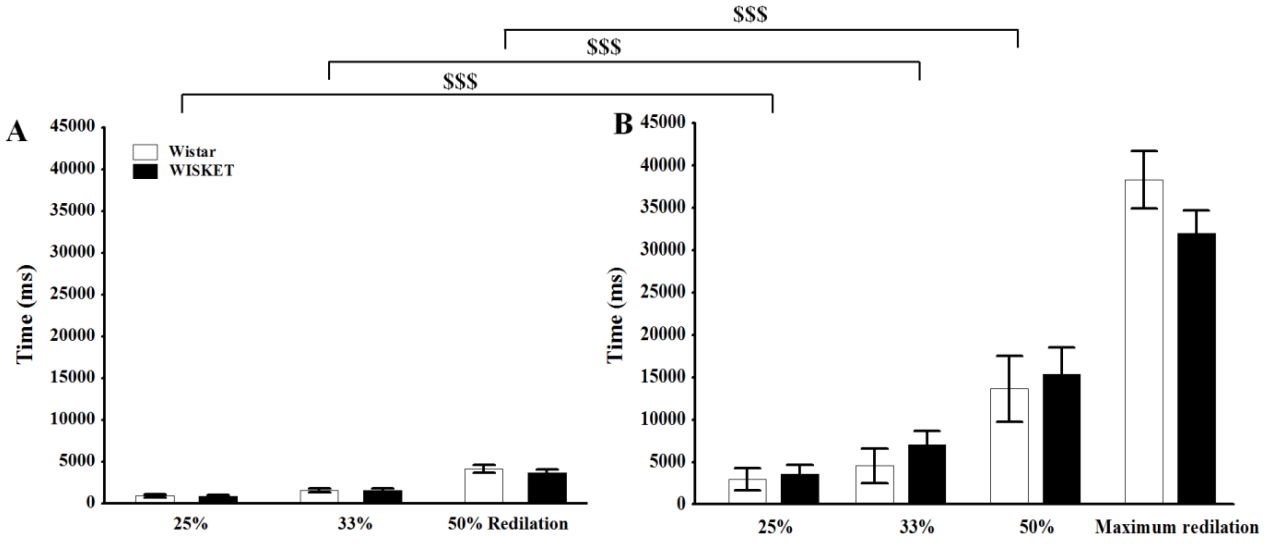

Figure 19. Analysis of time to 25%, 33% and 50% redilation in sedated animals (A) and 25%, 33%, 50% and maximum redilation in anesthetized rats (B). The symbol ($^{\$\$\$}p<0.0001$) indicates significant differences between conditions.

5.3.2.2 Pupillary function in anesthetized animals

Regarding the pupillary parameters during chloral hydrate anesthesia, ANOVA analysis showed only one significant difference between the two groups, i.e. the minimum pupil diameter was larger in the WISKET rats with higher variance (1.16 vs 0.24) compared to the Wistar rats (Table 7 and Fig. 16B). No significant differences were detected between the groups in the latency and duration of constriction and the flatness of the curve, but higher variance was observed in the WISKET group (flatness of the curve: 5953 vs 2320) (Fig. 17B). Thus, the duration of total constriction was also similar in both groups with higher variance in the WISKET group (7595 vs 3981, Fig. 17B). The amplitude of the constriction was similar, while the degree of the constriction showed close to significant difference ($p= 0.064$) between the two groups (Fig. 18B). Regarding the redilation process, the time to reach the 25%, 33% and 50% of the initial diameter were not significantly different between the two groups (Fig. 19B). The prolonged investigation of the pupillary reaction in this series made it possible to observe the redilation process for a longer period (Fig. 19B); a trend for a shorter redilation time was seen in the WISKET group.

5.3.2.3 Comparison of the two conditions (sedation vs anesthesia)

Regarding the comparison of the two conditions, all of the constriction-, and redilation-related parameters were significantly prolonged under chloral hydrate anesthesia, and the degree of constriction was significantly larger (Table 7 and Fig. 17, 18, 19). Correlations were revealed between the initial and the minimum pupil diameters and between the initial diameter and the amplitude of constriction in both groups and under both conditions (Table 8). Additionally, more significant correlations were detected in the WISKET compared to the control animals, especially during anesthesia (number of correlations: sedation: WISKET: 12, Wistar: 10; anaesthesia: WISKET: 26, Wistar: 15, Table 8).

Table 8. Significant correlations between different pupillary parameters in sedated (A) and anesthetized (B) Wistar and WISKET animals.

A

Wistar / **WISKET**	Initial diameter	Minimum diameter	Latency	Amplitude	Degree of constriction	Duration of constriction	Flatness of curve	Total constriction time	25% Redilation	33% Redilation	50% Redilation
Initial diameter		0.79	0.54	0.90							
Minimum diameter	0.79										
Latency				0.56							
Amplitude	0.74				0.61						
Degree of constriction		-0.48		0.77							
Duration of constriction		-0.54						0.90			
Flatness of curve									0.75	0.71	
Total constriction time		-0.52				0.91	0.56		0.59		
25% Redilation										0.89	
33% Redilation									0.87		
50% Redilation	0.58			0.60						0.50	

B

Wistar / **WISKET**	Initial diameter	Minimum diameter	Latency	Amplitude	Degree of constriction	Duration of constriction	Flatness of curve	Total constriction time	25% Redilation	33% Redilation	50% Redilation	Max. Redilation	Degree of redilation	Redilation time
Initial diameter		0.73		0.97	0.66	0.57		0.63				0.80		
Minimum diameter	0.82											0.69		
Latency	0.51	0.45												
Amplitude	0.52				0.80	0.66	0.61	0.71				0.74		
Degree of constriction		-0.84		0.56		0.61		0.63						-0.58
Duration of constriction				0.81	0.64			0.72						
Flatness of curve				0.51	0.53	0.50		0.96						
Total constriction time				0.61	0.6	0.64	0.98							
25% Redilation						0.55	0.82	0.84		0.97				
33% Redilation						0.55	0.90	0.91	0.95		0.57			
50% Redilation							0.73	0.66	0.61	0.70			0.58	
Max. Redilation	0.70	0.73			-0.59									
Degree of redilation			0.45	0.48							0.49			
Redilation time														

6 DISCUSSION

Accumulating evidence proved that our WISKET rat model has validity in several aspects of behaviors related to schizophrenia; therefore it might be appropriate for preclinical screening of putative antipsychotic agents. Besides the decreased acute heat pain sensitivity and sensory gating disturbance, we found reduced exploratory and locomotor activities, increased anxiety level, altered social interest accompanied with increased aggression and avoidance, especially in male WISKET animals. Furthermore, we observed reduced cognitive performace in two simple behavioral tests. At last, we showed that the dysfunction of the ANS lead to impairments not only in the thermoregulation, but also in the pupillomotor control [142].

6.1 General motor activity

Our study revealed that the general motor behavior, including rearing and locomotor activities were decreased especially in adult male WISKET rats. All these results are in agreement with the results obtained recently in NOR and the five-day long HB tests [202, 208]. The female and male animals in both group spent more time with rearing and grooming in the social interaction test compared to the HB test, which might be due to the different environmental circumstances and/or the appearance of a new counterpart, which required different behavioral patterns from the animals.

Motor abnormalities are common in schizophrenia, i.e. decreased activity is associated with negative and depressive symptom clusters, while excessive motor activity is often accompanied by positive symptoms [2, 226, 227]. Regarding the animal models of schizophrenia, increase/decrease or no change in locomotor activity has also been described. [228-238]. Some schizophrenia-related animal studies show 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 [235, 236, 239].

Generally juvenile and prepubertal animals are described as more anxious compared to adult rats; similar to that our rats also showed elevated grooming activity in younger age regardless group and sex in the social interaction test [240].

It is well known that stress and the development of a schizophrenic psychosis are inextricably related [241]. Even the decreased motor activity suggests enhanced anxiety in WISKET animals. Besides this, the enhanced grooming behavior in the social and HB tests and the

altered place preference (less time spent in central part of arena in HB test) further indicate the high level anxiety in adult male WISKET rats.

There are controversial findings in the sex-dependent response to stressful situation in control and schizophrenia animal models, as well [242-247]. Female rodents appear less anxious in the most tests, but it depends on the applied test and on stages of the oestrous cycle.

6.2 Cognitive performance

Our present results indicated that very simple and fast behavioral tests (HB and AMBITUS tests) are appropriate to detect the presence of cognitive disturbances.

In agreement with the results obtained recently WISKET rats showed decreased learning ability in both tests [202, 208]. The most efficient strategy in these tasks requires the subjects to run directly from box/hole to box/hole, collecting food rewards at each site. It was found that normal rats were able to learn the task to a high level of performance during a short period of time, and they exhibited improved performance at the second trial in the AMBITUS test, suggesting that these simple tasks could be acquired easily.

It is well-known that the cognitive functions significantly depend on the behavioral activity, which is also impaired in schizophrenic patients, and a close relationship was detected between reinforcement learning and general psychosocial functioning in schizophrenia [2, 248].

Cognitive deficits in several “two-hit” rat models of schizophrenia were recently investigated and some of them also paid attention on the sex differences. Combining of postweaning isolation rearing and postnatal MK-801 or PCP treatment have resulted more severe impairments in recognition and contextual memory in adulthood than the single interventions [150, 249, 250].

Regarding the sex differences in HB test, the performance of females was found to be less affected by the complex treatment, which is in agreement with findings that women are less vulnerable than men to schizophrenia [251]. Despite the large number of studies demonstrating sexual dimorphism in cognitive function in schizophrenia, other studies have shown no difference by sex [251]. The cognitive alterations are most likely supported by sexual dimorphism in brain morphology and neurochemistry found in schizophrenia [76, 252].

Wistar rats exposed to neonatal maternal separation and chronic adolescent corticosterone treatment have shown sex-specific behavior in adulthood: in male rats marked disruptions were detected in Y-maze and in the Morris water maze tests, which were absent in females

[253, 254]. These behavioral alterations were accompanied by region- and sex-specific long-term effects in brain-derived neurotrophic factor expression and signaling. Inescapable footshock exposure and corticosterone administration also led to the inability to ignore irrelevant stimuli in the male, but not the female offsprings [255]. Shionogi's mutant male rats treated with both a glutathione synthesis inhibitor and a dopamine uptake inhibitor have been resulted in impaired recognition memory in NOR test in adulthood, whereas females were not affected [256]. Furthermore, neonatal domoic acid treatment with social isolation rearing also impaired the attentional processing on latent inhibition in young adult male, but not female rats [257].

6.3 Social behavior

Our present experiment was the first attempt to shed light on sex-dependent affiliative and aggressive behavior in a "multi-hit" rat model of schizophrenia. The findings indicated decreased social interest in each group with the age. Adult male WISKET rats showed increased aggression and avoidance, while no differences were detected in the social behavior between female WISKET and Wistar animals.

In agreement with our results, a 2–10-fold increased incidence in aggression could be observed in schizophrenia patients, and sex differences in social interaction of schizophrenic patients have long been recognized [73, 258-260]. Decreased social interaction has been described in different animal models of schizophrenia [261, 262]. Thus, isolation reared rats show markedly more aggression than socially reared ones, which could be resistant to re-socialization procedures in a good correlation with our present findings [263]. Subchronic PCP treatment in male rats reduces the initiation of affiliative contacts and the time the spent with social interaction, and increases the aggressive responses [264].

As sex hormones play an important role in brain development, it is 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, only some studies investigated the sex differences in the social behavior [265-269]. Each of them has found that males, but not females show a significantly reduced duration of active social interactions and spent longer time in avoidance, but exposure to periadolescence social isolation can result in increased aggression also in females [265-270].

Few studies have investigated the social behavior in two-hit models [271-276]. Ketamine combined with maternal deprivation or sepsis resulted in increased latency to start social behavior in adult rats without significant differences in the number of contacts and the time

spent in social engagement [273]. So far only one experiment has revealed the sex-dependent alterations in social behavior in a double hit model: neonatal exposure to polyI:C with peripubertal unpredictable stress resulted in deficits in the social preference of male rats [269].

6.4 Autonomic function

The goal of the pupillary light reflex investigation was to determine the autonomic function of a new schizophrenia model by a simple and fast method, which can be applied in a large number of animals without any training and/or surgery. The values of pupillary parameters observed in the naive Wistar animals were comparable with earlier rat studies, which suggest that the applied method is appropriate for the characterization of pupillary function in rodents [217, 221, 277, 278].

The sedated WISKET rats had greater pupil diameters in the different phases of PLR compared to control animals, which were accompanied by shorter and lower-degree of constriction without alterations in redilation process. In contrast, chloral hydrate anesthesia provided ideal circumstances for the investigation of pupil functions for a longer period, it blunted the differences between the two groups and resulted in a prolonged pupillary reaction [216]. Based on these results, we suggest that sedation is more appropriate for the purposes of such investigations. In conclusion, both the impaired thermoregulation and pupillary function in WISKET animals suggest disturbed ANS balance [142].

Data concerning the pupillomotor control in schizophrenic patients are controversial. Regarding the initial pupil diameter (i.e. dark adapted pupil diameter) in PLR test, some human studies have found a smaller value in schizophrenic patients compared to control subjects, while a lack of difference in diameter has also been reported with prolonged reaction latency to light stimulus [48, 51-53, 279]. Most of these studies emphasize the decreased amplitude of constriction to light stimulation [48, 49, 52, 279]. Hakerem et al. (1964) reported no significant difference in constriction amplitude between control subjects and drug-free schizophrenic patients, which are in agreement with our results [51]. However, newer data show significantly increased resting pupil diameter in schizophrenic patients and lower degree of constriction without changes in the latency of PLR, similar to our findings [50]. Since the calculation of the “degree of constriction” is independent of the initial diameter, it may serve as a more reliable parameter for the analysis of constriction than the “amplitude of constriction”. In accordance with clinical data, which have found shorter constriction, and

therefore, an earlier redilation after visual stimulation in schizophrenic patients, a shortened flatness of the curve was detected in our sedated WISKET rats [49, 53, 279].

Several psychological and physiological variables can influence the pupil diameter and the reflex response to light stimulus [51, 215]. In healthy volunteers, anxiogenic stimuli influence this reflex, and patients with anxiety disorders show several alterations in pupillary function [51, 280, 281]. It is well-known that fear causes an increase in the initial pupil diameter and a decrease in the amplitude of the response to light, reflecting the active inhibition of the parasympathetic efferent pathway.

As was mentioned in the Introduction, schizophrenic patients exhibit an impaired ANS balance [36, 48, 50, 282]. Most of the studies investigating the heart rate variability indicate decreased parasympathetic tone [283]. Rubin (1974) discussed findings in which schizophrenia patients experienced difficulty exerting appropriate reciprocal parasympathetic control following stress cessation and throughout the restitution period [8]. Specifically, Spohn & Patterson (1979) identified “sluggish” parasympathetic function among schizophrenia patients [284]. It is well-known that the higher autonomic centers (e.g. the hypothalamus, the amygdala and the prefrontal cortex) play an important role in the changes of autonomic tone, and multiple pathways influence the activity of the autonomic preganglionic neurons. Several descending projections from these centers have inhibitory effects on the Edinger-Westphal nuclei of the midbrain, resulting in pupillary dilation. Either the inhibition of the parasympathetic fibers or the increased activity of the posterior hypothalamus via the sympathetic nervous system branch might be responsible for the increased pupillary diameter in schizophrenic patients.

In darkness, the active parasympathetic tone is minimal [285], thus the enhanced initial pupil diameter, but also the shortened flatness of the curve suggest enhanced sympathetic nervous sytem activity. Since constriction is primarily influenced by the parasympathetic nervous system, the lower degree of constriction suggests a decreased level of parasympathetic activity, too. Similarly to clinical findings from schizophrenic patients, a large variance was observed in several parameters in WISKET rats, suggesting that an impaired inhibitory control occurs by the higher centers [51].

The relatively moderate correlations among the pupillary parameters suggest that the initial pupil diameter determines only a part of the variance of the other measures. However, the number of significant correlations was higher in the WISKET rats hypothesizing that the altered central mechanisms (enhanced sympathetic tone) may contribute to these changes [49]. Surprisingly, anesthesia, which inhibits the sympathetic activity, increased the number

of correlations, too. These observations together indicate that the alteration of the balance between the parasympathetic and sympathetic tone (in any direction) may lead to increased correlation between the different pupillary parameters.

Regarding the determination of the action mechanisms of the observed changes require several new experiments, which might allow whether these changes are due to the alterations at the supraspinal, spinal or even postganglionic levels. These experiments should investigate both sympathetic and parasympathetic postganglionic neurons (including the processes of release, uptake and metabolisms of noradrenaline and acetylcholine).

All of the observed pupillary changes in this substrain suggest enhanced sympathetic and/or decreased parasympathetic activity, giving further support the validity of the WISKET substrain as a model of schizophrenia, through the general shift towards a sympathetic predominance in these animals [142].

6.5 Limitations

Our substrain was originally developed as a complex model of schizophrenia, and has been extensively investigated as such; however, it is well-known that several signs of schizophrenia overlap with autism spectrum disorders. Therefore, it can not be claimed that this model specific to schizophrenia.

The lack of appropriate controls – a socially-reared and without ketamine treatment WISKET control group; and a Wistar group with isolation rearing and ketamine administration – to determine the contribution of the different interventions to the face validity of the model, might be seen as a major limitation. However, as far as the validity of the animal model itself is concerned, this is not really an issue. Ample evidence was provided in a previous paper that the new substrain after the complex treatment has the highest validity as a schizophrenia model compared to the appropriate control groups, suggesting that the combination of genetic and environmental factors lead to the best model in this paradigm [202]. From that time characterization of this model was focused in several aspects [202, 208, 209, 225]. The contribution of the genetic or environmental factors separately to the different signs is indeed an interesting theoretical question, however, it also raises ethical concerns, and, from an animal welfare point of view, it would be difficult to argue for the necessity of extra experiments only to describe the individual contribution of the hits, once it has been proven that the model is valid.

Regarding the social interaction test, it is a fast and robust method providing information about the social interest, aggressive behavior and avoidence. We applied only this type of

social behavior test, however, it is preferable to use multiple methods (e.g. three chamber test, tube test, resident–indruder test) to show the altered social interaction [138]. The challenge with this version of social interaction test is that both animals are freely moving, making it difficult to disentangle factors such as, social novelty preference, sociability and social discrimination [286]. The three chamber test might be used to define the animal's preference for social novelty versus a familiar conspecific [287]. Distinct aspects of social dominance can be measured with the tube and resident–intruder tests [288].

The social interaction test that we used was conducted in a novel environment that might change the pattern of exploratory and anxiety–like activities in addition to social behaviors. Tests conducted in the home cage may solve such problems. However, forebrain-specific calcineurin mutant mice showed profound abnormalities in social behavior as assessed with an automated home–cage system similarly to the more conventional 10-min social interaction test under novel environment [289].

A limitation of series 2 investigating pupillary light reflex is that only male rats were used. However, earlier data did not show substantial sex differences in the pupillary responses obtained in healthy subjects and even no studies are available about the sex differences in the pupillary responses in schizophrenia [78].

7 CONCLUSION

The WISKET rats after the complex treatment showed several alterations relevant to schizophrenia. Both series of the experiments confirmed decreased acute heat pain sensitivity, impaired sensory gating, exploratory activity and cognitive function in WISKET rats.

The series 1 proved that these disturbances were sex-dependent and reduced social interest accompanied with increased aggression, avoidance and anxiety-like behavior were detected especially in adult male WISKET animals. Thus, the performance of females was found to be less affected by the complex treatment.

Furthermore, the data from series 2 demonstrated that not only the thermoregulation, but another autonomic function, the pupillary control also shows significant alterations in WISKET rats, suggesting the imbalance in sympathetic and parasympathetic divisions; however, the type of anesthesia significantly influence the difference between the groups.

7.1 Clinical relevance

The increased sympathetic activity and frequency of metabolic syndromes have been reported in untreated schizophrenic patients, which indicate a shortened life expectancy and an increased mortality ratio for cardiovascular disorders [292]. Worsening of the vascular risk factors has been signaled as a side effect of treatment with neuroleptic drugs [293].

Thus, we suggest the introduction of the quick and simple PLR test as a routine in the clinical monitoring to characterize the ANS status of schizophrenic patients.

The present findings obtained from the social and cognitive tests further increase the face and constructive validities of our WISKET rat model that reinforces its translational utility for animal-based preclinical drug discovery studies improving negative symptoms and cognitive deficits with potential antipsychotic efficacy.

8 SUMMARY

Our studies provide an intimate view of detailed characterization of a new, complex, chronic animal model of schizophrenia (named WISKET) through the following findings:

1. In agreement with our recent studies the Wistar and WISKET rats showed significant differences in basal behavioral parameters in both series of experiments.
2. WISKET male rats showed decreased learning capacity, low exploratory and locomotor activity.
3. Both male and female WISKET animals had higher level of anxiety compared to Wistar rats.
4. The social interest was decreased in each group by age; accompanied by enhanced aggressive and avoidance behavior in male WISKET rats.
5. The pupillary light reflex test performed under sedation proved that WISKET animal had significantly greater initial and minimum pupil diameters with significantly lower degree of the constriction and longer constriction time.
6. The anesthesia blunted the differences between the groups observed in the other condition; however, the minimum pupil diameter was significantly greater in WISKET animals.

In conclusion accumulating evidences suggest that our WISKET rat model has high degree of validity in several aspects to mimic abnormal behavioral and autonomic disturbances related to schizophrenia and it might be appropriate for preclinical screening of putative antipsychotic agents.

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Impaired pupillary control in "schizophrenia-like" WISKET rats

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ABSTRACT

Patients with schizophrenia show impairments in autonomic regulation, including pupillomotor control. The aim of this study was to explore the changes of pupillary light reflex in a new substrain (WISKET) with several schizophrenia-like alterations.

Male WISKET rats housed individually (for four weeks) and treated with ketamine (for 3 × 5 days) after weaning and naive group-housed Wistar rats (controls) were involved in the study. The pupillary light reflex was studied in two series after sedation (diazepam) or anesthesia (chloral hydrate). Video recordings were evaluated with custom made video analyzer software.

Several significant changes were observed between the two groups: the initial and minimum pupil diameters were greater, the degree of the constriction was lower, and the flatness of the curve and the total duration of constriction were shorter in the sedated WISKET rats. No other pupillary parameters (latency, amplitude and redilation) showed significant alterations.

Chloral hydrate anesthesia prolonged the constriction and redilation processes compared to the sedated animals, and diminished the differences between the groups.

In conclusion, WISKET rats showed disturbances in the pupillary light reflex, suggesting a general shift of autonomic balance towards a sympathetic predominance. The results provide further evidence to support the validity of WISKET rats as a complex, chronic animal model of schizophrenia.

1. Introduction

Patients with schizophrenia show not only behavioral impairments but also autonomic dysregulation, manifest as decreased variability of blood pressure and heart rate, abnormal thermoregulation and impaired pupillary function (Bar et al., 2007, 2008; Hermesh et al., 2000; Rubin and Barry, 1972; Shiloh et al., 2005, 2007; Zahn and Pickar, 2005). Pupil responses are sensitive and reliable sources of information about the function of the nervous system, including its autonomic division. Pupillometry, a simple, non-invasive technique, can be utilized for the objective characterization of the pathophysiology of pupillary functions, and it has long been applied in human diagnostics (Bremner, 2009; Neuhuber and Schrodl, 2011). Pupil diameter is determined by two antagonistic smooth muscle groups, the sphincter and dilator muscles within the iris. Pupillary constriction or miosis is brought about by the action of the sphincter muscle, whereas pupillary dilation or mydriasis happens by the contraction of the dilator muscle. The sphincter muscles receive primarily postganglionic parasympathetic fibers from the oculomotor nerve through the ciliary ganglia, and the preganglionic fibers originate from the Edinger-Westphal (EW) nucleus located in the midbrain. Generally, the parasympathetic pathway provides a tonic drive to the sphincter iridis muscle through the activation of M3 muscarinic receptors, and their enhanced activation causes miosis. The dilator muscles receive noradrenergic sympathetic input from the superior cervical ganglion, which acts primarily on α1-adrenoceptors; providing only little tonic drive. Thus, pupillary function is determined by a balance between the sympathetic and parasympathetic autonomic nervous system (ANS), such as reciprocal control, co-inhibition or co-activation which depend on a number of factors, including genetic influences, age, wakefulness, accommodative state and ambient lighting conditions (Neuhuber and Schrodl, 2011). Therefore, the resting pupil size may vary over a wide range even in healthy individuals (Bremner, 2009; Goldwater, 1972).

The pupillary light reflex (PLR) is a primitive, cross-species, bidirectional

Abbreviations: ANS, autonomic nervous system; ASD, autism spectrum disorder; C, control; EA, exploratory activity; EW, Edinger-Westphal; LC, learning capacity; PA, pulse alone; PLR, pupillary light reflex; PP, prepulse - pulse pair; PPI, prepulse inhibition; TF, tail-flick; W, WISKET

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reflex. Under normal circumstances, bright light shone into one or both eyes produces a brisk and transient contraction followed by a slow redilation (Goldwater, 1972). The reflex is mediated by a well-characterized and relatively simple neural circuit, involving the retinal ganglion cells, the pretectal nuclei, the mesencephalic EW nucleus, the ciliary ganglion, and the pupillary sphincter muscle (Neuhuber and Schrodl, 2011).

Those clinical studies that used pupillometry to investigate the autonomic function in schizophrenic patients yielded controversial results, but most of them suggested an increased sympathetic modulation and/or decreased parasympathetic activity (Bar et al., 2008; Hakerem et al., 1964; Lidsky et al., 1971; Morris et al., 1997; Okada et al., 1978; Rubin and Barry, 1972; Steinhauer and Hakerem, 1992; Steinhauer et al., 1992). The physiology, pathophysiology and pharmacology of pupillary functions were also investigated in rodents (Mohan et al., 2012; Young and Lund, 1994), however, no data on pupillary function are available from animal models of neuropsychiatric disorders, including schizophrenia.

Recently a complex, chronic rat substrain of schizophrenia has been derived, that shows several symptoms of the disease. The new substrain was named **WISKET**, since the original strain was **WIS**tar and the selective breeding was based on behavioral alterations after the combination of postweaning **IS**olation rearing and subchronic **KET**amine treatment (Horvath et al., 2016, 2017; Kekesi et al., 2015; Petrovszki et al., 2013). These animals exhibit disturbances in sensory gating and pain sensitivity, altered auditory evoked potentials, and impairments in various cognitive functions. Furthermore, the binding affinity of opioid and cannabinoid receptors in the brain of these animals is also significantly different from that of Wistar rats (Szűcs et al., 2016a, 2016b). In a recent study, we applied telemetric method for body temperature registration, and found that WISKET rats had higher body temperature during the active phase, and they showed a wider range of the body temperature alterations than the control Wistar rats, which suggests disturbed thermoregulation (Horvath et al., 2015). In the present study, we sought to further investigate the alterations of autonomic control in the new substrain by characterizing the changes of PLR.

It is well-known that the test procedure leads to a significant stress response in the animals, which can influence ANS functions. While anesthesia can prevent the stress and allows a convenient investigation of pupillary reactions for a longer period, it diminishes the autonomic responses (Hussain et al., 2009; Tayefeh et al., 1997). Therefore, the second goal of this study was to compare the pupillary responses in lightly sedated and anesthetized control and WISKET rats.

2. Materials and methods

2.1. Subjects

All experiments were carried out with the approval of the Hungarian Ethical Committee for Animal Research (registration number: XIV/2014). Animals were kept with a 12 h light/dark cycle under controlled temperature (22 ± 1 °C) with ad libitum water and food access (except for the experiments in the AMBITUS system, when the animals were food deprived for two days, see below, but water was always freely available).

2.2. Selective breeding

The paradigm for selective breeding, starting from a population of outbred Wistar rats, was described previously in detail (Petrovszki et al., 2013), and is shown in Table 1. Briefly, after weaning, at 3 weeks of age, the rats were tested with the tail-flick (TF) test to assess their basal pain sensitivity and then housed individually for 28 days (between 4 and 7 weeks of age). The animals in each generation were treated with ketamine (Calypsol, Gedeon Richter Plc., Budapest, Hungary; 30 mg/kg intraperitoneally [i.p.], 4 ml/1000 g body weight, daily, 5 times/week, 15 injections in total) from 5 to 7 weeks of age. Then the animals were re-housed in a group setting (3–4 rats per cage) and 1 week of recovery with no treatment followed. Starting at the age of 9 weeks, TF latency, sensory gating (pre-pulse inhibition), exploratory activity and cognitive functions were assessed (see below). Animals with the highest level of disturbance in these parameters were used for selective breeding throughout several generations (Horvath et al., 2015, 2016, 2017; Kekesi et al., 2015; Petrovszki et al., 2013; Szűcs et al., 2016a, 2016b).

2.3. The experimental paradigm

Two series of experiments were performed one week after the routinely executed behavioral tests in naive socially rearing/group-housed male Wistar rats without any interventions (Controls: C) and male WISKET (W) rats (Table 1) derived from multiple litters.

In the first series, the pupils were tested 15 min after diazepam-induced sedation (Seduxen, Gedeon Richter Plc, Budapest, Hungary; 2.5 mg/kg i.p.; W: n = 22, C: n = 17) for up to 15 s. The sedated animals had slow righting reflex and they accepted the slight restrain during the test period.

In the second series, the pupils were tested 15 min after chloral hydrate-induced anesthesia (Gedeon Richter Plc, Budapest, Hungary; 200 mg/kg i.p.; W: n = 20, C: n = 14) for up to 60 s (Lau et al., 1992). The anesthetized animals had no righting reflex and there was no response to mechanical stimuli.

The experiments were performed in each animal (in both series) between 8:30 and 12:30 without any training section.

2.4. Procedures

2.4.1. Nociceptive testing

Acute nociceptive threshold was assessed by the TF test at the 4th and 9th weeks of age (Table 1). 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: 20, 40 s, on the 4th and 9th weeks, respectively). TF latencies were obtained four times at 30 min intervals and were averaged to establish the pain threshold for each group.

2.4.2. Sensory gating testing

The degree of sensory gating (PPI) of the acoustic startle response was measured as described previously (Petrovszki et al., 2013). Briefly, after 10 min habituation in plexiglas startle chambers (12 × 17 × 15.3 cm) rats were exposed to two different trial types: the pulse alone (PA), in which a 40 ms 95 dB white noise burst was presented; and the prepulse - pulse pair (PP) in which prepulse stimuli (20 ms, 76 dB) were followed by the startle stimulus with a latency of 150 ms. Both types of stimuli were applied 20 times in random pattern. The interstimulus intervals ranged from 7 to 13 s. PPI was calculated as percentages using the following equation: PPI (%) = [1 − (*startle response for PP*) / (*startle response for PA*)] × 100.

2.4.3. Appetitively motivated cognitive task in the AMBITUS system

The AMBITUS system is a cognitive-behavioral test suitable for the detection of exploratory activities and learning capacity in rats (Horvath et al., 2017). Briefly, it is a square corridor of clear plexiglas on black floor (outer diameter of 80 cm, width of 8 cm and height of 50 cm), and all the eight walls have two equally spaced sites (side-boxes: 5 × 5 × 5 cm) with food reward in each of them (puffed rice, 20 mg) and equipped with infrared photocells for the detection of the exploratory activity (visits into the side-boxes) of the animals. The tests were also recorded using an infrared video device (WCM-21VF, CNB, China) fixed above the apparatus.

Trials commenced by placing the rats into the same starting point within the corridor; thereafter, the experimenter immediately left the room. The animals were allowed to explore the corridor and collect the 16 food rewards within 5 min (cut-off time). The number of food

Table 1
Schedule of the experimental protocol for selective breeding and behavioral testing.

Age (weeks)	4	4	5–7	8	9	10	12
Control group	TF0		Group housing (3–4 rats/cage)		TF1, PPI	AMBITUS	PLR
WISKET group		Social isolation	Social isolation + ketamine treatment (30 mg/kg/daily)	Group housing			

Abbreviations: TF: tail-flick; PPI: pre-pulse inhibition; PLR: pupillary light reflex.

rewards eaten was recorded at the end of each trial by the experimenter. The apparatus was cleaned with 70% alcohol after each trial. The trials were repeated two times with 1 min apart (1st and 2nd trials). The number of side-box visits was used to characterize the exploratory activity (EA), while learning capacity (LC) was calculated to determine the cognitive function of the animals (Horvath et al., 2017; Kekesi et al., 2015), as follows:

$$LC\ (\%) = \left[\frac{\text{number of collected food rewards} \times \text{cut–off time of the learning phase (300 s)}}{\text{number of food rewards (16)} \times \text{time required to complete the task (s)}}\right] \times 100.$$

2.4.4. Pupillary measurements

A modified digital camera (Nikon D7000) was used to record pupillary responses at a speed of 24 frames-per-second under infrared illumination. After the induction of sedation or anesthesia, a 10-minute dark adaptation period followed. Thereafter, the animals were positioned in front of the camera (the sedated animals were gently restrained), and the initial value of the pupil diameter was determined (the mean of 24 frames, 1 s before the stimulus). Then an intensive light stimulus (approximately 300 cd/m^2 for 600 ms) was applied to the left eye, along with an infrared flash.

The custom image and signal processing methods were developed, which allow the automatic measurement of the relative diameter of the pupil (as a ratio of the diameter of the iris, expressed in % (Blasiak et al., 2013; Dabisch et al., 2008; Thompson et al., 2011)) and extract the required parameters of the light impulse response on each recorded frame (Table 2, Fig. 1A). In video-recorded images, the exact definition of the pupil in the iris may be challenging due to scattering movements, significant blur, low contrast difference between the pupil and iris, noise and reflections. Besides the robust handling of these, the measurement process needs to be fast, accurate and reproducible. The solution is a novel, energy attenuation model-based ray propagation method, which uses mathematical, geometrical and physical relations to explore and analyze the structure of the iris and pupil regions and to estimate the latter's diameter. The evaluation of the proposed method on 20 manually processed video recordings showed that the overall diameter measurement error is less than ± 2%.

2.5. Statistical analysis

Data are expressed as means ± SEM. One-way or factorial (group and series) ANOVA were used to analyze data. For the post-hoc comparisons, the Newman-Keuls test was used. The relationships between pupil parameters were assessed by linear regression analysis and calculation of the Pearson correlation coefficient. Only probabilities lower than 0.05 were considered significant. For the analyses, STATISTICA 13.1 (Dell Inc. Round Rock, Texas, US) was used.

3. Results

In agreement with our recent studies (Horvath et al., 2015, 2016, 2017; Kekesi et al., 2015; Petrovszki et al., 2013; Szűcs et al., 2016a, 2016b), the control and WISKET rats showed significant differences in the behavioral tests in both series of experiments. The new substrain showed decreased acute heat pain sensitivity, impaired sensory gating, and motor and cognitive impairments (Table 3).

Regarding the pupillary parameters, the light stimulus caused significant pupillary constriction in both groups and in both series. The curves show fast constrictions followed by a slow recovery (Fig. 1B, C)

Table 2
Investigated pupillary parameters.

Parameters	Definition
Initial pupil diameter (%)	Pupil diameter before light stimulus related to iris diameter
Minimum diameter (%)	Minimum diameter after light stimulus related to iris diameter
Constriction latency (ms)	Time interval between light stimulus and constriction onset
Amplitude of constriction (%)	Initial diameter − Minimum diameter
Degree of constriction (%)	(1 − (Minimum pupil diameter / Initial pupil diameter)) × 100
Duration of constriction (ms)	Time interval between constriction onset and to reach the minimum diameter
Flatness of curve (ms)	The time the pupil remains at maximal constriction
Total constriction time (ms)	Duration of constriction + Flatness of curve
25% redilation time	Time to reach the 25% of the initial diameter
33% redilation time	Time to reach the 33% of the initial diameter
50% redilation time	Time to reach the 50% of the initial diameter
Maximum redilated pupil diameter (%)	Maximal redilation value related to iris diameter
Redilation time (ms)	Duration of maximum redilation

The calculated parameters based on earlier studies: (Canver et al., 2014; Clarke, 2007; Dabisch et al., 2008; Fan et al., 2009a, 2009b; Grozdanic et al., 2002; Hakerem et al., 1964; Hussain et al., 2009; Lau et al., 1992).

3.1. Pupillary function in sedated animals (series 1)

ANOVA showed significant differences between the two groups for the initial ($F_{(1,37)} = 12.54$, $p < 0.05$) and the minimum ($F_{(1,37)} = 32.94$, $p < 0.001$) pupil diameters (Fig. 2A), with a higher variance of the latter one in the WISKET rats (C: 0.16 and W: 0.31).

No significant differences were detected in the latency and the duration of constriction, while the flatness of the curve ($F_{(1,37)} = 10.04$, $p < 0.05$) and the duration of total constriction time ($F_{(1,37)} = 5.43$, $p < 0.05$; Fig. 3A) were significantly shorter in the WISKET group. The amplitude of constriction did not show significant differences between the groups, however, the degree of the constriction was significantly lower ($F_{(1,37)} = 19.94$, $p < 0.001$; Fig. 4A), and the variance of the degree of constriction was higher in the WISKET group compared to the control (C: 39.0 and W: 49.9). Regarding redilation, the time to reach the 25%, 33% and 50% of the initial diameter did not differ significantly (Fig. 5A), but the short period of recording did not allow a detailed analysis of the redilation process in the sedated animals.

3.2. Pupillary function in anesthetized animals (series 2)

Regarding the pupillary parameters during chloral hydrate anesthesia, ANOVA analysis showed no significant differences between the two groups in the initial pupil diameter, while the minimal pupil diameter was larger in the WISKET rats ($F_{(1,32)} = 5.57$, $p < 0.05$) with higher variance (C: 0.24 and W:1.16) (Fig. 2B). No significant differences were detected in the latency and duration of constriction and the flatness of the curve with higher variance in the WISKET group (C: 2320

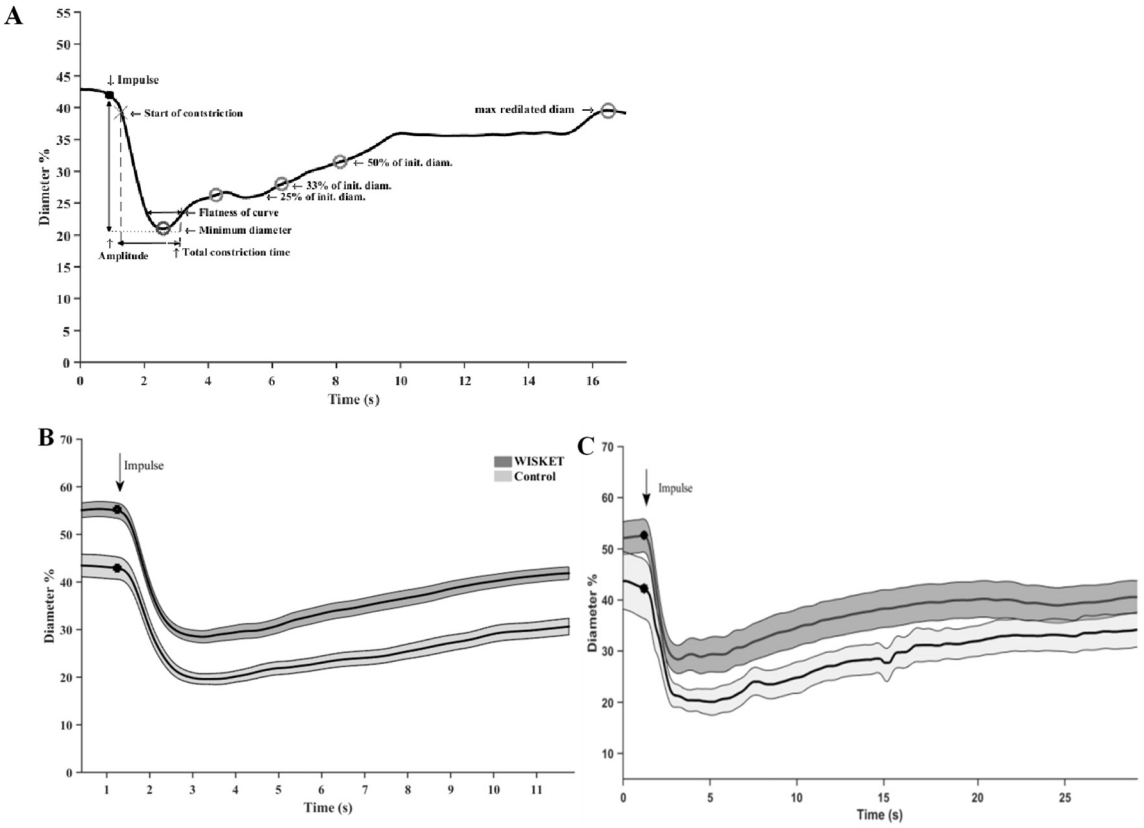

Fig. 1. Illustration of the pupillogram and the associated PLR parameters (A). Response to single light stimuli in sedated (B) and in anesthetized (C) animals.

and W: 5953). Thus, the duration of total constriction was similar in both groups with higher variance in the WISKET group (C: 3981 and W: 7595; Fig. 3B). The amplitude of the constriction was similar, while the degree of the constriction showed close to significant differences ($p = 0.064$) between the two groups (Fig. 4B). Regarding redilation, the time to reach the 25%, 33% and 50% of the initial diameter were not significantly different between the two groups (Fig. 5B). The prolonged investigation of the pupillary reaction in this series made it possible to observe the redilation process for a longer period: animals in both groups reached the initial value at about 35 s (Figs. 2B, 5B), but a slightly shorter redilation time was seen in the WISKET group ($p = 0.30$).

Regarding the comparison of the two series, all of the constriction-, and redilation-related parameters were significantly prolonged under chloral hydrate anesthesia, and the degree of constriction was significantly higher (Figs. 3, 4 and 5).

Data analysis revealed close correlations between the initial and the minimum pupil diameters and between the initial diameter and the amplitude of constriction in both groups in both series (Table 4.). Additionally, more significant correlations were detected in the WISKET compared to the control animals, especially during anesthesia (1st:C10, W: 12; 2nd: C: 15, W: 26) regarding the same parameters.

4. Discussion

The values of pupillary parameters observed in the control animals of this study were comparable with earlier rat studies (Clarke, 2007; Dabisch et al., 2008; Grozdanic et al., 2002; Lau et al., 1992), which suggests that the applied method is appropriate for the characterization of pupillary function in rodents. Our study revealed that the WISKET rats exhibit significant alterations in certain parameters of pupillary function, which suggest disturbed ANS balance, in agreement with our recent data (Horvath et al., 2015). The sedated WISKET rats had greater pupil diameters in the different phases of PLR, which were accompanied by shorter and lower-degree of constriction without any alterations in redilation process. Additionally, chloral hydrate anesthesia

Table 3
Results of behavioral parameters.

	1st series					2nd series				
	Control		WISKET			Control		WISKET		
	Mean	SEM	Mean	SEM	*p* value	Mean	SEM	Mean	SEM	*p* value
TF0	4.56	0.20	5.70	0.28	< 0.01	4.22	0.24	7.44	0.34	< 0.001
TF1	7.12	0.33	15.73	0.84	< 0.001	8.60	0.57	15.52	1.01	< 0.001
PPI	55.55	4.75	36.52	5.07	< 0.05	46.88	7.10	26.05	6.94	< 0.05
EA1	24.5	1.54	14.00	0.64	< 0.001	18.93	1.72	13.35	1.26	< 0.05
EA2	29.88	3.15	11.81	1.42	< 0.001	29.29	3.86	8.70	1.06	< 0.001
LC1	61.33	6.38	1.44	0.53	< 0.001	40.62	8.38	5.94	3.058	< 0.001
LC2	74.97	6.47	6.97	2.74	< 0.001	62.11	12.27	5.31	2.49	< 0.001

Abbreviations: EA: exploratory activity; LC: learning capacity; TF: tail-flick.

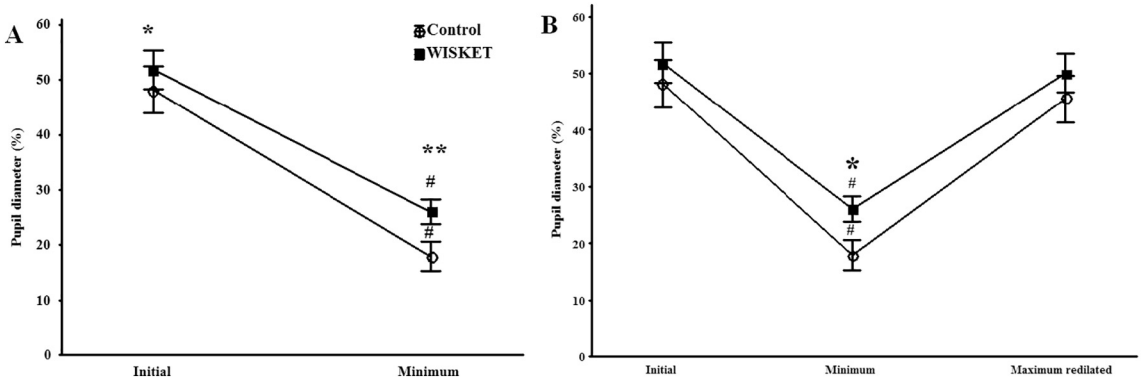

Fig. 2. A: initial and minimum pupil diameter in sedated animals. B: initial, minimum and maximum redilated pupil diameter in anesthetized animals. Data are presented as means ± SEM. The symbol (*) denotes significant differences by Newman-Keuls post-hoc test between the control group and WISKET rats. The symbol (#) denotes significant differences between the initial and the minimum diameter within a group. *, #: $p < 0.05$, **: $p < 0.01$.

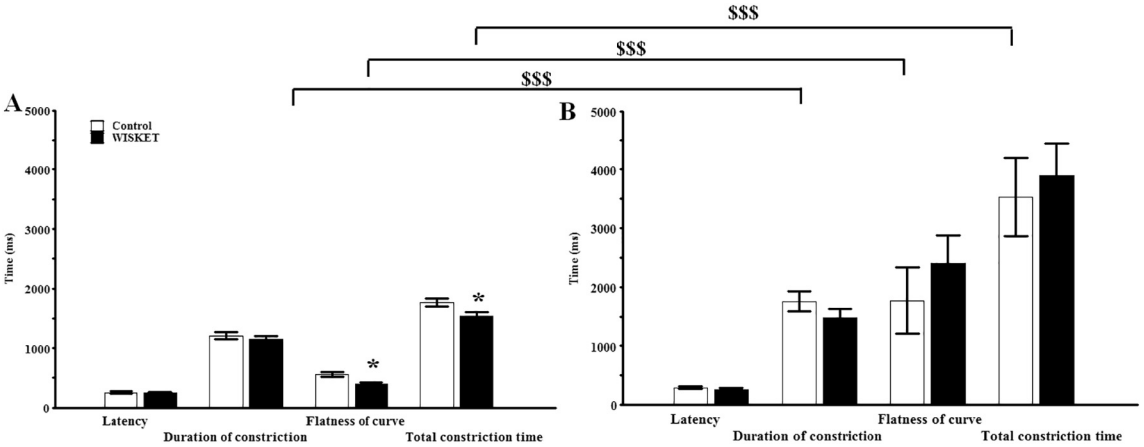

Fig. 3. Analysis of the time related parameters: latency, duration of constriction, duration of flatness of curve and the total constriction time in sedated (A) and in anesthetized (B) animals. Data are presented as means ± SEM. The symbol (*) denotes significant differences by Newman-Keuls post-hoc test between the healthy group and WISKET rats. The symbol ($) denotes significant differences between the series. *: $p < 0.05$, $$$: $p < 0.0001$.

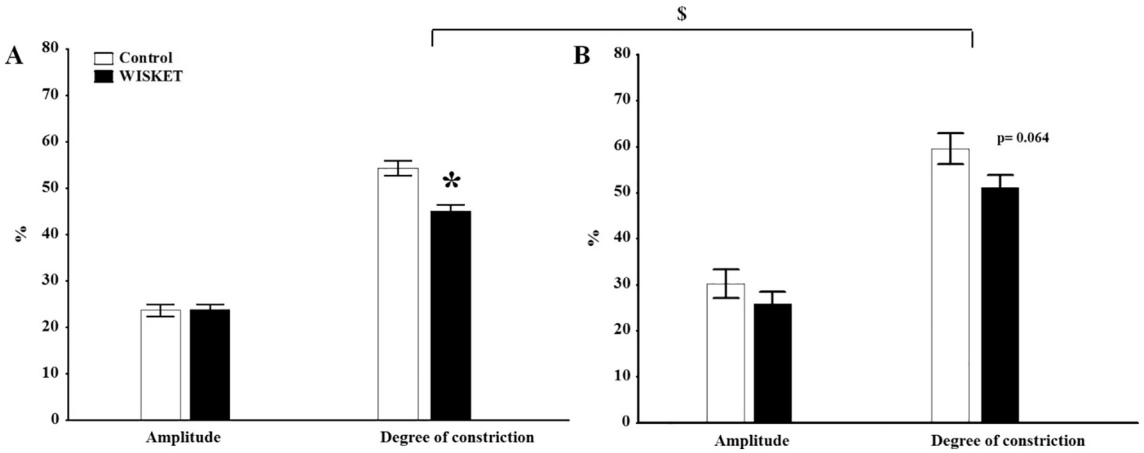

Fig. 4. Amplitude and degree of constriction in sedated (A) and in anesthetized (B) animals. Data are presented as means ± SEM. The symbol (*) denotes significant differences by Newman-Keuls post-hoc test between the control group and WISKET rats. The symbol ($) denotes significant differences between the series. *: $p < 0.05$, $: $p < 0.05$.

resulted in a prolonged pupillary reaction and blunted the differences between the two groups.

The data concerning the pupillomotor control in schizophrenic patients are controversial. Regarding the baseline (i.e. dark adapted pupil diameter), some human studies found a smaller value in schizophrenic patients compared to control subjects (Hakerem et al., 1964; Lidsky et al., 1971; Rubin and Barry, 1972; Steinhauer et al., 1992), while a lack of difference in diameter has also been reported with prolonged reaction latency to light stimulus (Okada et al., 1978). Most of these studies emphasized the decreased amplitude of constriction to light stimulation (Lidsky et al., 1971; Rubin and Barry, 1972; Steinhauer and Hakerem, 1992; Steinhauer et al., 1992). Hakerem et al. (1964)

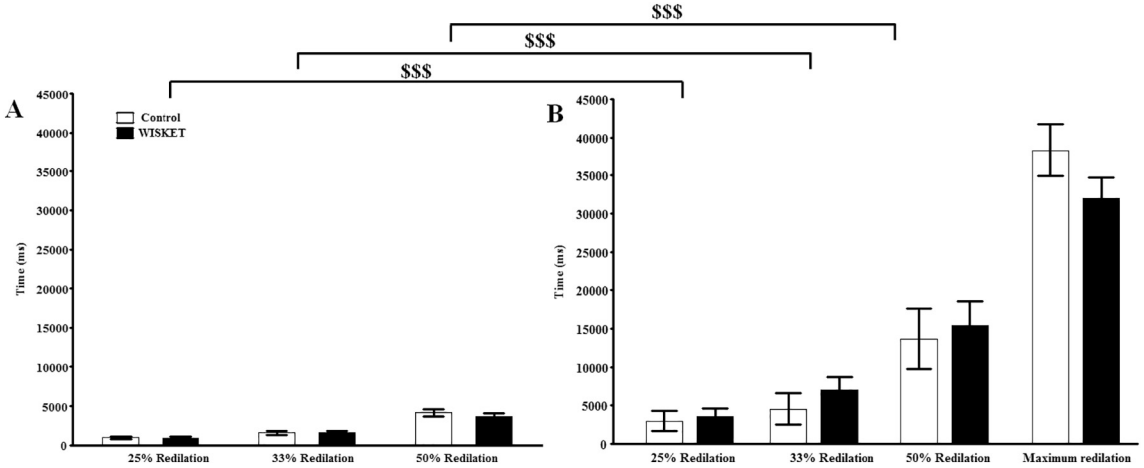

Fig. 5. A: analysis of redilation parameters 25%, 33% and 50% redilation in sedated animals. B: 25%, 33%, 50% and maximum redilation in anesthetized rats. Data are presented as means ± SEM. The symbol ($) denotes significant differences between the series. $$$: $p < 0.001$.

reported no significant difference in constriction amplitude between control subjects and drug-free schizophrenic patients, which is in agreement with our results. However, newer data showed significantly increased resting pupil diameter in schizophrenic patients and decreased degree of constriction without changes in the latency of PLR, which is in agreement with our results regarding WISKET rats (Bar et al., 2008). Since the degree of constriction is independent of the initial diameter, it may serve as a more reliable parameter for the analysis of constriction than its amplitude. In accordance with clinical data, which found shorter constriction and therefore an earlier redilation after visual stimulation in schizophrenic patients (Okada et al., 1978; Steinhauer and Hakerem, 1992; Steinhauer et al., 1992), a shortened flatness of curve was detected in our sedated WISKET rats.

As mentioned in the Introduction, schizophrenic patients exhibit an impaired ANS balance, which is reflected in the decreased variability of certain physiological parameters (Bar et al., 2008; Boettger et al., 2006; Lidsky et al., 1971; Zahn and Pickar, 2005). It is well-known that the higher autonomic centers (e.g. the hypothalamus, the amygdala or the prefrontal cortex) play an important role in the changes of autonomic tone, and multiple pathways influence the activity of the autonomic preganglionic neurons. Several descending pathways from these centers have inhibitory effects on the EW nuclei, resulting in pupillary dilation. Either the inhibition of the parasympathetic fibers or the increased activity of the posterior hypothalamus via the sympathetic ANS branch might be responsible for the increased pupillary diameter in schizophrenic patients.

Regarding the balance between the sympathetic and parasympathetic tones in schizophrenic patients, most of the studies investigating the heart rate variability suggest a decreased parasympathetic tone (Montaquila et al., 2015). Furthermore, several data suggest that anxiety symptoms can occur in up to 65% of patients with schizophrenia (Temmingh and Stein, 2015), and enhanced stress sensitivity is commonly found among them (Montaquila et al., 2015). The decreased exploratory activity in the Ambitus test also suggest an enhanced stress responsiveness in novel environment in our schizophrenia animal model which can influence significantly the balance between the sympathetic/parasympathetic tone. However, a further study is required to determine the tone of the sympathetic and/or parasympathetic branches of the autonomic nervous system by the investigation of the heart rate variability in our animal model.

In darkness, the active parasympathetic tone is minimal (Loewenfeld and Lowenstein, 1993), thus the enhanced initial pupil diameter, but also the shortened flatness of curve suggest enhanced sympathetic activity. Since constriction is primarily influenced by the parasympathetic system, the lower degree of constriction suggests a decreased level of parasympathetic activity, too. Similarly to clinical findings from schizophrenic patients, a large variance was observed in several parameters in WISKET rats, suggesting that both schizophrenic patients and this new substrain show an impaired inhibitory control by the higher centers (Hakerem et al., 1964).

The relatively moderate correlations among the pupillary parameters suggest that the initial pupil diameter determines only part of the variance of the other measures. However, the number of significant correlations was higher in the WISKET rats hypothesizing that the altered central mechanisms (enhanced sympathetic tone) may contribute to these changes (Steinhauer and Hakerem, 1992). Surprisingly, anesthesia, which inhibits the sympathetic activity, increased the number of correlations, too. These observations together suggest that the alteration of balance between the parasympathetic and sympathetic tone (in any direction) may lead to increased correlation between the different pupillary parameters.

4.1. Limitations

Several psychological and physiological variables can influence the baseline pupil diameter and the PLR (Giakoumaki et al., 2005; Hakerem et al., 1964). In healthy volunteers, anxiogenic stimuli influence this reflex, and patients with anxiety disorders show several alterations in pupillary function (Davis et al., 2013; Hakerem et al., 1964; Tavernor et al., 2000). It is well-known that fear causes an increase in the baseline pupil diameter and a decrease in the amplitude of the response to light, reflecting the active inhibition of the parasympathetic efferent pathway. The sedation and/or the anesthesia decreased the fear responses in these animals, but the sedation did not cancel the differences in the autonomic functions between the two groups. Chloral hydrate anesthesia provided ideal circumstances for the investigation of pupil functions for a longer period, and it significantly prolonged both constriction and redilation (Kim et al., 2015), however, it masked the differences between the groups.

The lack of appropriate controls - a WISKET control group without isolation housing and ketamine treatment; and a Wistar treatment group with isolation housing and ketamine - to determine the contribution of the different interventions to the face validity of the model might be seen as a major limitation. However, as far as the validity of the animal model itself is concerned, this is not really an issue. In our previous paper we provided ample evidence that the new substrain after the complex treatment has the highest validity as a schizophrenia model compared to the appropriate control groups, suggesting that the combination of genetic and environmental factors lead to best model in this paradigm (Petrovszki et al., 2013). From that time we concentrated

Table 4
A. Significant correlations between different pupillary parameters in sedated (A) and anesthetized (B) control and WISKET animals.

A

Control / WISKET	Initial diameter	Minimum diameter	Latency	Amplitude	Degree of constriction	Duration of constriction	Flatness of curve	Total constriction time	25% Redilation	33% Redilation	50% Redilation
Initial diameter		0.79	0.54	0.90							
Minimum diameter	0.79										
Latency				0.56							
Amplitude	0.74				0.61						
Degree of constriction		-0.48		0.77							
Duration of constriction		-0.54						0.90			
Flatness of curve									0.75	0.71	
Total constriction time		-0.52				0.91	0.56		0.59		
25% Redilation										0.89	
33% Redilation									0.87		
50% Redilation	0.58			0.60						0.50	

B

Control / WISKET	Initial diameter	Minimum diameter	Latency	Amplitude	Degree of constriction	Duration of constriction	Flatness of curve	Total constriction time	25% Redilation	33% Redilation	50% Redilation	Max. Redilation	Degree of redilation	Redilation time
Initial diameter		0.73		0.97	0.66	0.57		0.63				0.80		
Minimum diameter	0.82											0.69		
Latency	0.51	0.45												
Amplitude	0.52				0.80	0.66	0.61	0.71				0.74		
Degree of constriction		-0.84		0.56		0.61		0.63						-0.58
Duration of constriction				0.81	0.64			0.72						
Flatness of curve				0.51	0.53	0.50		0.96						
Total constriction time				0.61	**0**.6	0.64	0.98							
25% Redilation						0.55	0.82	0.84		0.97				
33% Redilation						0.55	0.90	0.91	0.95		0.57			
50% Redilation							0.73	0.66	0.61	0.70			0.58	
Max. Redilation	0.70	0.73			-0.59									
Degree of redilation			0.45	0.48							0.49			
Redilation time														

on the characterization of this model from several aspects (Horvath et al., 2015, 2016, 2017; Kekesi et al., 2015; Szűcs et al., 2016a, 2016b). The contribution of the genetic or environmental factors separately to the different signs is indeed an interesting theoretical question, however, it also raises ethical concerns, and, from an animal welfare point of view, it would be difficult to argue for the necessity of extra experiments only to describe the individual contribution of the hits, once it has been proven that the model is valid. While, the changes in the opioid and cannabinoid receptor functions in rat brain homogenate preparation suggest neurodevelopmental alterations in WISKET animals, further studies are required to reveal the supposed widespread changes in other receptor functions (e.g. dopamine, NMDA, etc) in different brain structures.

The goal of this study was to determine the autonomic function of a new schizophrenia model by a simple, fast method which can be applied in a large a number of animals without any training and or surgery. Regarding the determination of the action mechanisms of the observed changes require several new experiments, which might allow whether these changes are due to alterations at the supraspinal, spinal or even postganglionic levels. These experiments should investigate both sympathetic and parasympathetic postganglionic neurons (including the processes of release, uptake and metabolisms of noradrenaline and acetylcholine).

A further limitation might be that only male rats were investigated. It is well-known that schizophrenia affects both human sexes, and sex-dependent differences exist in age of onset, clinical characteristics, treatment response, the course and prognosis of the disease (Häfner, 2003). Earlier data did not show substantial gender differences in the pupillary responses obtained in healthy subjects, and even no rodent studies are available in this regard (Fan et al., 2009a, 2009b). Furthermore, no human studies exist regarding the gender differences in the pupillary responses in schizophrenic patients. Therefore, future human and rodent studies warrant further investigation to reveal the potential gender differences in the pupillary responses.

Our substrain was originally developed as a complex model of schizophrenia, and has been extensively investigated as such; however,

it is well-known that several signs of schizophrenia overlap with autism spectrum disorders (ASD), including autonomic dysfunction. For instance, an increased sympathetic and a decreased parasympathetic tone have been reported in children with autism (Daluwatte et al., 2013; Ming et al., 2005). Similarly to schizophrenic patients, altered pupillary function has been reported in ASD patients, but the data are controversial (Anderson and Colombo, 2009; Anderson et al., 2013; Daluwatte et al., 2013; Martineau et al., 2011). According to some studies, the initial pupil size is larger in ASD (Anderson and Colombo, 2009; Anderson et al., 2013; Daluwatte et al., 2013), while others report a smaller size (Martineau et al., 2011), or no difference compared to healthy individuals, however, a significantly larger variation of data were reported in the ASD group (Fan et al., 2009a, 2009b). Children with ASD also showed significantly longer PLR latency, smaller constriction amplitude and lower constriction velocity to light stimuli (Daluwatte et al., 2013; Fan et al., 2009a, 2009b) than healthy participants. Some of these parameters are definitely in agreement with our results, which suggest that the WISKET substrain might be a good model of ASD, too.

4.2. Conclusion

In summary, these data demonstrate that not only the thermoregulation, but another autonomic function, pupillary control, also shows significant alterations in WISKET rats. All of the observed pupillary changes in this substrain suggest enhanced sympathetic and/or decreased parasympathetic activity. It gives further support to the validity of the WISKET substrain as a model of schizophrenia, through the general shift towards a sympathetic predominance in these animals (Horvath et al., 2015). As anesthesia blunted the differences between the groups, we suggest that sedation is more appropriate for the purposes of such investigations.

We suggest that this quick and simple test might be introduced as a routine for the characterization of autonomic function in different animal models. Although the strengths and weaknesses of this substrain should be evaluated in the future by molecular biological methods, we conclude that our animal model may provide additional opportunity for the translational research of schizophrenia and ASD.

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II.

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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

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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

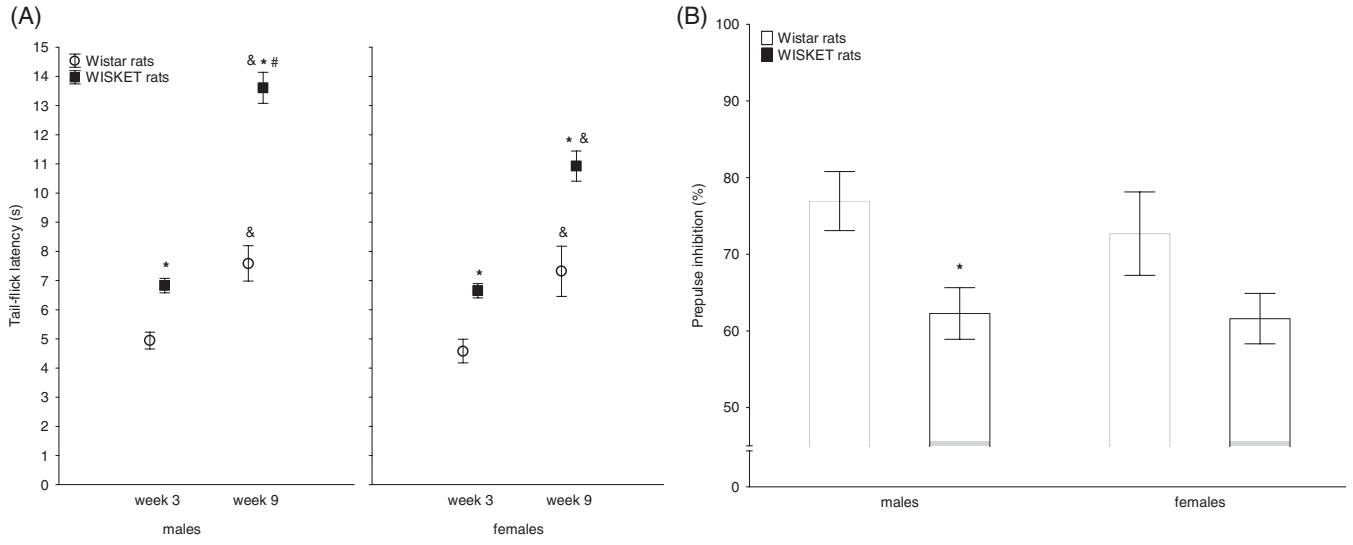

FIGURE 1 Pain sensitivity (A) and sensory gating process (B) indicated by the tail-flick latency and prepulse inhibition (%PPI) values in Wistar and WISKET rats by age and sex. Trials were performed at the age of 3 and 9 weeks, respectively. The symbols indicate significant differences ($P < 0.05$) by 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

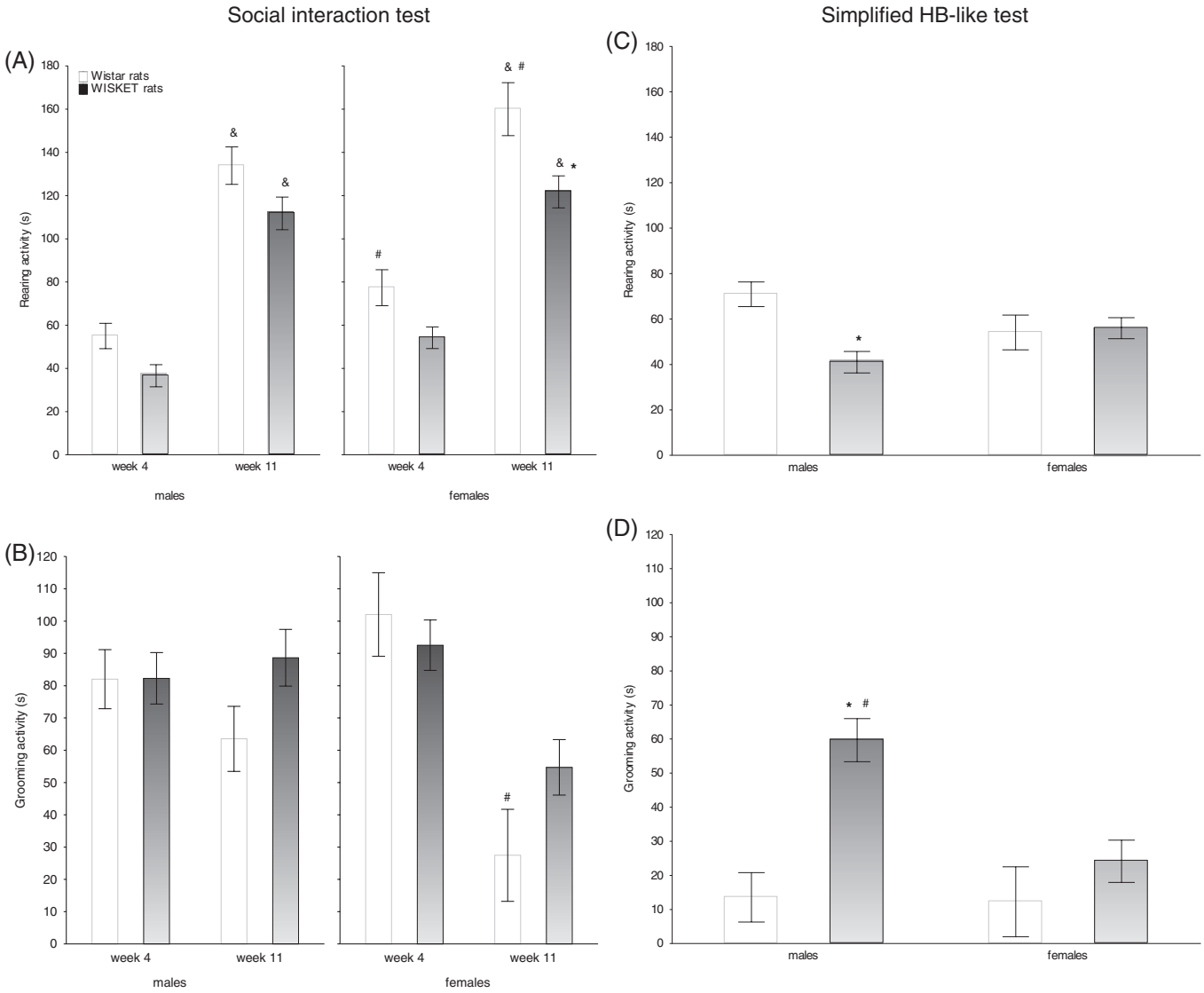

FIGURE 2 Basic activities (rearing and grooming) during the social interaction (A and B) and simplified holeboard tests (C and D) indicating the exploratory activity and stress-related behavior. The symbols indicate significant differences ($P < 0.05$) by Newman-Keuls post hoc test between: the corresponding 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

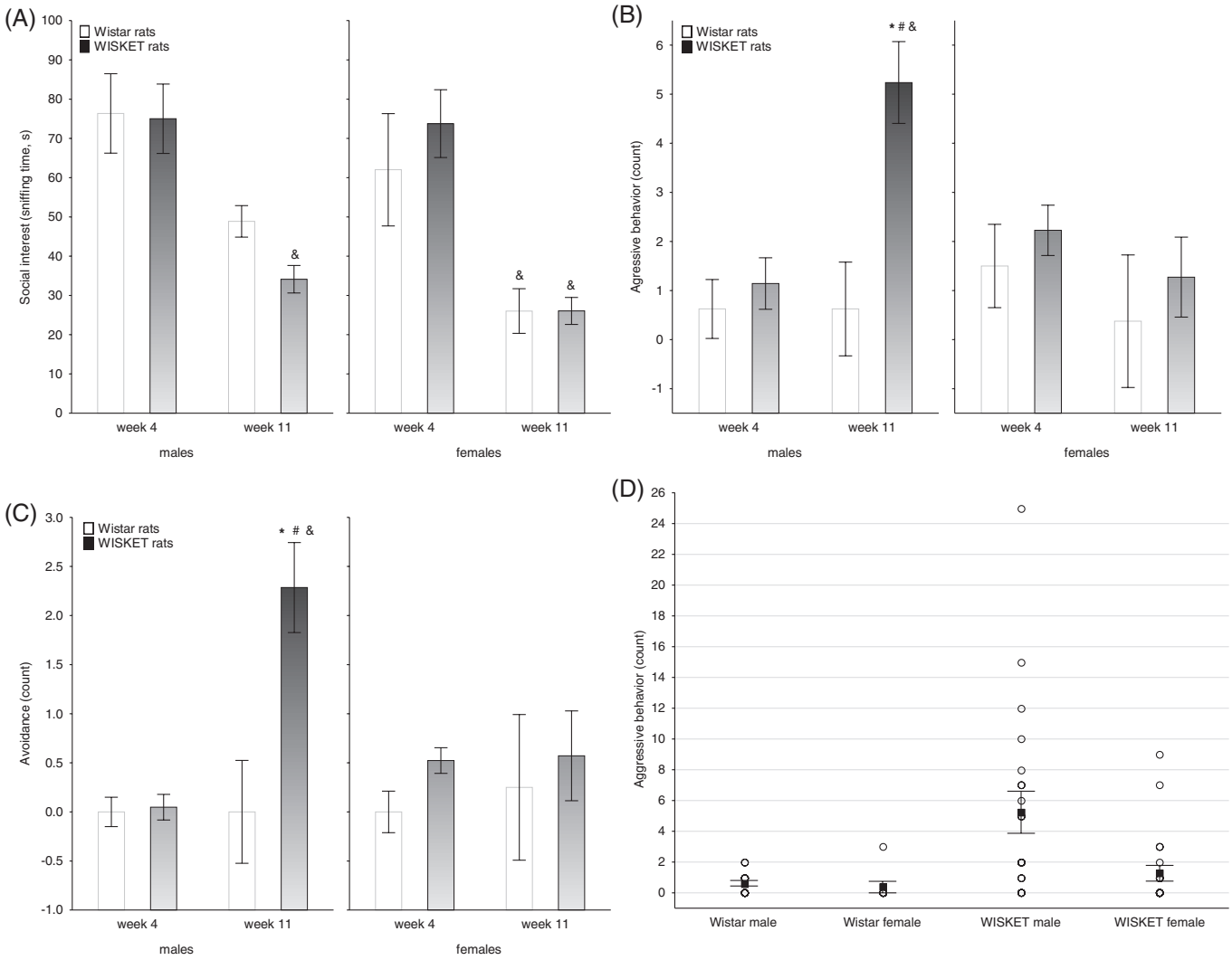

FIGURE 3 The social interest (A), aggressive behavior (B), and avoidance (C) of the animals during the social interaction test. Scatterplot with means ± SEM showing aggression behavior from individual animals at 11 weeks of age by groups (D). The symbols indicate significant differences ($P < 0.05$) 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

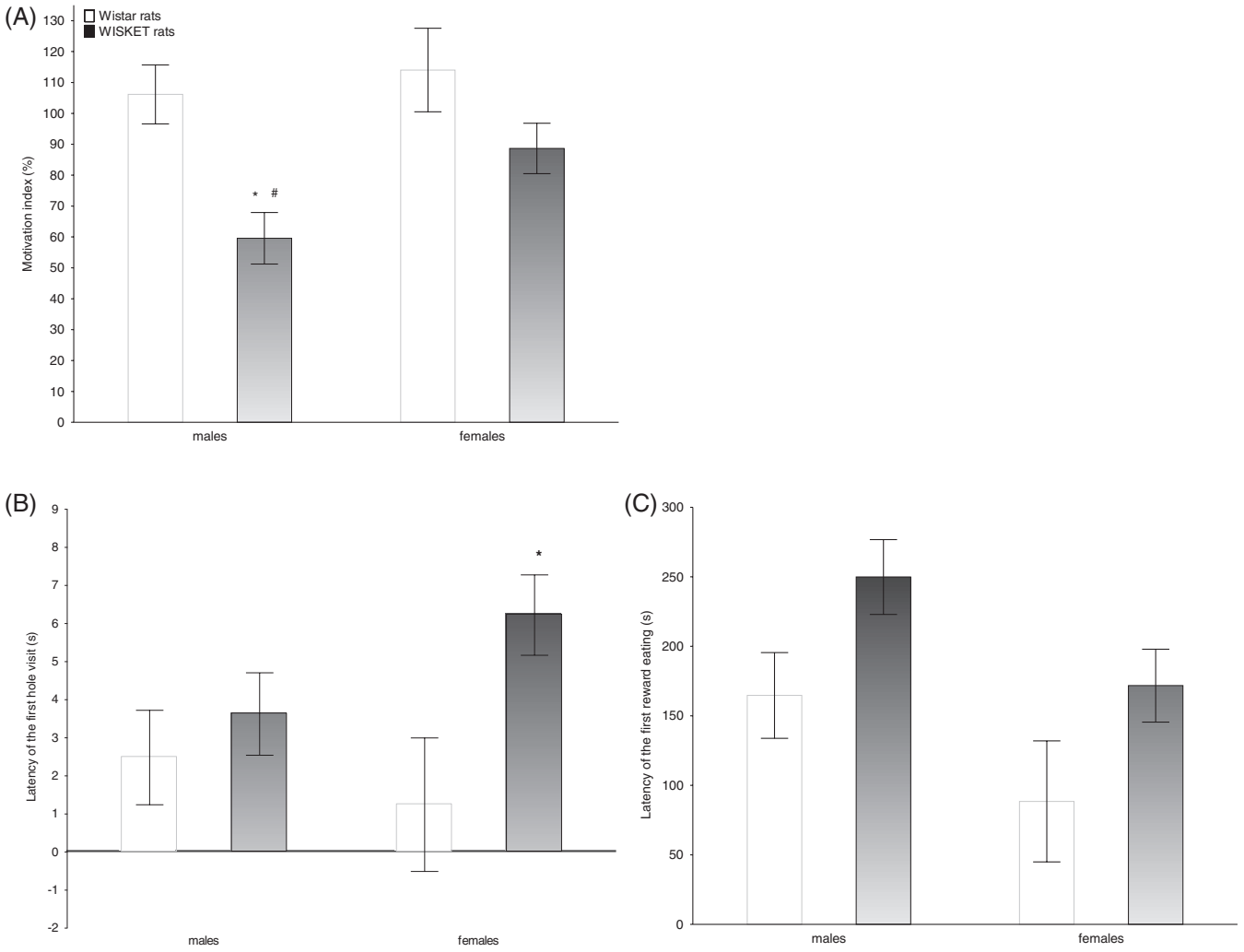

FIGURE 4 The motivation of the animals in the simplified holeboard test represented by the motivation index (A), the latency of the first hole visit (B), and eating (C). The symbols indicate significant differences ($p<0.05$) by Newman–Keuls post hoc test between: the corresponding Wistar and WISKET 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]

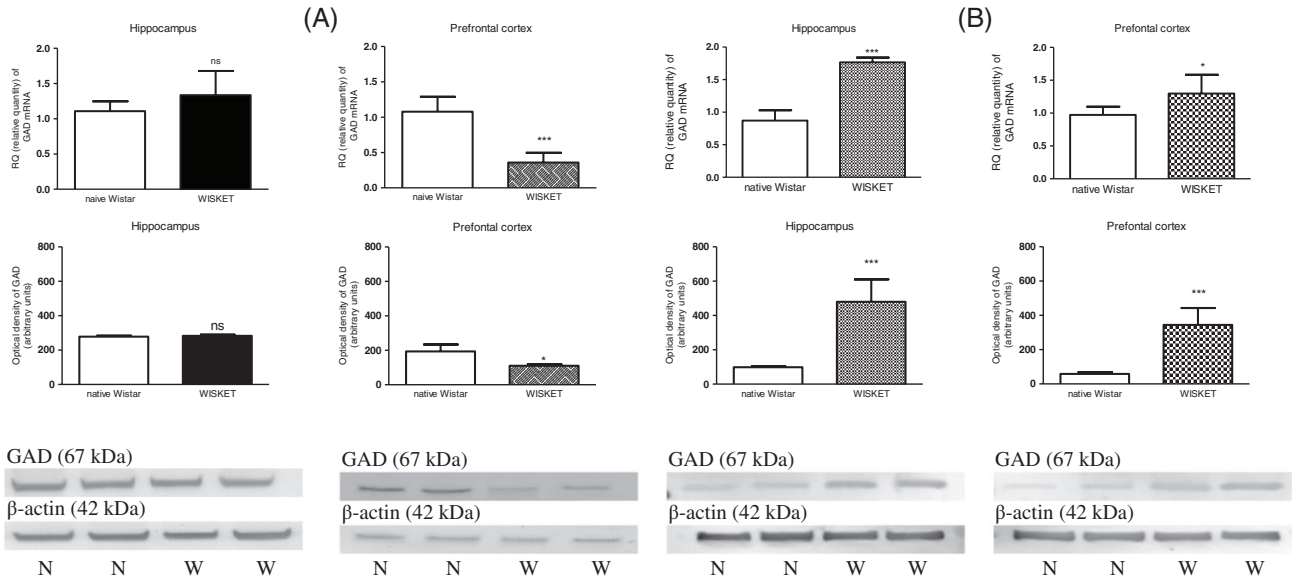

FIGURE 5 Results of RT-PCR and western immunoblotting experiments. The changes of mRNA and protein expressions of GAD1 in the hippocampal region and prefrontal cortex samples of male (A) and female (B) naive Wistar (N) and WISKET (W) rat brain. * $P < 0.05$, ***$P < 0.001$ as compared with the Wistar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

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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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