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

Summary of Ph.D. Thesis

Alexandra Büki

Supervisors: Gyöngyi Horváth, MD, DSc

Gabriella Kékesi, PharmD, Phd

Doctoral School of Theoretical Medicine

Department of Physiology

Faculty of Medicine

University of Szeged

Szeged

2019

PUBLICATIONS

Full papers related to the Thesis

I. **Büki A**, Kalmár G, Kekesi G, Benedek G, Nyúl L. G, Horvath G, (2018) Impaired pupillary control in “schizophrenia-like” WISKET rats, Auton. Neurosci. 213:34–42. Q2

IF: 2.605

II. **Büki A**, Horvath G, Benedek G, Ducza E, Kekesi G, (2018) Impaired GAD1 expression in schizophrenia-related WISKET rat model with sex-dependent aggressive behavior and motivational deficit, Genes Brain Behav. e12507. Q1

IF: 3.496

Total IF of full papers related to the thesis: 6.101

Full papers, not involved in the Thesis

1) Horvath G, Liszli P, Kekesi G, Büki A, Benedek G, (2019) Cognitive training improves the disturbed behavioral architecture of schizophrenia-like rats, "Wisket". Physiol. Behav. 201:70-82. Q1
   IF: 2.517

2) Horvath G, Liszli P, Kekesi G, **Büki A**, Benedek G, (2017) Characterization of exploratory activity and learning ability of healthy and "schizophrenia-like" rats in a square corridor system (AMBITUS). Physiol. Behav. 169:155-164. Q1
   IF: 2.517

3) Kalmár G, **Büki A**, Kekesi G, Horvath G, Nyúl L. G. (2017) Image processing-based automatic pupillometry on infrared videos. Acta Cybern. 23(2):599-613. Q4
   IF: 0.87

4) Szűcs E, **Büki A**, Kékesi G, Horváth G, Benyhe S, (2016) Mu-Opioid (MOP) receptor mediated G-protein signaling is impaired in specific brain regions in a rat model of schizophrenia. Neurosci. Lett. 619:29-33. Q2
   IF: 2.159

5) Szűcs E, Dvorácskó S, Tömböly C, **Büki A**, Kékesi G, Horváth G, Benyhe S, (2016) Decreased CB receptor binding and cannabinoid signaling in three brain regions of a rat model of schizophrenia. Neurosci. Lett. 633:87-93. Q2
   IF: 2.159

6) Bohár Z, Nagy-Grócz G, Fejes-Szabó A, Tar L, László AM, **Büki A,** Szabadi N, Vraukó V, Vécsei L, Párdutz A, (2015) Diverse effects of Brilliant Blue G administration in models of trigeminal activation in the rat. J. Neural. Transm. (Vienna). 122(12):1621-1631. Q1

   IF: 2.392

   Cumulative IF: 18.818

1. INTRODUCTION

Schizophrenia is a complex multifactorial psychiatric disorder characterized by wide variety of symptoms, which are generally classified into positive (hallucinations, delusions and thought disorder), negative (deficits in social interaction, emotional expression and motivation) symptoms and cognitive dysfunctions (impaired attention, information processing, problem-solving, learning or memory) and other, non-specific sings such as decreased pain sensitivity, sensory gating disturbance, and alterations of the autonomic nervous system (decreased heart rate variability, disturbed thermoregulation and pupillomotor control). These abnormalities are differentially expressed across patients and through the course of the illness.

Schizophrenia has an onset in early adulthood with sex differences in its clinical feature. Females show greater frequency of neurotic disorders, but men are more frequently affected than women and consistently express more severe cognitive deficits from the premorbid phase of the illness. Moreover, men show higher degree of social dysfunctioning, antisocial personality, aggression, which is mostly pronounced in adolescence and early adulthood.

Various prenatal and postnatal environmental factors, genetic susceptibility and their interaction may lead to disturbed maturation of the brain and thus abnormal neuroplasticity and deficits in several neurotransmitter systems. Disinhibition of the mesolimbic dopaminergic system generates the positive symptoms, while the negative and cognitive symptoms develop due to the hypoactivity of the mesocortical pathway.

To investigate the different features and neurobiological basis of this disease, and to identify new drug targets providing more effective treatment in the future, valid animal models are necessary, which fulfill the criteria of the appropriate triad of validities: face (symptom homology), constructive (replicating the theoretical neurobiological rationale and pathology) and predictive (adequate pharmacological response to treatment by known antipsychotics) validities. Although, it is impossible to model completely a complex human mental disease such as schizophrenia in rodents, they can be expected to display some abnormalities that are found in schizophrenia patients, e.g. disturbed sensory gating and cognitive functions.

Several animal models of schizophrenia have been developed, and they fit into three different induction categories: neurodevelopmental, pharmacological and genetic manipulations. However, recently it is supported that animal models with the combination of these different

procedures may help to produce more reliable animal models than only one applied intervention.

Our research group developed a complex, chronic “three hit” rat model, named WISKET originating from Wistar strain. The first hit is pharmacological, namely subchronic ketamine treatment; the second one is postweaning social isolation as an environmental stress. The third hit is selective breeding as a genetic factor, based on behavioral phenotypes.

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 electroencephalography pattern. Furthermore, molecular-biology studies revealed disturbed functions in opioid and cannabinoid receptor systems.

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

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). Wistar and selectively bred WISKET animals from both sexes were used. The body weight of rats was measured weekly throughout the whole investigation period. After weaning (postnatal day 21) the acute heat pain sensitivity of all rats were tested 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, Calypsol). 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, the acute heat pain sensitivity and the sensory gating of the animals were measured. The basal parameters of the animals include the results from these tests.

To further characterize our model in new aspects we tested the animals in two series. In the series 1 besides the routinely executed behavioral tests the social behavior at the age of 3 and 11 weeks and the cognitive skills at the age of 10 weeks were assessed. 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.

In the series 2 one week after the routinely executed behavioral tests the cognitive and two weeks later the pupillary function were studied in male naive Wistar (n=31) and WISKET (n=42) rats under two conditions: sedation (WISKET n=22, Wistar n=17) or chloral hydrate anesthesia (WISKET n=20, Wistar n=14).

Basal tests

Nociceptive test

The acute heat pain sensitivity was assessed with tail flick 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). Tail flick latencies were obtained four times with 30 min intervals and were averaged to establish the pain threshold for each group.

Sensory gating test

The degree of sensory gating of the acoustic startle response was measured with pre-pulse inhibition (PPI) test, where the rats were exposed to two different trial types: the pulse alone (PA) (40 ms 95 dB white noise burst) 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. Degree of prepulse inhibition was calculated as percentages using the following equation:

PPI (%) = *[1 − (startle response for PP) / (startle response for PA)] × 100.*

Procedures in series 1

Holeboard test

The one-phase holeboard test is an appetitively motivated test (an 80×80 cm square arena with 40 cm high black walls contained 16 cylinders [5×5 cm diameter] in a 4×4 array with food reward (puffed rice, 20 mg) in each of them. The animals were placed into the center of the arena, and their behaviors were recorded for 600s with an overhead infrared video device (WCM-21VF, CNB, China) and analyzed offline.

The durations of locomotor (horizontal) and rearing (vertical) behaviors were defined as basic activities. The anxiety behavior was characterized with the grooming and the place preference (time ratio spent in the central area). The data about the latency of the first hole–visit, the first reward eating were also collected and the learning capacity of the animals was determined as:

Learning capacity (%) = [*number of collected food rewards x cut-off time of the task (600 s) / number of food rewards (16) x time required to complete the task (s)] x 100.*

Social interaction test

The social interaction test was applied to evaluate the social behavior of the rats, in which the behavior of weight- and sex-matched, unfamiliar pairs of rats from the same group was evaluated. The animals' behavior was recorded for 600 s with an infrared video camera (WCM-21VF, CNB, China) and analyzed offline. 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.

Procedures in series 2

AMBITUS test

The AMBITUS test as a special combination of holeboard and corridor tests is a newly developed appetitively motivated cognitive-behavioral test (outer diameter of 80 cm, width of 8 cm and height of 50 cm). 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 of the animals. The behavior of the animals was recorded for 300 s with an infrared video device (WCM-21VF, CNB, China) and analyzed offline. 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 holeboard test.

Pupillary light reflex test

The pupillary light reflex (PLR) test is a simple and non-invasive technique for evaluating and assessing autonomic function. It was conducted 15 minutes after diazepam-induced sedation (2.5 mg/kg i.p., Seduxen) or chloral hydrate anesthesia (200 mg/kg i.p.). Under infrared illumination an intensive light stimulus (approximately 300 cd/m^2 for 600 ms) was applied in the eye of the animals and the response was recorded with a modified digital camera (Nikon D7000). From the offline analysis the automated feature extraction method produced 13 features: initial diameter, minimum diameter; maximum of the redilated diameter, amplitude of constriction, degree of constriction, constriction latency, duration of constriction, the flatness of the curve, total constriction time, and in the redilation phase: time required reaching different percentages (25 %, 33 %, 50 % and 100 %) of the initial size of the pupil.

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

Basal parameters

In agreement with our recent studies, WISKET rats showed significant differences in body weight and in basal behavior parameters in both series of the experiments.

Body weight

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

Pain sensitivity

The pain threshold increased in each group with the age, but it increased in WISKET rats compared to Wistar animals in both series and in both trials. The 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.

Sensory gating

Regarding the sensory gating, the WISKET rats had lower PPI compared to the Wistar animals in both series, significant differences were observed between male WISKET and Wistar rats.

Series 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. Male WISKET rats spent shorter time with locomotion and rearing compared to the Wistar ones, 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. Furthermore, both sexes of the WISKET animals showed significant reductions in the time spent in central area compared to the controls.

ANOVA revealed significant effect of the group with a lower cognitive performance in WISKET animals, and close to significant effect of sex ($p=0.072$). 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, and the first reward eating were significantly increased in WISKET animals compared to the control animals.

Social interaction test

The rearing and grooming activities were influenced by the different factors (age, group and sex) or their interactions. The rearing activity increased with age in all groups, but it decreased in WISKET animals in both trials compared to control ones. The Wistar female animals had higher level of rearing activity, but not the WISKET animals. Grooming activity decreased with age especially in females, and the post hoc analysis revealed significant decrease in adult Wistar female animals compared to males.

The social interest decreased in each group with the age. In contrast, the aggressive and avoidance behavior increased with age in male WISKET rats, but it was not characteristic for the other groups. The female WISKET rats showed similar social behavior as the Wistar ones.

Series 2

AMBITUS test

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

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. In the sedated animals ANOVA showed significant differences between the two groups in the initial and the minimum pupil diameters. The flatness of the curve and the duration of total constriction time were significantly shorter in the WISKET group. The degree of the constriction was significantly lower. Regarding redilation, the time to reach the 25 %, 33 % and 50 % of the initial diameter did not differ significantly; the short period of recording did not allow a detailed analysis of the redilation process in the sedated 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. The degree of the constriction showed close to

significant difference (p= 0.064) between the two groups. The prolonged investigation of the pupillary reaction in this series made it possible to observe the redilation process for a longer period, a trend for a shorter redilation time was seen in the WISKET group.

Regarding the comparison of the two conditions, all of the constriction-, and redilation-related parameters were significantly prolonged, and the degree of constriction increased under chloral hydrate anesthesia. 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. Additionally, more significant correlations were detected in the WISKET compared to the control animals, especially during anesthesia (number of correlations under sedation: WISKET: 12, Wistar: 10; anesthesia: WISKET: 26, Wistar: 15).

6. DISCUSSION

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 novel object recognition and the five-day long hole board tests. Motor abnormalities are common in schizophrenia, i.e. reduced activity is associated with negative and depressive symptom clusters, while excessive motor activity is often accompanied by positive symptoms. Regarding the animal models of schizophrenia, increase/decrease or no change in locomotor activity have also been described.

Sex difference in locomotor activity has been observed, thus the activity is higher in females than in males, but in our study the control animals showed sex differences only in the rearing activity in social interaction test.

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.

Stress and the development of a schizophrenic psychosis are inextricably related. The enhanced grooming behavior and the altered place preference (less time spent in central part of arena in holeboard test) further indicate the high level anxiety in adult male WISKET rats.

Cognitive performance

Our present results indicated that very simple and fast behavioral tests (holeboard and AMBITUS test) are appropriate to detect the presence of cognitive disturbances instead of using really complex and time-consuming tests. In agreement with the results obtained recently WISKET rats showed decreased learning ability in both tests.

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 memory in adulthood than the single interventions. 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 than men to schizophrenia. The cognitive alterations are most likely supported by sexual dimorphism in brain morphology and neurochemistry found in schizophrenia.

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 and revealed disturbances in the social behavior of male WISKET rats, but not in the female ones.

As sex hormones play 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. Each of them has found that males, but not the females show a significantly reduced duration of active social interactions, and spent longer time in avoidance.

Autonomic function

The values of pupillary parameters observed in Wistar animals were comparable with earlier rat studies, suggesting that the applied method is appropriate for the characterization of pupillary function in rodents.

The impaired thermoregulation (earlier paper) and pupillary function in WISKET animals suggest disturbed autonomic nervous system balance giving further support for the validity of the WISKET substrain as a model of schizophrenia.

Data concerning the pupillomotor control in schizophrenic patients are controversial, e.g. the initial pupil diameter, some human studies have found smaller or the same value compared to healthy controls. Newer data show significantly increased resting pupil diameter with lower degree of constriction and unchanged latency in patients, shorter constriction time and an earlier redilation after visual stimulation.

Patients with schizophrenia exhibit an impaired autonomic nervous system balance, thus they have difficulty in exerting appropriate reciprocal parasympathetic control following stress cessation and throughout the restitution period. Most of the studies investigating the heart rate variability indicated decreased parasympathetic tone. The higher autonomic centers play an important role in the changes of autonomic tone, and multiple pathways influence the activity of the autonomic preganglionic neurons. Either the inhibition of the parasympathetic fibers or the increased activity of the posterior hypothalamus via the sympathetic branch might be responsible for the altered pupillary control in schizophrenic patients.

Limitations

Our substrain was originally developed as a complex model of schizophrenia; however, it is well-known that several signs of schizophrenia overlap with autism spectrum disorders. Therefore, it cannot be claimed that this model is perfectly specific to schizophrenia.

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, as with many behavioral dimension, it is preferable to use further methods to show decreased social interaction.

A limitation of series 2 investigating pupillary light reflex is that only male rats were used, but there is no human study in this respect.

7. CONCLUSION

The WISKET rats after the complex treatment showed several alterations relevant to schizophrenia. Both series confirmed the decreased acute heat pain sensitivity, the impaired sensory gating, exploratory activity and cognitive function in WISKET rats. The series 1 proved that these disturbances were sex-dependent, 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 influences the difference between the groups.

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.

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

The present findings 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 low exploratory and locomotor activities.
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 PLR test performed under sedation proved that WISKET animal had significantly greater initial and minimum pupil diameters with significantly lower degree of 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.

9. ACKNOWLEDGEMENT

First of all, I would like to thank my supervisors Professor Gyöngyi Horváth and Dr. Gabriella Kékesi for their not just scientific, but personal guidance, encouragement and continuous support throughout my PhD studies.

I am grateful to Professor Gábor Jancsó supporting my participation in the Neurosciences Doctoral program.

I would like to express my thanks to Professor Gyula Sáry for providing me the opportunity for conducting research at the Department of Physiology.

I would also like to thank to Ágnes Ábrahám-Tandari for her patience and technical guidance in managing laboratory measurements.

I would like to express my gratitude to Péter Liszli for the great help in setting up the experimental protocol and devices.

I would like to give my sincere thanks to our coworkers from the Department of Image Processing and Computer Graphics, György Kalmár, Dr. László G. Nyúl for their help in the video and image processing.

I owe a lot of thanks to all the former and current colleagues in the Department of Physiology.

Finally, I wish to thank my family and my friends for their love and all their support.

The presented studies have been partially supported by the by the Hungarian Research Grant (GINOP-2.3.3-15-2016-00031) and by the “UNKP-17-3 New National Excellence Program of The Ministry of Human Capacities” (UNKP-17-3-IV-SZTE-5).