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Electrophysiological and pharmacological characterization of a new schizophrenia rat model (Wisket)

Summary of Ph.D. Thesis

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1. INTRODUCTION

Schizophrenia is a complex, severe psychiatric disorder, which affects about 1 % of the population worldwide. It is characterized by positive (hallucinations, delusions), negative (alogia, apathy), and cognitive symptoms (impaired attention, learning, and memory functions). Apart from the classical traits, there are several non-specific features of schizophrenia, such as altered sensory gating, pain threshold, sleep-wake rhythm, and electroencephalographic (EEG) oscillatory pattern. All of the disturbances may arise from the combination of environmental, genetic, and developmental factors. Several neurotransmitter system changes were observed in the pathomechanism of schizophrenia and the abnormal dopaminergic pathways have particular importance. The altered functions of dopamine 1 receptor (D_1R) may contribute to the cognitive deficits of schizophrenia.

Due to the various signs and the numerous neurotransmitter systems involved, treating schizophrenia is demanding. The first-line drugs applied in schizophrenia are the antipsychotics. The currently available medication for schizophrenia has several limitations, such as most antipsychotics have moderate effects on the negative and cognitive signs, and can cause metabolic syndrome. Clozapine (CZP) is a second-generation antipsychotic drug, which is mainly effective in treating positive symptoms, but the available results are controversial regarding the CZP's effect on the negative and cognitive symptoms. The combination of antipsychotics with drugs that can blunt their side effects might have clinical significance. Metformin (MTF) is a well-known antidiabetic medication, it has neuroprotective effects in neurodegenerative diseases; however, there is no clinical study regarding schizophrenia.

To examine complex psychiatric disorders (e.g., schizophrenia), and to determine the neurological underpinnings, and/or novel drug targets, relevant animal models are required. All of the existing animal models should satisfy three validation criteria: face (similar symptoms), construct (reproduction of pathological and neurobiological reasons), and predictive (animal model is appropriate for acquiring new information about the disease and reveals possible therapies) validities. The animal models of schizophrenia are classified into four main categories: developmental, pharmacological, genetic, and combinational. Since the etiology of schizophrenia seems to be based on gene-environmental interactions, research suggests that the combination of genetic, developmental, and environmental factors can provide a more reliable model of schizophrenia. A triple-hit schizophrenia rat model, called Wisket, was developed in our laboratory from the Wistar rat strain with the combination of post-weaning social isolation (developmental, environmental), ketamine treatment (pharmacological), and selective breeding

based on behavioral parameters (genetic). Wisket animals demonstrated decreased acute and chronic pain sensitivity, impaired sensory gating, locomotor activity, social behavior, cognitive functions, disruption of thermoregulation and pupillary light reflex, alterations in electroencephalographic patterns, furthermore, receptor abnormalities in the opioid, cannabinoid, and D_2 receptors, as well as disturbances of the GABA-ergic and oxytocinergic systems. Recently, our results showed that caffeine treatment ameliorated the learning of Wisket rats with prolonged alterations in the dopaminergic system. Furthermore, the regulation of cerebral metabolism was also disturbed in Wisket animals with delayed response to caffeine. Altogether, apart from our Wisket model as far as we know, there is no article with the combination of social isolation, ketamine treatment, and behavior-based selective breeding.

2. AIMS

The purpose of the thesis was to further characterize the predictive and face validities of the Wisket schizophrenia rat model by different techniques including:

1. the evaluation of the effects of chronic CZP or MTF administration alone, and their combination on locomotor and exploratory parameters, cognitive functions,
2. the investigation of the D_1R-mediated signaling, ligand binding, and mRNA expression in the different brain regions,
3. the description of changes in the D_1R-mediated signaling, ligand binding, and mRNA expression in various brain structures after chronic administration of CZP, MTF, and their combination,
4. the analysis of the sleep-wake rhythm,
5. the determination of electroencephalographic oscillatory pattern.

3. MATERIALS AND METHODS

3.1. Animals

Male, adult 4-6-month-old Wistar (control), and Wisket rats were used in the study. All of the experiments were performed with the permission of the Hungarian Ethical Committee for Animal Research (registration number: XIV/1248/2018) and in accordance with the guidelines established by the Government of Hungary and EU Directive 2010/63EU for animal experiments. The animals were housed in controlled temperature (23±1°C) and humidity (55±10%) conditions with a 12 h light/dark cycle. The behavioral tests were carried out between

8 a.m. and 4 p.m., under dim lighting. The body weight of the animals was monitored throughout the entire experiment.

3.2. Behavioral tests

3.2.1. Tail-flick

The tail-flick (TF) test was used to assess baseline acute heat pain sensitivity. By immersing the tail's distal 5 cm in hot water (48 °C) a tail-withdrawal response was noticed, and reaction time was calculated (cut-off time: 20 s or 40 s at the age of 3 or 9 weeks, respectively). TF latencies were measured four times (at 0, 30, 60, and 90 min) to ascertain the pain threshold for each group.

3.2.2. Ambitus

The Ambitus apparatus (rectangular corridor) is a reward-based learning test, which was developed to detect the exploratory, locomotor activity, and learning capacity of rats. There are four equal-sized side-boxes (5x5x5 cm) 2-2 on the interior and external walls (a total of 16) and they contain food rewards (puffed rice: 20 mg). For five minutes (cut-off time: 300 s) the animals were free to explore the corridor and gather food rewards. All experiments were recorded with an infrared video camera (WCM-21VF, CNB, China). During the study, the animals performed three different tasks. In Task 1 (trials 1 and 2 during the baseline measurements) all of the boxes (internal and external) contained food rewards (total of 16), in Task 2 (trials 3 and 4 during the baseline measurements and at Days 3–4 in the 4-day-long experiment of Series I.) 8 rewards were available only in the inside boxes, finally only the outside boxes (8 rewards) were baited for Task 3 (Days 1-2 during the 4-day-long experiment in Series I.). Each rat performed two sessions (two trials per session, one minute apart) of the tasks per day, one in the morning and the second around three hours later (4 trials/day). The means of the four trials that were conducted each day were further examined after the completion of the 16 total trials in Series I.

3.2.3. Sensory gating test

The pre-pulse inhibition (PPI) of the acoustic startle response was assessed using the Startle and Fear Conditioning System (Panlab, S.L., Harvard Apparatus, Barcelona, Spain). Rats were habituated for 7.5 minutes in startle chambers with a background noise of 60 dB, then completed two different trials. The first trial type was the pulse alone, applying a 40 ms, 115 dB white noise burst; the other type was the prepulse-pulse pair, which contained prepulse

stimuli (20 ms, 85 dB) followed by the startle stimulus with a latency of 150 ms. There were 20 random applications of each kind of stimuli. The interstimulus intervals were between 7 and 13 seconds. The following equation was used to convert the PPI to a percentage: PPI (%) = [1 − (startle response for prepulse-pulse pair) / (startle response for pulse alone)] × 100.

3.3. Series I.

3.3.1. Experimental paradigm

One week following the behavioral tests, Wisket and control rats were divided into 4–4 groups: water, MTF, CZP, or MTF_CZP combination drinking groups. Water was used as a solvent and a diluent of MTF and CZP during the experiments. MTF had a concentration of 1 mg/mL, while CZP had a concentration of 0.33 mg/mL of both the single and combined drug-treated groups. The experiment lasted for 28 days, and the animals were kept in pairs. Throughout the study, the body weight was measured and the relative amounts of food and fluid consumption were calculated and fresh solutions were provided twice a week. After 2 days of food withdrawal, a 4-day-long Ambitus test (Ambitus 2) was carried out on week 4 of the drug treatments by performing Tasks 3 and 2. The moderate level of food restriction (10–15 g/day) was performed to ensure the adequate motivation to perform the task. Drinking solutions were freely available throughout the experiment.

3.3.2. D_1R functional activity, binding characteristics, and mRNA expression

The animals were terminated one day following the Ambitus test 2 (Day 29), the brains were removed. The D_1R binding and signaling were analyzed with [^{35}S]GTPγS binding tests and in vitro receptor binding assay in the cerebral cortex (CTX), olfactory bulb (OB), brainstem, and diencephalon. In the [^{35}S]GTPγS binding assays, the maximal stimulation (efficacy [E_{max}]) of G-protein and the negative logarithm of ligand potency (pEC_{50}) were established following agonist occupation of the D_1Rs. In equilibrium-saturation-binding assays, the maximal number of specific radioligand binding sites (capacity, B_{max}) and the affinity of the ligand–receptor interaction (dissociation constant [Kd]) were determined. The D_1R mRNA expression was observed in the prefrontal cortex (PFC), striatum, cerebellum, hippocampus, and CTX with quantitative real-time polymerase chain reaction experiments following RNA extraction (qRTPCR).

3.4. Series II.

3.4.1. Experimental paradigm

For recordings of the EEG, surgical procedure was performed under anesthesia with an intraperitoneal injection of xylazine (CP-Xylazin, Produlab Pharma B.V.Raamsdonksveer, Netherlands, 8 mg/kg) and ketamine hydrochloride (Calypsol, Gedeon Richter Plc., Budapest, Hungary; 72 mg/kg) combination. Electrodes were implanted into the parietal cortex (from bregma: −2.3 and 2.4 mm right to the midline), occipital cortex (ground electrode: −6.1 and 2.4 mm right to the midline), and the cerebellum (reference: −10.5 and 0.5 mm right to the midline). After a one-week recovery period, the animals were allowed to habituate to the experimental environment and connected to the recording tether, then a total of 21 hours of EEG recordings were evaluated, covering the full length of the light phase (12 h) and 9 hours of the dark phase. Wisket and control rats (n=8/group) were recorded in the experiments. Data were examined offline in 8-s epochs. Three vigilance stages were identified over 8 seconds of epochs: wakefulness, NREM sleep, and REM sleep. Wakefulness was described as less regular theta activity, frequent body movement, and it was further divided into two substages: active and quiet/inactive awake. The motor activity served as the basis for the subdivision; any movement lasting longer than 1 s during an 8 s interval was classified as an active substage. NREM sleep was defined with high-amplitude slow waves and low level of body movements, and REM sleep was characterized as highly regular theta activity in the EEG, low level of movement with occasional twitches. Power spectra were divided and assessed in the following frequency ranges: delta (0.5−4 Hz), theta (4−8 Hz), alpha (8−12 Hz), beta (12−30 Hz), and low gamma (30–48 Hz).

4. STATISTICAL ANALYSIS

All data were presented as means ± S.E.M., and significance was determined at $P < 0.05$ level. STATISTICA 13.4.0.14 (TIBCO Software Inc., USA) was used to conduct the statistical analyses. Data from in vivo and in vitro experiments were analyzed using factorial or repeated measures ANOVA, where the repeated measurements were days and the factors were group (control, Wisket) and treatment (water, MTF, CZP, and MTF/CZP). Furthermore, repeated and/or factorial analysis of ANOVA was used to compare the parameters of the EEG recordings. Fisher's LSD test was used to perform post hoc comparisons. The [35S]GTPγS binding data and radioreceptor binding were analyzed with a specialized curve-fitting program (GraphPad Prism 5.0.; Inc., San Diego, CA) utilizing a non-linear regression analysis. The

unpaired t-test was additionally performed to establish differences between the various groups for the mRNA expression data.

5. RESULTS

5.1. Results of Series I.

5.1.1. The effects of chronic treatment on behavioral parameters (motor activity and learning function)

Wisket animals demonstrated reduced pain sensitivity, sensory gating, locomotor and, exploratory activies, and learning capacity compared with the control rats. There were no significant differences in these basal parameters between pharmacologically treated groups. In comparison to the water-drinking animals, MTF treatment alone did not significantly affect the observed behavioral parameters. Nevertheless, in comparison with the water-drinking group, CZP treatment reduced the exploration, which was associated with a reduced learning capacity. The combined treatment of MTF_CZP improved all of the investigated parameters to a level that is comparable to the water-drinking group.

5.1.2. D_1R activation, binding, and gene expression in different brain structures

Wisket rats had significant reductions in binding capacity, G-protein activation, and gene expression in the CTX. None of the treatments had significant effects on the D_1R mRNA expression in this area. The E_{max} and B_{max} values were significantly improved by the combination therapy in this region. Wisket animals had higher levels of D_1R binding and G-protein-mediated transmembrane signaling in the OB. Additionally, the combination treatment led to a significant improvement in the efficacy of G-protein activation in these animals, and a comparable pattern was found in the control rats, albeit it was associated with a lower level of pEC50. In the brainstem, no alterations were found, whereas the diencephalon showed a higher ligand binding potency in Wisket rats (lower pEC50 values). Additionally, the enhanced B_{max} and unchanged E_{max} values caused by combined treatment were primarily found in control animals.

Regarding the D_1R mRNA expression in the striatum and hippocampus, no significant alterations were demonstrated between the two groups. Furthermore, chronic CZP treatment did not change this parameter in rodents. MTF treatment alone led to a significant enhancement in D_1R expression in the striatum in the Wisket and control groups. In the PFC and

hippocampus, no significant effects were revealed. Concerning the cerebellum, significantly lower D_1R expression was observed in Wisket animals, and the post hoc analysis revealed that it was also reduced in the MTF-treated Wisket rats compared with their matched controls.

5.2. Results of Series II.

5.2.1. Sleep-wake rhythm

The Wisket animals displayed almost normal diurnal fluctuation in their sleep-wake pattern. The amount of time that Wisket rats spent awake during the light phase was modestly reduced and this was followed by a moderately long period of NREM sleep. In addition, a tendency toward shorter REM sleep cycles was noted in comparison to the control group, and this was seen along with an overall slight decline in the number of episodes in all stages.

5.2.2. Oscillatory pattern

A tendency of increased delta frequency power was displayed in Wisket animals at the awake stage (at the active and quiet substages) relative to controls. Regarding the activity of low-frequency delta, it was increased in Wisket rats during NREM, while the higher frequency delta wave was reduced at both NREM and REM. Concerning the theta band, the relative power of its higher frequency band was reduced during the awake (both in the active and quiet substages) and REM stages, while during NREM a significantly decreased power of the lower frequency range of theta waves was revealed in our animal model. Wisket rats exhibited significant enhancement of relative power in low-frequency alpha waves in awake (mainly during active substage) and REM stages. In comparison to control animals, Wisket rats displayed significantly higher beta power at lower frequencies at all stages, but this difference was most pronounced during the active awake substage. Wisket rats exhibited a significant reduction in relative power of the higher band of gamma oscillation (between 39 and 48 Hz) and it was dominant during the awake stage (mainly in the active substage).

6. DISCUSSION

6.1. The effects of chronic treatment on behavioral parameters (motor activity and learning function)

The presented data revealed that antipsychotic treatment (CZP) alone induced further behavioral abnormalities. MTF had no significant influence on behavioral parameters by itself, although, some animal studies reported that MTF administration alone was able to ameliorate pharmacologically induced cognitive deficits in rats. Co-administration significantly reduced

the cognitive deficit generated by CZP in Wisket animals. In line with our data combined treatment (with two antidepressants, CZP, and MTF) enhanced the MK-801-generated impaired behavior.

6.2. D_1R activation, binding, and gene expression in different brain structures

Regarding the DA-ergic system, reduced level of D_1R binding, signaling, and gene expression was detected in the CTX of Wisket rats. Neuroimaging studies have shown contradictory results about the density and/or activity of the D_1Rs in schizophrenia. In a recent study, D_1R upregulation was demonstrated in the striatum and CTX, while other studies revealed no alterations in the various cortical regions, even discovered a lower D_1R-binding potential in the frontal cortex of patients with schizophrenia. A study showed that chronic CZP injection moderately elevated the D_1R mRNA expression in rats, while our study demonstrated that none of the treatments affected these parameters. The E_{max} and B_{max} values were significantly improved by the combined drug treatment in the CTX, which may have contributed to the treatment's positive impacts on the behavior of Wisket rats. Clinical data have shown lower expression of the D_1R transcript in the PFC of patients with schizophrenia, although the hippocampus and caudate nucleus did not demonstrate alterations. Additionally, downregulation of the D_1R mRNA in the PFC was observed after subchronic treatment of an NMDA receptor antagonist (as a schizophrenia model), which is consistent with the pattern observed in Wisket rats. A study demonstrated that subchronic administration of CZP did not prevent the decline in frontal cortex D_1R density, as shown post-weaning isolation rearing, but rather increased its affinity. Due to the differences in the models and/or treatments, we were unable to detect any effects of CZP treatment on D_1R mRNA expression in the PFC.

OB is rich in D_1Rs and also important in cognitive functions, which may be contributed, at least in part, by D_1Rs. Wisket animals had higher levels of D_1R binding and signaling in the OB, indicating an enhanced D_1R density in this region. Additionally, the combination treatment led to a significant improvement in the efficacy of G-protein activation in these animals, and a comparable pattern was found in the control rats, albeit it was associated with a lower level of ligand potency. These effects may also have contributed to the positive outcomes of combined treatments seen in the Wisket group.

Numerous physiological processes (such as eating, pain perception, sensory gating, and circadian rhythm) may be regulated by the activation of D_1Rs in several brainstem and diencephalon nuclei, which are affected in schizophrenia patients. In the brainstem, no

alterations were found, whereas the diencephalon showed a higher ligand binding potency in Wisket rats (lower pEC50 values). Additionally, the enhanced B_{max} and unchanged E_{max} values caused by combined treatment were primarily found in control animals. These results suggest that the increased binding site density was associated with a reduced level of G-protein activity in the diencephalon.

In line with previous findings, the D_1R mRNA expression in the striatum and hippocampus did not show significant alterations in the Wisket animals. Furthermore, chronic CZP treatment did not change this parameter. However, without having any impact on the behavioral data, MTF treatment alone led to a significant enhancement in D_1R expression in the striatum in both groups. The cerebellum has an important role in motor coordination and cognition too, changes in cerebellar function have been linked to schizophrenia. Thus, the reduced D_1R mRNA expression of Wisket rats might also contribute to their behavioral deficiencies.

6.3. Sleep-wake rhythm

The circadian rhythm of the Wisket animals showed nearly normal diurnal variations. Different schizophrenia models (including disrupted-in-schizophrenia-1 [DISC1] gene mutant mice and rats with prenatal intervention or neonatal hippocampal lesion) also demonstrated normal diurnal rhythm. Wisket rats exhibited only a tendency of longer NREM sleep duration and shorter REM sleep phases. The studies obtained in mGLUR5 (metabotropic glutamate receptor 5) mutant mice revealed similar results. Some preclinical data (i.e., stable tubule-only polypeptide [STOP] mutant mice and methylazoxymethanol acetate exposure on embryonic day 17 mutant rats) found opposite results, i.e., sleeping duration was reduced, sleep-wake periods were fragmented, and the animals exhibited reduced NREM and increased awake time. The controversies might be due to the differences in models and experimental paradigms. The underlying mechanism might be the result of the chronic imbalance between the ascending and descending systems, whose functions are known to either precipitate or inhibit the onset of NREM and REM sleep states. Therefore, the poor cognitive functions observed in Wisket animals may be related to the less consecutive transitions of NREM-REM sleep cycles. Overall, the circadian rhythm phenotype of Wisket animals may reflect a modest abnormality seen in a small subset of schizophrenic patients, but does not generally correspond to sleep alterations associated with schizophrenia.

6.4. Oscillatory pattern

In the present study, a tendency of increased delta frequency power was displayed in Wisket animals at the awake stage (both substages) relative to controls. The activity of low-frequency delta was increased in Wisket rats during NREM, while the higher frequency delta wave was reduced at both NREM and REM sleeping. In agreement with human studies, enhanced low-frequency oscillations were noted in schizophrenic patients. Some other schizophrenia rat model showed similar findings to Wisket rats (i.e. neonatal hippocampal lesion schizophrenia model, STOP mutant mice, α-Amino-3-hydroxy-5-methyl-4- isoxazolepropionic acid [AMPA] receptor mutant mice), while mGLUR5 receptor mutant mice showed decreased power in the higher frequency range of the delta band during NREM sleeping.

Regarding theta wave, the relative power of its higher frequency band was reduced during the awake (both in the active and quiet substages) and REM stages, while during NREM a significantly decreased power of the lower frequency range was revealed in Wisket rats. Conflicting results are available regarding human and rodent models. Based on the leading symptoms, the theta oscillatory pattern remains unchanged or higher in patients. In line with our data, in STOP or AMPA receptor mutant mice reduced relative power in the theta band was observed, indicating decreased arousal level during the active stage, while in neonatal hippocampal lesioned rats theta power was increased during awake and NREM.

Wisket rats exhibited significant enhancement of relative power in low-frequency alpha waves in awake (mainly during active substage) and REM stages, which may be involved in negative symptoms of schizophrenia. A preclinical study reported similar findings as it was observed in Wisket rats, i.e. neonatal hippocampal lesion animals showed enhanced alpha power in the awake stage. In contrast, AMPA or STOP mutant mice had reduced alpha power in REM sleeping.

In comparison to control animals, Wisket rats displayed significantly higher beta power at lower frequencies at all stages, but this difference was most pronounced during the active awake substage. Similarly, patients with schizophrenia also demonstrated enhanced beta band oscillations, which might arise from the hyperexcitable CTX or disrupted attention. Regarding the preclinical data, STOP, AMPA, and voltage-gated calcium channel mutant mice exhibited decreased beta power in NREM and/or REM sleeping. AMPA knock-out animals showed enhanced beta power in awake phase, but calcium channel knock-out animals demonstrated decreased power.

Our study showed that Wisket rats exhibited a significant reduction in relative power of the higher band of gamma oscillation (between 39 and 48 Hz) and it was dominant during the awake stage (mainly in the active substage). This result may point to a reduced level of arousal during wakefulness, but it may also be connected to the poor cognitive abilities of the Wisket animals. Similar results were observed in patients with schizophrenia and it may be associated with negative symptoms. In agreement with our data, preclinical studies revealed that chronic administration of NMDA antagonists or mutation of voltage-gated calcium channels can cause diminished or unchanged gamma power, but mGLUR5 mutant mice showed enhanced gamma activity during sleep.

Limitations

There are some limitations of the presented experiments. In Series I., CZP and MTF have a considerable impact on carbohydrate metabolism, and the fact that glucose metabolism was not examined here is a notable disadvantage of this study. Therefore, it cannot be completely ruled out that the interaction of MTF and CZP on metabolic parameters, such as glucagon-like peptide regulation, may be a factor in the detected improved cognition. For in vitro measures, particularly for radioligand binding tests, we collected tissue samples from a lot of animals within the same experimental group. In experiments conducted for biochemical purposes, it is usual practice to combine tissue samples from various animals in this manner.

In Series II., the absence of deep hippocampus electrodes, the constrained number of channels, tethered option instead of wireless recording were restrictions. It should be noted that we conducted our investigation using a narrow gamma frequency range, thus, we cannot rule out other Wisket animal deficits related to the higher frequency range. The sampling rate of the EEG recordings was reduced (128 Hz), thus it might have have prevented precise analyses of the oscillation pattern in the upper gamma frequency range.

7. CONCLUSION

Taken together, in Series I. the obtained results revealed that CZP led to further behavioral deficits in Wisket animals, and this effect was mitigated by MTF co-treatment. Regarding the D_1R functions among the investigated regions, changes in CTX were the most prominent in Wisket rats, gene expression, binding capacity, and G-protein activation were reduced in this region of the brain. D_1R binding and activity in the CTX were enhanced by the MTF and CZP combined treatment of both groups. The results of Series II. displayed that the Wisket rats showed only a trend of changes in their sleep-wake rhythm, while oscillatory pattern analyses

demonstrated complex alterations. It might be supposed that the disrupted D_1R receptor system and brain oscillation pattern in Wisket rats plays a role in the development of behavioral symptoms resembling schizophrenia. Thus, the obtained findings clearly show that our multiple-hit schizophrenia rat model has deficits at electrophysiological and D_1R levels too. These alterations contribute to the Wisket model's utility in translational research, which may help to identify novel drug targets to treat this disorder.

Clinical relevance

It is a well-known phenomenon, that antipsychotic agents have serious adverse effects such as metabolic syndrome, therefore, adjuvant therapies, including MTF may be beneficial in the course of treating schizophrenia. Growing evidence indicates that MTF may have positive effects on cognitive processes. In Series I. the MTF was able to attenuate the deteriorative effects of CZP in Wisket rats. Numerous studies revealed that individuals with schizophrenia experience sleep disturbances, and EEG revealed alterations in oscillation patterns. Wisket animals exhibited numerous abnormalities in the different oscillatory bands, and these alterations can be involved in the impaired cognitive functions found in previous studies. The present findings may improve our understanding of the underlying connections between behavioral, neurochemical, and electrophysiological alterations of schizophrenia. In conclusion, our data provided additional insights into the characteristics of Wisket rats. Additionally, the rat model created after complex manipulations offers new perspectives on the disease and may provide a clinically relevant treatment method.

8. SUMMARY

The presented studies aimed to characterize our chronic schizophrenia rat model, Wisket, with the following findings:

1. The Wisket rats displayed deficits in motivation, attention, and cognitive functions, which were accompanied by a reduced level of D_1R primarily in the CTX.
2. CZP administration had further deteriorative effects on the examined behavioral parameters.
3. MTF treatment improved the CZP-generated behavioral impairments.
4. The combined treatment of CZP and MTF significantly affected different brain regions with a significant enhancement of the G-protein activity and D_1R binding in the cerebral cortex in both groups.
5. Regarding the circadian rhythm, Wisket rats demonstrated a similar light-dark cycle to the control animals.

6. A trend was detected in terms of NREM sleeping being prolonged, while REM stages were shortened in Wisket rats.
7. Wisket rats showed altered oscillation patterns in several frequency ranges, particularly during the active awake substage.

Therefore, the observed results suggest that our multiple-hit schizophrenia rat model mimics several schizophrenia-related abnormalities, which may contribute to the validation of this animal model.

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