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

Leatitia Gabriella Adlan

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ABBREVIATIONS

[^{35}S]GTPγS	guanosine 5′-O-(3-[^{35}S] thio)triphosphate)
5-HT	serotonin (5-hydroxytryptamine)
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
B_{max}	maximal number of specific radioligand binding sites capacity
CTX	cerebral cortex
CZP	clozapine
D_1R	dopamine D_1 receptor
D_2R	dopamine D_2 receptor
DA	dopamine
DISC1	disrupted-in-schizophrenia-1 gene
EEG	electroencephalography
E_{max}	maximal stimulation efficacy
GABA	gamma-aminobutyric acid
mGLUR5	metabotropic glutamate receptor 5
MTF	metformin
MTF_CZP	combination of clozapine and metformin
NMDAR	N-methyl-D-aspartate receptor
NREM	non-rapid eye movement
OB	olfactory bulb
PCP	phencyclidine
pEC_{50}	the negative logarithm of half maximal effective concentration (ligand potency)
PFC	prefrontal cortex
PPI	prepulse inhibition
REM	rapid eye movement
STOP	stable tubule only polypeptide
TF	tail-flick

1 INTRODUCTION

1.1 Schizophrenia

Schizophrenia is a complex, severe psychiatric disorder with hallucinations, delusions, cognitive impairments, alogia, apathy, and bizarre behavior [1]. Kraepelin was the first who distinguished 'manic depression' from 'dementia praecox', which referred to the condition of schizophrenia [2]. In 1911 Bleuler developed the term schizophrenia from the combination of the Greek word for 'splitting' (schizo) and 'mind' (phren) [3]. Throughout the ages, the definition varies across the different editions of the Diagnostic and Statistical Manual of Mental Disorders [4,5]. The fifth version suggests a spectrum approach instead of a categorical approach to describe schizophrenia and other psychotic disorders (e.g., schizoaffective disorder) [6].

1.2 Symptoms

Generally, schizophrenia is classified into 3 core features: positive (hallucinations, delusions), negative (alogia, apathy), and cognitive symptoms (impaired attention, learning, and memory functions) [7]. Patients usually experience the symptoms in early adulthood or late adolescence, men tend to develop the disorder at a younger age and experience a more serious form [8]. Besides, patients with schizophrenia may display affective (depression, mania), and aggressive (verbal-, object-, self-, physical aggression) symptoms as well [9,10]. Apart from the classical symptoms, there are several non-specific features of schizophrenia, such as reduced sensory gating, elevated pain threshold, and impaired sleep-wake rhythm [11,12].

1.2.1 Cognitive dysfunction

Abnormal cognitive functions are considered a key component of schizophrenia, it has an essential effect on the patient's quality of life [13]. In patients, cognitive decline varies across several different cognitive domains, including attention, general intelligence, executive function, and memory, and most of these signs are associated with impairments of specific alleles [13–15]. Executive functions consist of mental abilities including memory, cognitive flexibility/set-shifting, and inhibition, which are crucial for environmental adaptation [16]. Neuropsychological studies revealed that many components of executive functioning are impaired in schizophrenia. The most typical features are the inability to encode and retain verbally presented information and poor planning capability [13,17–20].

1.2.2 Changes in circadian rhythm and electroencephalographic (EEG) pattern

The connection between schizophrenia and sleep disturbances has been reported since Kraepelin's first description of the disease [21,22]. Sleep abnormalities and dysregulation of circadian rhythm are well documented in other mental disorders for example in Alzheimer's disease as well, but its prevalence is prominent in schizophrenia [23–25]. In general, schizophrenic patients suffer from difficulties in initiating and maintaining sleep, as well as advanced sleep phase syndrome and hypersomnia with short naps are common, additionally, decreased rapid eye movement (REM) sleep density and increased slow-wave sleep are also observed [23,24,26–28]. These patients also exhibit differences in circadian rhythm, impaired phasing, and fragmentation of rest-activity patterns [26–32]. Disturbed sleep and circadian rhythm in schizophrenia affect the quality of life (impaired coping, increasing suicidal ideation), cognitive performance (impaired sleep-dependent memory consolidation), and positive symptoms (persecutory delusions, hallucinations) [33–38]. The abnormalities could arise from impaired neural (irregularly elevated thalamocortical functional connectivity), and molecular mechanisms (e.g., altered expression of the calcium voltage-gated channel subunit alpha1 I gene) [39,40].

Abnormal oscillation patterns in EEG frequency bands play a role in impaired sensory and cognitive functions, which has been observed in schizophrenic patients [41–51]. It seems that negative symptoms correlated with higher delta band power, additionally, patients with positive forms of schizophrenia had lower delta band power [52]. Theta oscillations may be connected with both positive and negative symptoms in patients, and the changes were influenced by the leading symptoms of schizophrenia [49,53–56]. Both enhanced and reduced alpha power were linked to negative and positive signs [48,52,53,57,58]. Regarding beta activities, inconsistent findings were detected at both lower and higher beta powers [51,52,55,58,59]. Evidence indicates that gamma oscillations are connected to cognitive and sensory processing [57,59–62]. Numerous studies found diminished gamma oscillatory activity, which might be associated with the negative symptoms of schizophrenia, but findings also demonstrated enhanced gamma activities, which were related to positive domains of schizophrenia [45,49,53,55,59,63–68].

1.3 Epidemiology and etiology

Schizophrenia affects about 1 % of the population worldwide and has an increasing prevalence globally [69]. Regardless of the numerous studies in the past, the exact etiology and pathophysiology

of the disease remain ambiguous [70]. Systematic reviews revealed sex differences; schizophrenia is more common in men, and the incidence approximates 1.4 male:1 female [71]. Familial, twin, and adoption studies show that the heritability rate of schizophrenia is approximately 66–83% [72–75]. There are several schizophrenia-associated genes, including, dysbindin, DISC1 (disrupted-in-schizophrenia-1), neuregulin-1, and reelin [73]. All of the related genes have various roles, such as dysbindin contributes to cognitive functioning, DISC1 has a regulatory effect on glutamate functioning and it may be involved in dopamine (DA) homeostasis, and reelin is important in the synaptic formation and plasticity [76–81]. Evidence suggests that the development of schizophrenia is influenced by several environmental and social risk factors [82]. It is more frequent in people who live in big cities; furthermore, migrant status could also increase the liability of developing this disorder [82–85]. Additionally, prenatal and birth complications (fetal hypoxia, parental age at conception) and different types of infections, like influenza, toxoplasma, and herpes simplex virus type 2, also contribute to the onset of schizophrenia [86–89]. Epidemiological studies have shown that cannabis or other substance use has been linked to developing schizophrenia [90–92].

1.4 Neurobiological alterations

Several neurotransmitter system impairment was observed in the pathology of schizophrenia. DA imbalance has been primarily involved in the pathomechanism of schizophrenia, and conventional treatments mainly act on the DA pathways in the brain [93]. However, increasing evidence has shown that the development of schizophrenia might involve other neurotransmitter system impairments, such as glutamatergic, and gamma-aminobutyric acid (GABA) [93].

The ‘classical’ DA hypothesis originated in the 1960s when it was discovered that psychostimulants, such as amphetamine or cocaine, increase DA levels and could be responsible for psychotic symptoms [94]. Between the main DA-ergic pathways, subcortical/cortical imbalance is hypothesized. Many studies suggested that, subcortically, the mesolimbic areas are overactivated (i.e. the ventral tegmental area, the ventral striatum, the hippocampus, and the amygdala) and may contribute to the positive symptoms (Fig. 1) [94–96]. On the other hand, hypoactivation of DA transmission in the mesocortical regions results in negative, affective, and cognitive symptoms (Fig. 1) [94–96].

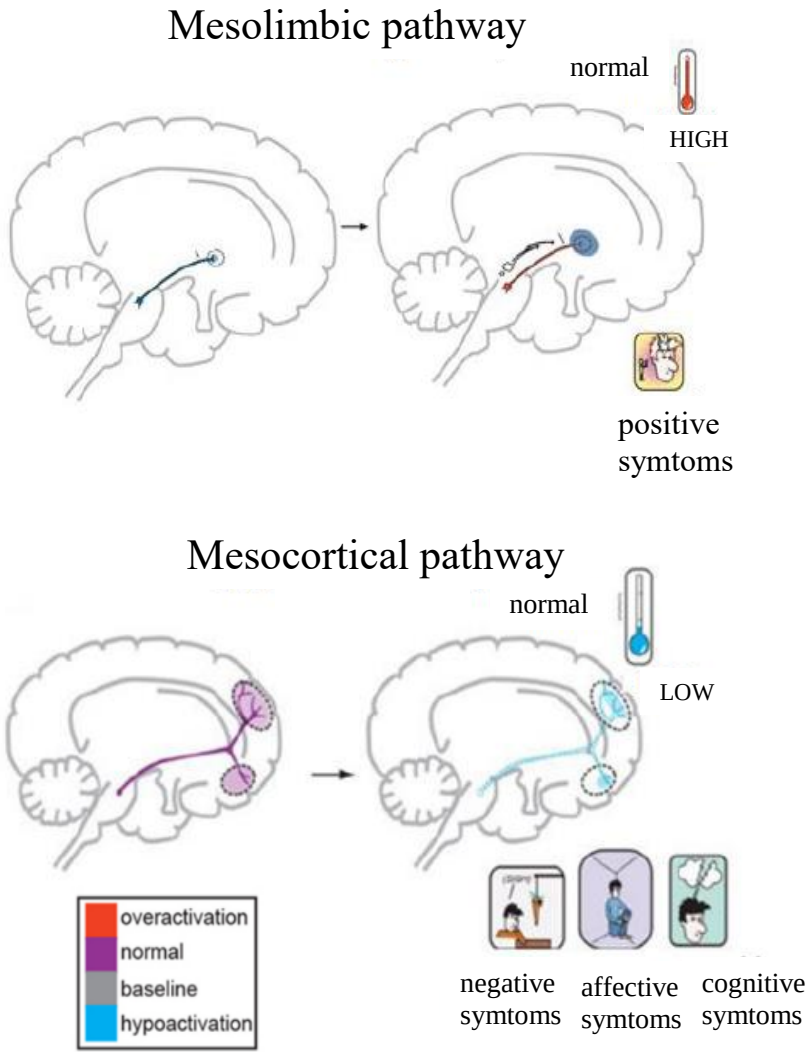

Figure 1 *The mesolimbic pathway hyperactivation, and hypoactivation of the mesocortical pathway*

Based on the modulatory effect of the adenylate cyclase enzyme, DA receptors can be classified into two major groups, D_1-like and D_2-like receptors [97–100]. The D_1-like receptors (D_1R and D_5) can activate adenylate cyclase, while D_2 subgroups block the enzyme (D_2R, D_3, and D_4) [97]. Several data suggest the role of alterations in the different DA receptors in schizophrenia [101–104]. D_1R is primarily associated with cognitive functions such as working memory; therefore, impairments of this receptor functioning may contribute to the cognitive and negative symptoms of schizophrenia [95,105]. Antipsychotic drugs, such as clozapine (CZP), can bind to DA receptors, however, it can affect other neurotransmitter systems as well as, serotonin, histamine, adrenaline, and acetylcholine, which also may contribute to its beneficial effects [106–108]. It was suggested that the

combination of drugs with different DA receptor antagonists (D_1 or D_2) may be effective in schizophrenia treatment [109]. According to a recent study, the efficacy of antipsychotic medications was linked to the regulation of D_1R (rather than D_2R) -expressing neurons in the striatum, suggesting a role for abnormal D_1R activity in the onset of psychosis [110]. Regarding the D_1R density in patients with schizophrenia, evidence from positron emission tomography studies revealed conflicting results. Some of them reported increased, decreased, or unchanged receptor density compared to the controls [111–119].

However, acting on DA receptors is insufficient for treating all symptom domains related to schizophrenia including cognitive dysfunctions (96). Glutamate is known as one of the major excitatory neurotransmitters in the mammalian brain [93]. Glutamate transmission is mediated by several different receptors. An important subtype of schizophrenia's aspect is the ionotropic N-methyl-D-aspartate receptor (NMDAR) [120]. Patients who abused NMDAR antagonist drugs, such as phencyclidine (PCP) or ketamine, often experienced psychotic symptoms [121]. In addition, the hypoactivation of NMDARs results in schizophrenia-like behavioral and cognitive deficits in normal subjects [122,123]. Postmortem studies also revealed altered glutamate receptor density in different brain regions (prefrontal cortex [PFC], thalamus, and temporal lobe) in schizophrenic patients [124]. Thus, novel antipsychotic medications that modulate glutamatergic neurotransmission can be promising for treating schizophrenia, particularly for the cognitive and negative symptoms [120].

The GABA-ergic interneurons' major act is the inhibition of the central nervous system [125]. The GABA-ergic pathways are related to the synchronization and oscillations of neuronal activity, which is essential for perception, memory, and consciousness [125]. Therefore, abnormal functioning of GABA circuits can lead to changes in neural synchrony [50], irregular gamma oscillations [126], and working memory impairments, which are manifested in schizophrenia generally [93,127]. Nevertheless, the interplay of GABA and other neurotransmitter systems remains unsolved, and further investigations are required to analyze the underlying therapeutic role of GABA-related drugs in treating schizophrenia [93].

Accumulating evidence indicates that other neurotransmitters are also involved in the pathophysiology of schizophrenia such as serotonin and acetylcholine [93]. Psychedelic drugs, such as lysergic acid diethylamide or psilocybin, acting on serotonin (5-hydroxytryptamine; 5-HT)

receptors, generate psychotic symptoms in healthy individuals (92). Schizophrenic patients are often heavy smokers to alleviate negative symptoms, the cholinergic system may be involved in schizophrenia [93,128]. Furthermore, it was found that sensory gating impairment in patients can be eased by the desensitization of the nicotinic acetylcholine receptors [93]. Additionally, changes in muscarinic receptors are also observed in schizophrenia, notably, antagonizing (with atropine or scopolamine) this receptor can induce psychotic symptoms, like delirium and hallucinations, or treatment with antagonists can create cognitive, negative symptoms, while treating with agonists (e.g., xanomeline), improve all of the symptom domains [108,129–134].

1.5 Treatment of schizophrenia

Regarding the various symptoms and the numerous neurotransmitter systems involved, treating schizophrenia is demanding. The currently available medication for schizophrenia has several limitations [108]. Generally, most antipsychotics have higher efficacy in mitigating positive symptoms, but they are less effective for cognitive and negative symptoms, and it is important to mention their severe side effects [135].

1.5.1 Antipsychotics

There are three main types of drugs applied in schizophrenia, i.e. the first, second, and third generation of antipsychotics. Typical or first-generation antipsychotics are mainly D_2R antagonists, such as chlorpromazine, but these drugs are not selective for the DA receptors in the mesolimbic pathways, which results in various side effects [108]. The most prevalent extrapyramidal side effects, including dyskinesia, dystonia, unwanted movements, tremors, and rigidity [108]. Furthermore, acting on the tuberoinfundibular pathway, these drugs induce hyperprolactinemia [108]. In addition, typical antipsychotics contribute to cardiovascular side effects, including tachycardia, palpitation, arrhythmia, or chest pain [108]. Adverse effects may be associated with the liver, like jaundice, reversible liver cell hyperplasia, and necrosis [108].

Atypical or second-generation drugs have different receptor binding profiles compared to first-generation drugs; they display higher affinity to 5-HT_2-A receptors than D_2Rs [108]. However, these drugs may also antagonize the adrenergic, cholinergic, and histaminergic receptors [108]. The primary effect of atypical antipsychotics is also to diminish positive symptoms [108]. Despite the lower risk of extrapyramidal symptoms, these medications can lead to severe metabolic side

effects, including type 2 diabetes mellitus and obesity. Currently approved drugs for clinical practice, including CZP, olanzapine, aripiprazole, cariprazine, and brexpiprazole [108,136]. The last three are the newest type of antipsychotics called ‘third-generation’ drugs.

The third-generation class of antipsychotics acts as partial agonists of D_2 and D_3, and 5-HT_1-A receptors [108,137]. Clinical data demonstrated that this type of drug can reduce the negative symptoms of schizophrenia, furthermore, brexpiprazole has a procognitive effect, too [108,138,139]. Despite their beneficial impacts, they have also side effects, including akathisia, weight gain, insomnia, gastrointestinal symptoms, somnolence, nasopharyngitis, sedation, and anxiety [108,140–143].

1.5.1.1 Clozapine

CZP was the first developed second-generation antipsychotic agent [108]. It has a potential role in treating positive symptoms, which is effective mainly in treatment-resistant patients [108,144]. Because of its wide-range receptor antagonistic character, it was suggested that CZP might be potent in reducing negative symptoms of schizophrenia [145]. Controversial results are available regarding the CZP’s effect on cognitive abnormalities, some data demonstrated improvements, whereas others found adverse effects [145–151].

Most of the body systems are involved in the side effects of CZP, among various hematological side effects (leukocytosis, leukopenia, eosinophilia) the most severe one is agranulocytosis, which may cause death [108,145]. CZP treatment may also be linked to various cardiovascular symptoms, including tachycardia, postural hypotension, hypertension, and myocarditis [145]. Furthermore, CZP administration may be related to obesity, hyperglycemia, diabetes mellitus, constipation, or sedation [145].

1.5.2 Adjuvant treatments

The conventional treatment of schizophrenia is based on the administration of antipsychotics, which are appropriate primarily to treat positive symptoms, but they also cause different side effects [152]. Therefore, adjuvant therapies (e.g., physical exercise, cognitive behavioral therapy, and different drugs) play an increasing role in clinical practice to decrease negative symptoms and/or antipsychotic-induced adverse effects [152–154].

1.5.2.1 Metformin

One of the most prevalent adverse effects of antipsychotics is the metabolic syndrome with disrupted lipid and glucose metabolism, weight gain, and hyperprolactinemia. Therefore, the combination of antipsychotics with drugs that can blunt these side effects might have clinical significance [155,156].

Metformin (MTF) is a well-known antidiabetic medication and it is derived from *Galega officinalis* or Goat's rue plant [157]. It can penetrate various cell types throughout the body, including hepatocytes, adipocytes, enterocytes, and neurons [158]. MTF controls the adenosine monophosphate-activated protein kinase enzyme and suppresses the mechanistic/mammalian target of rapamycin enzyme, which are both essential in cellular metabolism, cell growth, and survival through regulating various pathways [158,159]. Since these 2 enzymatic pathways play a crucial role in cell metabolism, impairment of these systems may lead to cell death and the development of different mental or neurodegenerative disorders [159]. MTF has been demonstrated to have neuroprotective aspects, thus, in diabetes-related dementia MTF was able to enhance verbal learning, working memory, and executive function [160]. In neurodegenerative disorders, such as Parkinson's and Alzheimer's the long-term application of MTF prevented cognitive decline; however, there is no data regarding schizophrenia [161].

1.6 Animal models

To examine complex psychiatric disorders (e.g., schizophrenia), and to determine the neurological underpinnings, and/or novel drug targets, relevant animal models are required [80]. The main challenge is to replicate the human characteristics of schizophrenia, such as hallucinations [80]. All of the existing animal models should satisfy three validation criteria: face, construct, and predictive ones [80]. Face validity refers to symptom homology or how well an animal model replicates the symptoms observed in humans. Construct validity means "the degree to which a test or instrument is capable of measuring a concept, trait, or other theoretical entity" [162]. Finally, predictive validity indicates whether the animal model is appropriate for acquiring new information about the disease and reveals possible therapies [80]. The animal models of schizophrenia are grouped into four main categories: developmental, pharmacological, genetic, and combinational.

A common theory of schizophrenia hypothesizes that exposure of early-life adverse events in people with genetic susceptibility to schizophrenia may lead to abnormal neuronal growth and

connection, which contribute to the emergence of a schizophrenic phenotype [80]. The neurodevelopmental models of schizophrenia contain environmental factors during pregnancy, which can induce schizophrenia-like symptoms, including maternal stress, malnutrition, infection, and birth complications [80].

Rats generally live in a colony with a social hierarchy, which is essential to healthy development [80]. Social isolation contributes to the onset of depression or schizophrenia and impairs brain development and behavior, causing decreased sensorimotor gating, hyperactivity, neophobia, and cognitive dysfunctions [163–166]. The social isolation is accompanied by altered dopaminergic and serotonergic neurotransmission in various brain areas [163]. Thus, the animal models generated with post-weaning social isolation largely satisfy construct validity [80].

Many drugs that affect the various neurotransmitter systems have been proposed as pharmacological models for schizophrenia [129]. DA agonists, such as amphetamine and cocaine, are utilized to cause positive symptoms and stereotyped behavior in rodents [129]. Lysergic acid diethylamide and psilocybin, serotonergic models of schizophrenia, directly disrupt the 5-HT_2-A receptors, resulting in impaired pre-pulse inhibition (PPI) [129,167,168]. Regarding the GABA-ergic system, evidence suggests that antagonism of the GABA-A receptor is associated with impaired PPI in rodents [129,169]. Animal studies demonstrated that NMDAR antagonist drugs, including PCP and MK-801 (dizocilpine) treatment can induce hyperactivity, hyperlocomotion, social withdrawal, impairment of PPI, and cognitive dysfunction [170–181]. Both PCP and MK-801 exposure can cause structural changes in the brain, including a reduced number of synaptic spines in frontal cortex neurons and cortical and hippocampal parvalbumin-immunoreactive neurons [80,182–188].

Ketamine, another NMDAR antagonist is used for anesthesia and analgesia in both humans and animals, but acute administration produced a variety of symptoms in healthy participants, including hyperactivity, paranoia, hallucinations, and cognitive deficits [189–192]. Furthermore, ketamine treatment can aggravate the schizophrenia-related positive and negative symptoms [193]. Similarly to PCP, ketamine can induce several cognitive disruptions in animals, such as impaired sensory auditory gating, reduced performance in attentional set-shifting, novel-object recognition, and social novelty detection tasks, social behavior deficit, and hyperactivity; therefore ketamine is frequently used for schizophrenia modeling [194–197]. Alterations due to ketamine application are also

presented at the neuroanatomical level, including the reduced number of parvalbumin-expressing neurons and cortical fast-spiking inhibitory neurons [198]. Moreover, ketamine administration was also associated with the deficient expression of a GABA-producing enzyme, glutamate decarboxylase 67 [198].

As was stated in Section 1.3, there are abundant genes associated with a high risk of developing schizophrenia, but it is still unknown what are the roles these genes play in the onset of the illness (75). Several genetic animal models of schizophrenia have been created using many of the identified genes [170]. Genetic models (even knockout or knockin) can be suitable for examining how specific proteins contribute to the onset of this disease and for the investigation of behavioral alterations [199]. These types of models have several limitations, such as the genetic manipulations for a single gene are unable to mimic all of the symptoms, rather, the complexity of these genetic components appears to be more likely to develop schizophrenia [199]. Furthermore, this process lasts a long time, it is complicated and also expensive [199].

Selective breeding is an alternative 'genetic' procedure based on selecting animals that show a higher level of the features relevant to the disorder and breeding it through generations, thus, this method might be also effective in generating an animal model [200,201]. In various studies, new rat lines in psychiatric conditions such as epilepsy, depression, anxiety, or schizophrenia were developed using the selective breeding process [202–204].

Since the etiology of schizophrenia seems to be multifactorial, and based on gene-environmental interactions, research suggests that the combination of genetic, developmental, and environmental factors can provide a more reliable model of schizophrenia [205,206]. The environmental risks in themselves appear insufficient in developing the disease [205]. According to the 'dual hit' theory of schizophrenia, the genetic aspect (hit 1) prepares the susceptibility to a second hit (environmental factor) in later life [205,207].

Some schizophrenia animal models operate with 'three-hits', which integrate genetic, pharmacological, and environmental risks [208–210]. Chen et al. produced a three-hit schizophrenia rat model by maternal separation, avoidance conditioning, and PCP administration [208]. The effects of the hits on PPI during two developmental stages (adolescence and adulthood) were evaluated. The authors found that multiple hit during adolescence and adulthood in rats was associated with

disturbed PPI [208]. Thus, 'multiple-hit' models may be appropriate for investigating behavioral and neurobiological mechanisms of schizophrenia [208].

A recent study revealed that the three-hit mice (hit 1: partial deletion of microtubule-associated protein 6, hit 2: maternal separation, and hit 3: MK-801 administration), exhibited increased locomotion and cognitive deficits [209]. Another study used a combination of genetic predisposition (hit 1: partial deletion of microtubule-associated protein 6), early postnatal stress (hit 2: 24 h maternal separation), and pharmacological exposure (hit 3: cannabinoid exposure during adolescence). This model displayed several landmarks mimicking negative and cognitive symptoms of schizophrenia, such as decreased activity, increased anxiety-like behavior, and structural alterations (reduced hippocampal volume) [210].

1.6.1 Wisket animal model

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) [211]. The selective breeding process has been ongoing since 2008 and we are currently breeding the 47th generations. Wisket animals demonstrated decreased acute and chronic pain sensitivity, impaired sensory gating, locomotor activity, and cognitive abilities [212–215]. Abnormalities of the autonomic nervous system were also evidenced by disturbances of thermoregulation and pupillary light reflex [216,217]. Neurophysiological alterations were also observed in the auditory evoked potentials [218]. Additionally, Wisket rats displayed changes in short-term EEG, including increased theta, alpha, and beta power, decreased delta and gamma oscillations, and increased gamma band power in both control and Wisket rats following acute ketamine application [218]. Molecular-biological studies have proven alterations of mu-opioid, cannabinoid 1, and D_2R, as well as disturbances of the GABA-ergic and oxytocinergic systems in different brain structures [211,219–221]. Recently, caffeine treatment was also investigated, and our results showed that caffeine injections ameliorated learning in Wisket rats with prolonged alterations in the dopaminergic system [222]. Furthermore, the regulation of cerebral metabolism was also disrupted in Wisket animals, with delayed response to the caffeine treatment [223]. 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 OF THE STUDY

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 involved 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-hour 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 (www.deakdelta.hu) is a rectangular corridor made of clear plexiglass with a black floor that has an external diameter of 80 cm, a width of 8 cm, and a height of 50 cm (Fig. 2). This system allows the rats to move forward and backward between the walls. 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). In each side-box, infrared beams were placed to detect exploratory behavior (nose pokes), while locomotor activity was measured with infrared beams which are located in the middle of each corridor. The experimenter placed the food rewards into the side-boxes and began the trials by positioning the rats at the same starting point within the corridor, then, left the room (Fig. 2) (231). For five minutes (cut-off time: 300 s) the animals were free to explore the corridor and gather food rewards. The amount of food rewards consumed by the animals was counted at the end of each trial, and the Ambitus was cleared with 70% ethanol. An infrared video camera (WCM-21VF, CNB, China) was fixed above the Ambitus to record all of the experiments. Offline analysis was carried out to assign the time required to complete the

task in case the animal had consumed all of the food rewards. During the study the animals performed three different tasks (Fig. 3b). 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, separated by one minute) 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. The definitions of the analyzed parameters are shown in Table 1, in the Results Section.

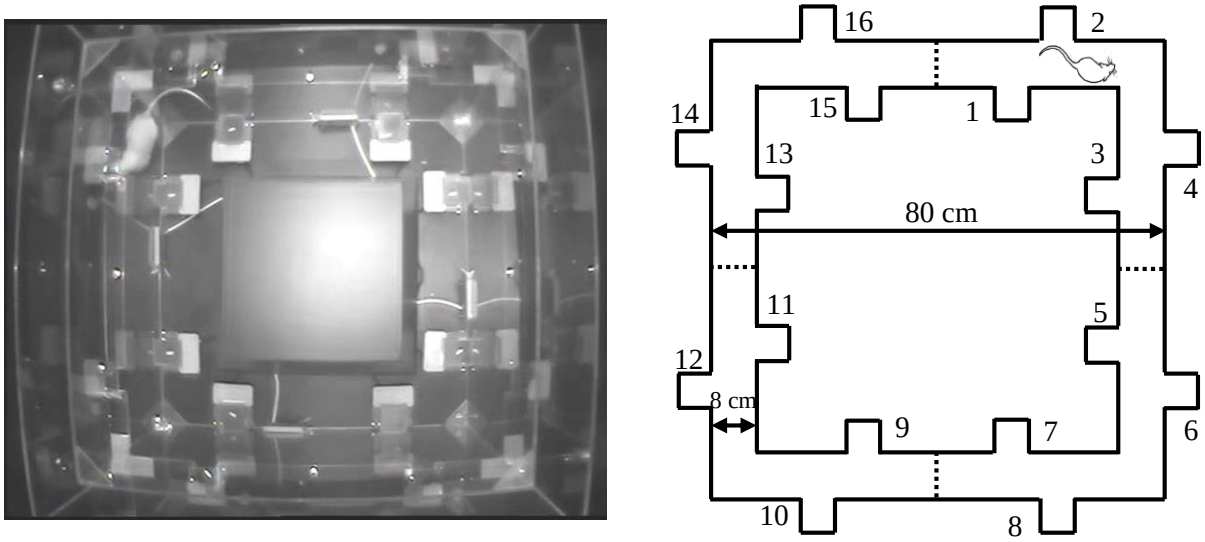

Figure 2 *Left: real photo of the AMBITUS system captured from an offline video. Rat is sniffing in Box 14 in the left upper corner. Right: the scheme of the Ambitus apparatus. The corridor with 16 side-boxes equipped with photo beams, a rat is at the starting point.*

3.2.3 Sensory gating test

After 12 hours of food deprivation, the 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 times 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 Drugs

Water was used as solvent and diluent of MTF and CZP. According to the basal test performance and body weight of Wisket and control animals, the rats were divided into pharmacologically treated groups. 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 dosing technique was based on earlier studies [148,224].

3.3.2 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. The experiment with the different treatments lasted for 28 days (Fig. 3a). The drugs were administered via the oral route (drinking water), which is the preferred translational method in humans. Even though a daily gavage would be a more reliable option for drug administration, we chose the above-mentioned way to avoid recurrent stress for this prolonged (28-day) period. During the experiment, the animals were kept in pairs as previously mentioned [225,226]. The present experiment required exact monitoring of fluid and food consumption of the individual animals, but we attempted to prevent the effects of social isolation. Throughout the study, the body weight and relative amounts of food and fluid consumption were measured twice a week, and fresh solutions were available. Due to the Ambitus test's restricted food availability (2 days before and during the test, see below), the amount of food consumed was analyzed during the phases with freely available food between Day 1–Day 25. 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 (see below and Fig. 3b). The moderate level of food restriction (10–15 g/day) was performed to ensure the adequate motivation to perform the task. Free voluntary drinking was available throughout the experiment.

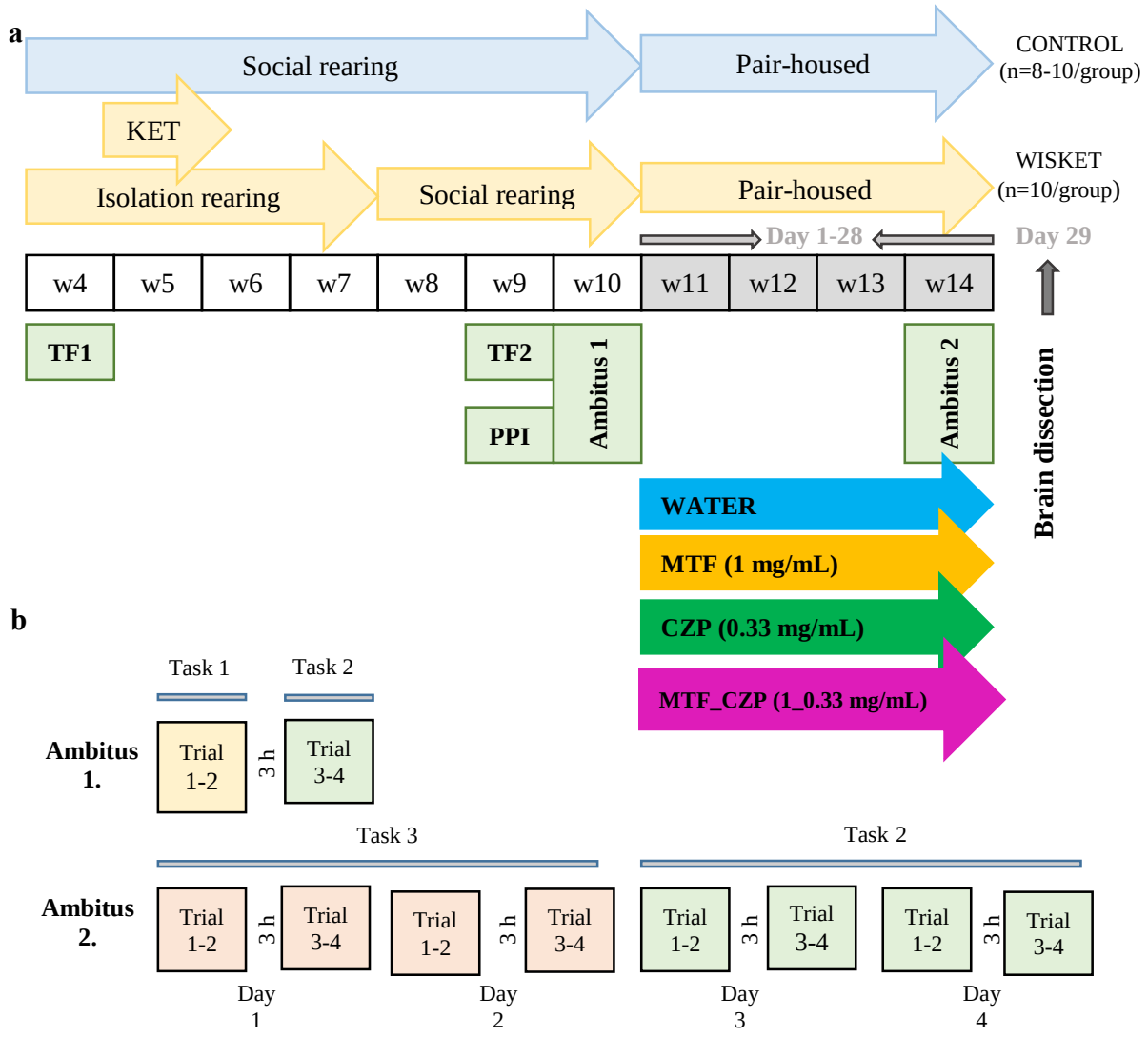

Figure 3 *Experimental paradigm of Series I. (**a**). Experimental paradigm in the Ambitus system (**b**). Trial 1 and Trial 2 (also Trial 3 and Trial 4) were repeated at intervals of 1 min, with an interval of 3 h between Trial 2 and Trial 3. Abbreviations: w, age in weeks; KET, ketamine; TF, tail-flick test; PPI, pre-pulse inhibition test; MTF, metformin; CZP, clozapine.*

3.3.3 D_1R functional activity, binding characteristics, and mRNA expression

The animals were terminated one day following the Ambitus 2 test (Day 29, Fig. 3a), the brains were removed, then dissected on dry ice, frozen in liquid nitrogen, and stored at − 80 °C until additional analysis. The following sections were made in collaboration with Sándor Benyhe and Edina Szűcs from the Laboratory of Opioid Research, Institute of Biochemistry, Biological Research Centre, Szeged, Hungary. The D_1R binding and signaling experiments were conducted

in the cerebral cortex (CTX), olfactory bulb (OB), brainstem, and diencephalon. In accordance with our earlier findings [221,227], neuronal membrane fractions from frozen brains were made for functional guanosine 5′-O-(3-[^{35}S] thio)triphosphate) ([^{35}S]GTPγS) binding tests and in vitro receptor binding (specific activity: 1000 Ci/mmol; purchased from Hartmann Analytic, Braunschweig, Germany). Using BSA as a standard, the protein content of the membrane preparation was assessed with the Bradford method [228], and the UltimaGold MV scintillation reagent was provided by PerkinElmer (Boston, USA). As previously published, the functional [^{35}S]GTPγS binding studies were carried out with a few modifications to improve the binding assay stimulated by a D_1R agonist [229–231]. Membrane homogenates were incubated at 30 °C for 60 minutes in buffer (pH 7.4) consisting of 25 mM HEPES, 120 mM NaCl, 20 mM $MgCl_2$, 1.8 mM KCl, and 1 mM sodium deoxycholate containing 20 MBq/0.05 cm [^{35}S]GTPγS (0.05 nM) and increasing concentrations ($10^{—}$ to $10^{—}$M) of the selective D_1R full agonist, SKF81297 (Tocris Bioscience, Bristol, United Kingdom) [146,155,213,232]. The experiments were conducted in a final volume of 1 mL of excess GDP (10 μM). Total binding was assessed in the absence of test compounds, while non-specific binding was evaluated in the presence of 10 μM unlabeled GTPγS and subtracted from the total binding. The basal activity was represented by the difference. The reaction was finished by fast filtering under vacuum (Brandel M24R Cell Harvester) and washed three times through Whatman GF/B glass fibers with 5 mL of ice-cold 0.1 M phosphate (pH 7.4) buffer. The radioactivity of the dried filters was detected using a Packard Tricarb 2300TR liquid scintillation counter and an UltimaGold MV aqueous scintillation cocktail. [^{35}S]GTPγS binding assays were carried out in triplicate and were repeated at least three times. Aliquots of frozen rat brain membrane homogenates were centrifuged, and thawed, and a 50 mM Tris-HCl solution (pH 7.4) was used for suspension. Since the D_1R antagonist [H]SCH 23,390 (Tocris Bioscience, Bristol, United Kingdom) also has a high affinity for the 5-HT_2 receptors, they were inhibited by adding 1 μM ketanserin, a selective 5-HT_2 ligand, to the buffer [146,233,234]. The membranes were incubated at 25 °C for 1 hour, and increasing concentrations of [H]SCH 23,390 (0.29–12.01 nM) were added to it [146]. The non-specific and total binding was assessed in the presence and absence of 10 μM unlabeled SCH 23,390, respectively. The reaction was finished by fast filtering under vacuum (Brandel M24R Cell Harvester) and washed three times through Whatman GF/B glass fibers with 5 mL of ice-cold 50 mM Tris–HCl (pH 7.4) buffer. The radioactivity of the dried filters was detected using a Packard Tricarb 2300TR liquid scintillation counter and an UltimaGold MV

aqueous scintillation cocktail. Saturation-binding assays were carried out in duplicate and were repeated at least three times.

The RNA extraction and quantitative real-time polymerase chain reaction experiments (qRT-PCR) were conducted by Gyöngyi Kis and Heni El Heni from the Laboratory of Sensory research group at our department. The D_1R mRNA expression was observed in the CTX, PFC, striatum, cerebellum, and hippocampus. From tissue samples that had been homogenized with TriXtract reagent (G-Biosciences, St. Louis, MO, USA), total RNA was extracted. Then, chloroform was added to the RNA to separate it into an aqueous phase. The RNA pellet was dissolved in RNAse-free water after precipitation with isopropyl alcohol and a wash with 70% ethanol. A Genova Nano micro-volume spectrophotometer (Jenway) was used to examine the quantity and quality of the extracted RNA at optical densities of 260 and 260/280 nm, respectively. The samples that were employed in further tests displayed an absorbance ratio between 1.6 and 2.0. Using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), equal amounts of RNA were used to synthesize cDNA in each experiment. PCR was performed in a thermo cycler (Bio-Rad CFX96 Optics Module) by preparing triplicates of reactions of 10 µl using the iQ SYBR Green Supermix (Bio-Rad). The thermal cycling process involved an initial denaturation step at 95°C for 30 seconds and 40 cycles of denaturation at 95°C for 10 seconds, annealing at 59°C for 30 seconds, and extension at 72°C for 20 seconds. A melting curve analysis was performed on the amplicons. The pair of primers previously created by Bangaru et al. was used in-house to determine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the endogenous control (forward: 5′–AAGAAGGTGGTGAAGCAGGCG–3′ and reverse: 5′–AGCAATGCCAGCCCCAGCAT–3′) [235]. To amplify a 92 bp fragment of the D_1R mRNA, (forward: 5′-GATCTCTTGGTGGCTGTCCTG-3′ and reverse: 5′-ACCCAGATGTTACAAAAGGGAC-3′) two primer pairs were created using the National Centre of Biotechnology Information reference sequence database [236]. In the negative controls, RNAse-free water was used instead of cDNA in the reaction. To accomplish relative quantification, the threshold cycle values were utilized as reference points, and the comparative threshold cycle method (ΔΔCt method) was implemented. Using the control groups as normalizers, $2^{-\Delta\Delta Ct}$ values were utilized to determine fold changes in target gene expression [237].

3.3.4 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 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). Definitions and denotes the significance of the examined behavioral parameters are shown in Table 1. Fisher's LSD test was used to perform post hoc comparisons. The [^{35}S]GTPγS binding data (sigmoid dose-response stimulation) and radioreceptor binding (saturation curves, one binding site model) were analyzed with a specialized curve-fitting program (GraphPad Prism 5.0.; Inc., San Diego, CA) utilizing a non-linear regression analysis. To ascertain the significance level of groups and treatments for the acquired in vitro parameters, a factorial ANOVA was carried out. The unpaired t-test was additionally utilized to establish differences between the various groups in comparison to the water-drinking control animals for the mRNA expression data.

3.4 Series II.

3.4.1 Experimental paradigm

The following sections were made in co-operation with Balázs Bodosi and Attila Nagy from the Sensorimotor Research Group at the Department of Physiology. For recordings of the EEG, the surgical procedure was comparable to that mentioned earlier [218,238,239]. The surgery 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. Then the rats were placed into a stereotaxic frame, and stainless steel screws were implanted. 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). A thermistor was positioned over the left parietal cortex to measure the cortical temperature. Subsequently, a miniature connector was attached to the skull with dental cement and linked to the electrodes through enamel-coated copper wires. After the procedure, animals were kept individually in special clear plexiglass cages (25 x 28 x 50 cm), to prevent the implanted devices from being removed by other rats in the cages. The visual, auditory, and olfactory

connections were provided between rats to reduce the impact of isolated rearing [240]. After a one-week recovery period, the animals were allowed to habituate to the experimental environment and connected to the recording tether, then 23 hours (since 1 hour was spent caring for the animals) EEG recordings were made. Nevertheless, 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 due to technical issues (the animals often damaged the recording cables close to the light period). There were conducted two series of experiments. Four rats (both control and Wisket; n=8/group) were recorded in the first series (matched) concurrently during each recording and five controls and six Wisket animals were represented in the second series (unmatched). The unmatched group of animals was different and observed on other days to detect if the alterations found between the control and Wisket group in the first series could be replicated independently of the environmental conditions. Balázs Bodosi created and built the following devices, together with all of the PC and microcontroller applications. Electrode cables were attached to a rechargeable Li-ion battery-powered small microcontroller-based transmitter unit and tied to the cables. The cable housing was connected to a plastic bearing (on the top of the cage) that enables free rotation. An internal analog-to-digital converter filtered (0.5−48 Hz), amplified (2000 x), and digitized the signals with a sampling rate of 128 Hz, and it had a 12-bit resolution. The animal's motor activity was measured with an internal 3-axis accelerometer of the device, (type: LIS3LV02DQ) with a range of 0–6 G, as explained previously [238,239]. The signals were transmitted at 2.4 GHz to a receiver unit connected to the PC via a USB link for scoring.

3.4.2 Data and statistical analysis

Data were examined offline in an 8-s epoch. The special preprocesses were developed by György Kalmár and Mátyás Csordás-Nagy (the Department of Technical Informatics, Department of Image Processing and Computer Graphics, Faculty of Science and Informatics, Institute of Informatics, University of Szeged). Preprocessed EEG signals were used in order to fix these sections. The clipping signals were first identified after they were evaluated and replaced. The substitution signal was created as a well-formed (colored) white Gaussian noise, and the noise's spectrum was formed to match the spectrum of the EEG sign surrounding the faulty part. It was generated with the same length as the faulty part. Therefore, the aggregated spectrum-based features that were derived later in the analysis remain unaffected by the substitution. 2% of the EEG dataset was adjusted by the pre-processing technique. Afterward, three vigilance stages were

identified over 8 seconds of epochs: wakefulness, NREM sleep, and REM sleep. Wakefulness was described as less regular theta activity, and frequent body movement. Based on earlier findings, the awake stage was further divided into two substages: active and quiet/inactive awake [241,242]. 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. Following the classification of vigilance states, power spectra for each stage and substage under a condition of 0.125 Hz resolution were calculated using the Fast Fourier Transform method with a Hanning window. The sum of the squares of all the frequency values included in a certain band was used to compute total absolute power. Total absolute power was computed by the sum of squares of all frequency values included in a certain band, while the power ratios of each frequency band to the overall (z-score) in each 8 s bin were used to express relative band powers. 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). The parameters were quantified and evaluated as means of values for each hour for 21 hours (daily) and divided into light and dark phases of the day (diurnal). The number and length of episodes in various stages, as well as the mean duration per hour, were analyzed. A distinct or even opposing tendency was shown during preliminary data analysis in relative powers within the small frequency ranges about the detailed investigation of the oscillatory pattern. In order to identify fine-grained differences between the two groups, we carried out statistical analysis for all of the data for each frequency band (delta, theta, alpha, beta, and gamma) based on previous findings (262). All data are presented as means ± SEM. Repeated and/or factorial analysis of ANOVA was used to compare the parameters. Fisher's LSD post hoc test was applied to assess the impact of various parameters when the global test was significant. STATISTICA 13.5.0.17 (TIBCO Software Inc., USA) was used for the statistical analysis, the alterations were determined significant for $P < 0.05$.

4 RESULTS

4.1 Results of Series I.

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

In agreement with our current studies [211,213], Wisket animals demonstrated reduced pain sensitivity ($F_{(1,74)} = 81.46$; $P < 0.001$), sensory gating ($F_{(1,74)} = 5.14$; $P < 0.05$), locomotor activity ($F_{(1,74)} = 4.69$; $P < 0.05$), exploratory activity ($F_{(1,74)} = 24.05$; $P < 0.001$), and learning capacity ($F_{(1,74)} = 14.89$; $P < 0.001$) compared with the control rats. There were no significant differences regarding these basal parameters between the two groups treated with different drugs. Concerning variations in body weight during the treatment interval between Weeks 11–14 (Fig. 3), ANOVA showed significant effects of group ($F_{(1,68)} = 21.27$; $P < 0.001$) and time ($F_{(8,544)} = 256.76$; $P < 0.001$), but not of treatment; i.e., the body weight enhanced steadily in all of the groups until the restricted-feeding condition (Day 25, Fig. 4a). Thus, the Wisket animals had lower body weight than Wistar rats, but the treatments did not significantly affect this parameter. Food consumption was comparable in all of the animal groups (on days without restriction), but it reduced significantly with time ($F_{(5,150)} = 61.43$; $P < 0.001$; Fig. 4b).

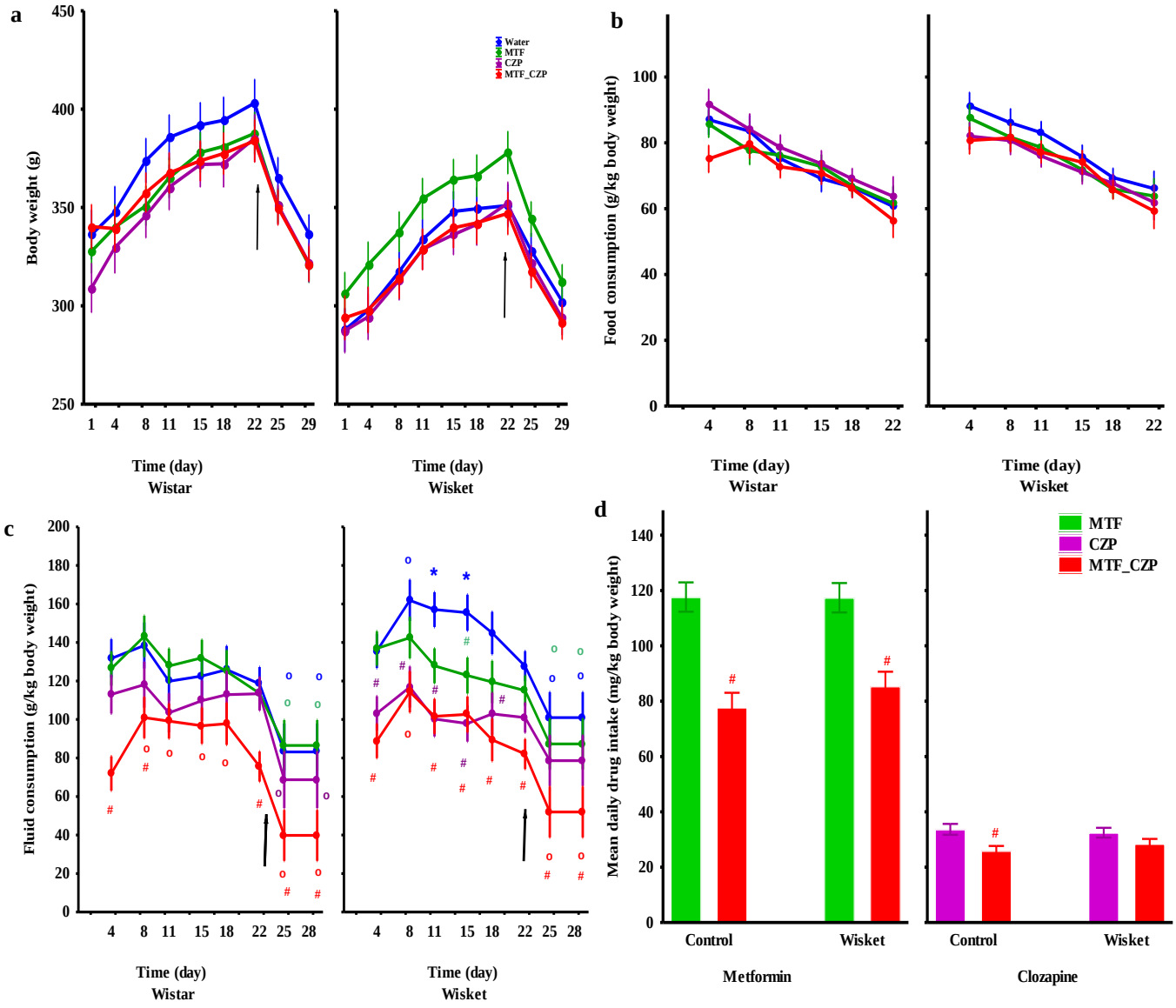

Figure 4 *Time-course curves of body weight* ***(a)****, food consumption* ***(b)****, and fluid consumption* ***(c)*** *according to group. The symbols indicate significant differences between the Wistar and Wisket groups (*), compared with the water-drinking animals (#), the CZP-alone group (x), and the Day 1 values (o). The arrows indicate the starting of the food restriction. Differences in MTF (left side) and CZP (right side) uptake* ***(d)*** *were observed between the single and combined treatments during the whole investigation period. The symbol # indicates significant differences between the single and combined treatment groups.*

The treatment ($F_{(3,30)} = 16.98$; $P < 0.001$) and time ($F_{(7,210)} = 40.30$; $P < 0.001$) had significant impact on fluid intake. Reduced fluid consumption was associated with food restriction in all groups. The post hoc test disclosed a significantly greater relative volume of water drinking in Wisket in comparison to Wistar rats between Days 8 and 15 (Fig. 4c). Additionally, the control,

but not the Wisket group, treated with drug combination showed a significantly lower fluid consumption contrasted with the rats treated with CZP alone observed on Days 4 and 22. As regards the analysis of the computed daily drug intake during the whole examined period, significant impacts of treatments were noticed for both MTF and CZP ($F_{(1,36)} = 62.60$; $P < 0.001$ and $F_{(1,34)} = 17.97$; $P < 0.001$, respectively; Fig. 4d). According to the post hoc comparison, the MTF intake was significantly lower in the combined treatment compared to the fluid comprising MTF alone in both control and Wisket rats. In addition, the CZP consumption in the combined treatment was lower than the CZP alone in the control, but not in the Wisket animals.

The repeated measures of ANOVA disclosed significant impacts of group, day, and/or group and-day interaction on all of the behavioral parameters determined here (Table 1). However, most of the obtained parameters (exception for locomotor activity) were significantly affected by the treatment or time and treatment interaction in the Wisket group (Table 2).

Table 1 *Definitions and ANOVA results for the parameters obtained in the Ambitus test*

Parameter	**Definition**	**ANOVA analysis for Wistar and Wisket animals**	**Significance: F;(df);p**
Locomotor activity	number of entries into the corridors up to 5 min	Group	30.14;(1,68);<0.001
		Day	9.76;(7,476);<0.001
Overall exploratory activity	number of box visits up to 5 min	Group	28.78;(1,68);<0.001
		Day	4.31;(7,476);<0.001
		Group/Day	2.1;(7,476);<0.05
Baited box exploration	number of visits into the baited boxes up to eating all rewards related to eating time	Group	18.15;(1,68);<0.001
		Day	20.25;(7,476);<0.001
		Group/Day	3.13;(7,476);<0.005
Non-baited box exploration	number of visits into the non-baited boxes visits up to eating all rewards related to eating time	Group	22.27;(1,68);<0.001
		Day	21.19;(3,204);<0.001
		Group/Day	5.98;(3,204);<0.001
Learning capacity (%)	[(eating count)x(300)x100]/ [number of rewards)x(eating time)]	Group	16.66;(1,68);<0.001
		Day	25.76;(7,476);<0.001
		Group/Day	4.32;(7,476);<0.001

Table 2 *ANOVA results for the parameters obtained in the Ambitus test*

Parameter	**ANOVA analysis during treatments**	**Significance: F;(df);p**
Locomotor activity	Treatment	NS
Overall exploratory activity	Treatment/Day	1.75;(7,252);<0.05
Baited box exploration	Treatment/Day	1.72;(7,252);<0.05
Non-baited box exploration	Treatment/Day	2.08;(9,108);<0.05
Learning capacity (%)	Treatment/Day	1.72;(21,252);<0.05

No significant effects of treatment were demonstrated by the separate analysis of data from the control group (Fig. 5). Therefore, only data from the four Wisket groups were involved in the detailed analyses. In comparison to the water-drinking animals, it was shown that MTF treatment alone did not significantly affect the observed parameters. Nevertheless, in comparison with the water-drinking group, CZP treatment reduced the exploration, which was associated with a reduced learning capacity (Fig. 5 b–e). The combined treatment of MTF_CZP ameliorated all of the investigated parameters to a level that is comparable to the water-drinking group.

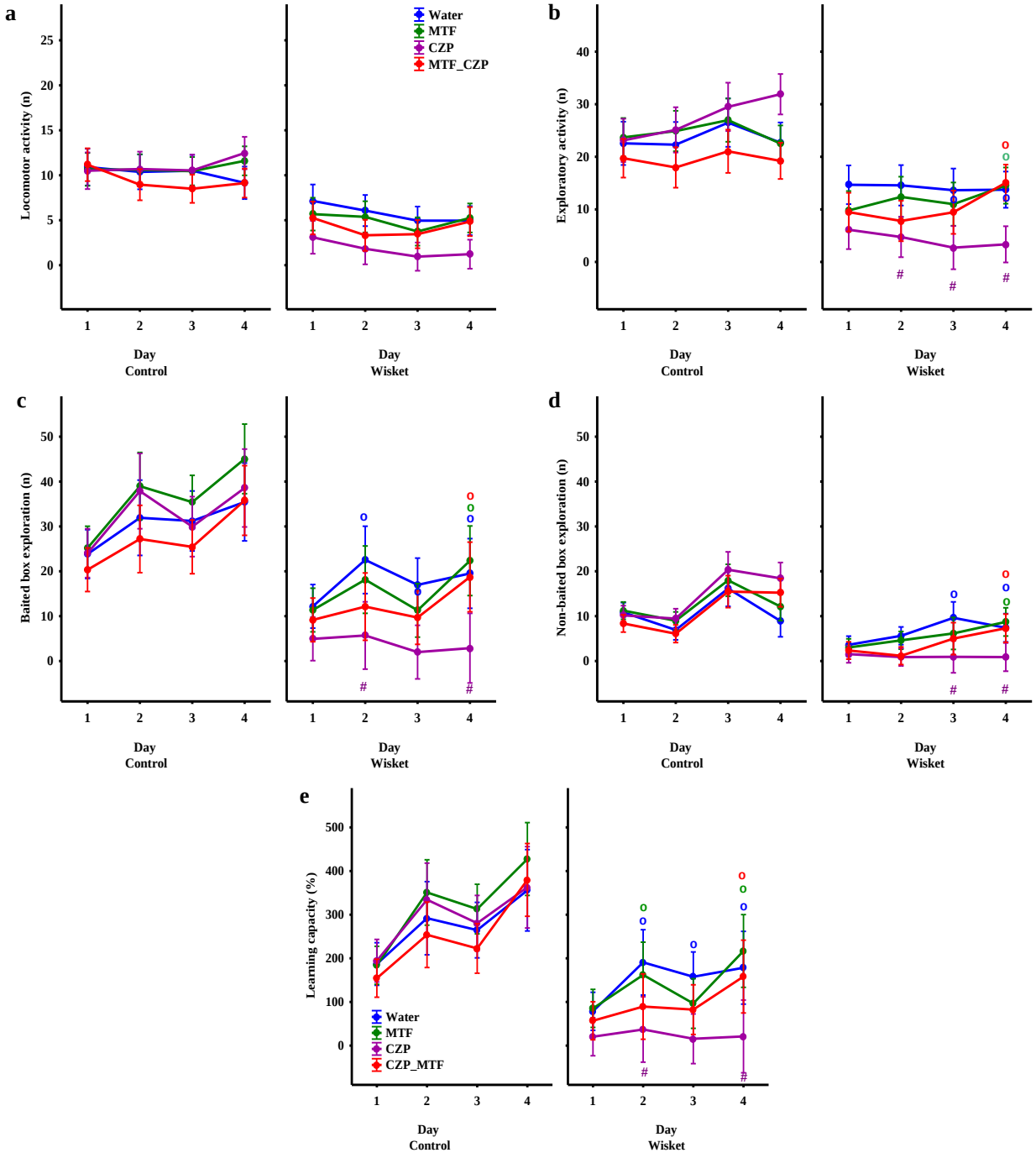

Figure 5 *Time-course curves of the locomotor (**a**) and exploratory (**b**–**d**) activities, as well as the learning capacity (**e**), according to the group. The symbols indicate significant differences between the Wistar and Wisket groups (*), compared with the water-drinking group (#), and Day 1 values (o).*

4.1.2 D_1R activation, binding, and gene expression in different brain structures

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. Concerning the G-protein activation in the CTX, the E_{max} values disclosed significant effects of group ($F_{(1,15)} = 8.17$; $P < 0.05$) and treatment ($F_{(3,15)} = 3.71$; $P < 0.05$; Fig. 6a). The post hoc test demonstrated that both MTF_CZP groups had a significantly higher E_{max} level than their MTF-treated matched pairs. Similarly to the CTX, the E_{max} values exhibited significant effects of group ($F_{(1,15)} = 12.57$; $P < 0.005$) and treatment ($F_{(3,15)} = 4.97$; $P < 0.05$; Fig. 6a) in the OB. According to the post hoc analysis, the Wisket rats treated with the MTF_CZP combination showed a significantly higher level of maximal G-protein activation in comparison with their matched control rats and to water-drinking Wisket rats. None of the treatments had any effect on the brainstem and diencephalon in this respect. Concerning the pEC_{50} values (Fig. 6b), significant effects of the treatment in the OB were detected ($F_{(3,15)} = 4.82$; $P < 0.05$), and the post hoc analysis disclosed that the MTF_CZP-treated groups had greater values compared with their water-drinking counterparts. Additionally, this parameter was significantly ($F_{(1,16)} = 6.95$; $P < 0.05$) affected by the group in the diencephalon with lower values observed in Wisket rats. In the CTX and brainstem, no significant impacts were detected.

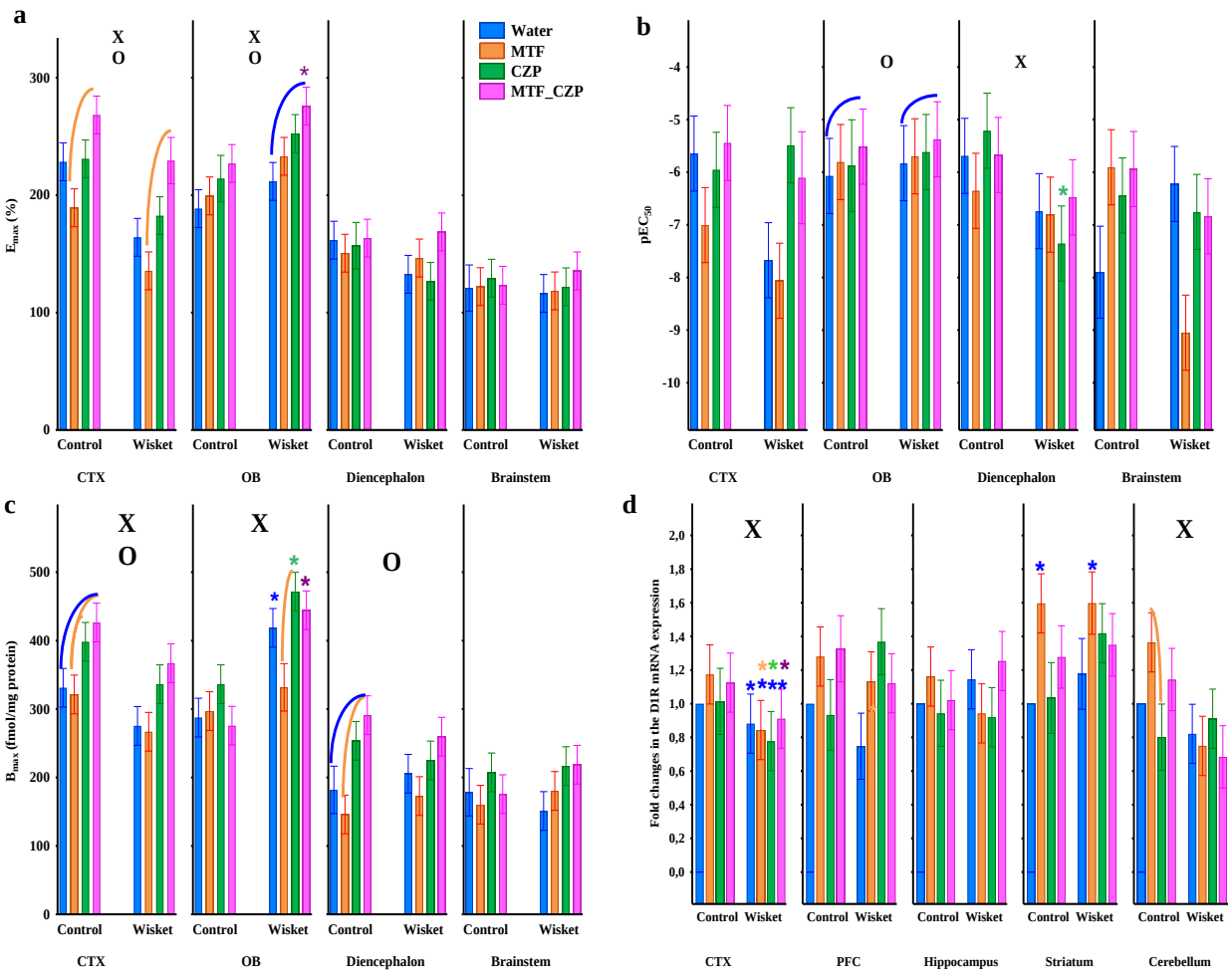

Figure 6 *Results of D_1R signaling, binding, and mRNA expression assays, as indicated by the changes in E_{max}* ***(a)****, pEC_{50}* ***(b)****, and B_{max}* ***(c)*** *values and mRNA expression* ***(d)*** *in the different rat brain structures. The symbols indicate the significant effects of group (X) and treatment (O). The symbols also indicate post hoc significant differences between groups (*) and treatments (arc). The blue stars pinpoint significant differences compared with the water-drinking Wistar rats, whereas the remaining stars indicate significant differences compared with the control matched pairs.*

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 [K_d]) were determined. Regarding the saturation-binding experiments, significant effects of group ($F_{(1,16)} = 12.77$; $P < 0.005$) and treatment ($F_{(3,16)} = 9.42$; $P < 0.05$; Fig. 6c) in the B_{max} values of the CTX were observed, with a lower level of binding detected in the Wisket rats. The post hoc test revealed significantly higher values in the MTF_CZP-treated Wistar animals than its water- or MTF-drinking counterparts, and the same trend was observed in the Wisket animals. The ANOVA of B_{max} values was significantly affected by group ($F_{(1,15)} = 20.22$; $P < 0.001$) in the OB, with a higher level observed in the Wisket group. The post hoc comparison demonstrated significant

changes between the control and Wisket group for the water, CZP, or MTF_CZP treatments, with significantly lower B_{max} values disclosed in the MTF- compared with CZP-treated Wisket group (Fig. 6c). Concerning the diencephalon, treatment had significant effects ($F_{(3,15)} = 5.20$; $P < 0.05$), i.e. significantly higher binding capacity was observed in the MTF_CZP-treated Wistar group than water- or MTF-drinking groups. In the brainstem, no significant effects were detected. None of the treatments had a significant effect on the K_d values (data not shown).

The factorial ANOVA of relative mRNA expression values exhibited significant effects of the group in the CTX ($F_{(1,68)} = 22.04$; $P < 0.001$) and cerebellum ($F_{(1,66)} = 5.05$; $P < 0.05$), with a lower level observed in the Wisket rats (Fig. 6d). The post hoc analysis revealed that the D_1R mRNA expression was significantly lower in the cerebellum of the MTF-treated Wisket rats compared with their matched controls. According to an unpaired *t*-test, the MTF administration increased the D_1R mRNA expression in the striatum in both Wistar and Wisket rats compared with their water-drinking counterparts. In the PFC and hippocampus, no significant effects were revealed.

4.2 Results of Series II.

4.2.1 Sleep-wake rhythm

Factorial ANOVA displayed that the time spent in the different stages (awake, NREM, and REM) during the entire investigated period was significantly affected by the stage ($F_{(2,42)} = 282.62$; $P < 0.001$; Table 3). Thus, both Wistar and Wisket rats spent significantly less time in the REM stage compared to the awake or NREM stages. The separate analysis of the active and quiet substages demonstrated significant effects of substage ($F_{(1,28)} = 29.82$; $P < 0.001$), therefore, both groups of the rats spent a shorter time in quiet awake than in active awake substage (Table 3). With regards to the light: dark (diurnal) rhythm (phases), the ANOVA revealed that stage ($F_{(2,42)} = 268.94$; $P < 0.001$) and phase and stage interaction ($F_{(2,42)} = 81.49$; $P < 0.001$) had significant effects, therefore, the rats in both groups spent a longer time in awake and shorter in NREM and REM stages of sleep during the dark phase compared to the light condition without significant differences between the two groups (Table 3). In addition, the separate analysis of the diurnal rhythm of the active and quiet substages was significantly affected by the substage ($F_{(1,28)} = 29.92$; $P < 0.001$), phase ($F_{(1,28)} = 48.87$; $P < 0.001$), and substage and phase interaction ($F_{(1,28)} = 13.05$; $P < 0.005$). Therefore, both groups of rats spent more time in both types of awake substages during the dark compared to the light phase (Table 3).

Table 3 *The mean per hour ± SEM duration of different stages and substages, the number and length of episodes in control and Wisket animals for the whole period, and by light and dark phases. Bolded values: significant differences between NREM and awake stages. Italicized data: significant changes compared to the light phase. Underlined values: significant changes compared to active substages*

STAGES/substages		**Group**	**Duration (min)**		**No. of episodes**		**Length of episodes (s)**		**Total power**	
AWAKE		Control	26.5±0.89		25.9±1.54		89.7±11.17		0.021±0.0017	
		Wisket	25.4±1.01		23.9±1.35		107.7±22.72		0.025±0.0019	
	active	Control	17.8±1.76						0.022±0.0018	
		Wisket	17.3±1.96						0.025±0.0020	
	quiet	Control	8.7±1.46						0.019±0.0017	
		Wisket	8.1±1.47						0.023±0.0017	
NREM		Control	26.4±1.46		25.5±1.65		64.0±5.46		0.041±0.0020	
		Wisket	28.2±0.73		23.3±1.42		73.9±3.58		0.044±0.0043	
REM		Control	**7.2±0.94**		20.2±3.77		21.8±1.49		0.021±0.0018	
		Wisket	**6.4±0.46**		17.2±1.86		22.5±2.57		0.026±0.0024	
			Light	***Dark***	***Light***	***Dark***	***Light***	***Dark***	***Light***	***Dark***
AWAKE		Control	20.2±0.94	*34.8±2.04*	27.5±1.56	***23.7±1.90***	60.5±14.02	128.7±16.08	0.021±0.0017	0.021±0.0017
		Wisket	17.1±1.90	*36.6±2.57*	25.6±1.43	***21.5±1.57***	49.2±10.00	185.7±22.85	0.024±0.0021	0.025±0.0018
	active	Control	12.9±1.58	*24.3±2.61*					0.022±0.0019	0.021±0.0017
		Wisket	11.1±1.54	*25.6±3.11*					0.025±0.0022	0.026±0.0019
	quiet	Control	7.3±1.08	*10.5±2.12*					0.019±0.0014	0.019±0.0015
		Wisket	5.9±1.00	*11.0±3.10*					0.023±0.0017	0.023±0.0018
NREM		Control	31.1±1.20	*20.1±2.09*	28.4±2.09	*21.7±1.38*	69.4±5.31	56.7±6.05	0.040±0.0021	0.042±0.0022
		Wisket	34.4±1.29	*19.9±1.94*	26.2±1.17	*19.5±1.92*	81.7±4.85	63.4±4.34	0.044±0.0044	0.046±0.0043
REM		Control	8.7±1.29	*5.1±0.75*	22.7±4.41	*16.8±3.05*	24.6±2.12	**18.1±1.74**	0.021±0.0020	0.021±0.0017
		Wisket	8.5±0.76	*3.5±0.72*	21.0±2.02	*12.0±2.06*	26.2±3.56	**16.9±1.51**	0.027±0.0025	0.025±0.0022

For the entire investigation period, there was no significant difference in the number of episodes or their length between the two groups (Table 3). The results of the ANOVA revealed that the stage ($F_{(2,42)} = 5.81$; $P < 0.01$) and phase ($F_{(1,42)} = 91.71$; $P < 0.001$) had significant effects concerning the diurnal rhythm in the number of episodes in awake, NREM, and REM stages. Thus, both groups of rats exhibited fewer numbers in bouts of all stages during the dark phase, with a moderate tendency of reduced numbers in Wisket rats (Table 3). The examination of the mean length of the episodes also demonstrated significant effects of the stage ($F_{(2,42)} = 24.75$; $P < 0.001$), phase ($F_{(1,42)} = 7.18$; $P < 0.05$), and the stage and phase interaction ($F_{(2,42)} = 15.01$; $P < 0.001$). Therefore, both groups of rats exhibited shorter REM sleep episodes than awake and NREM stages, and longer awake bouts during the dark phase (Table 3). Concerning, similar changes were observed in the non-matched animals as well (data are not shown).

4.2.2 Oscillatory pattern

Regarding the oscillatory pattern analysis of the EEG, no diurnal differences were in the oscillations, therefore, power spectra analysis was carried out for the entire session irrespectively for the stages, for the three sleep-wake stages (REM, NREM, and awake) and for the active and quiet awake substages, separately. To exhibit the fine-grained distribution of the relative power, ANOVA was used for all the parameters within the various frequency bands (delta, theta, alpha, beta, and gamma). Concerning the examination of total absolute power, the Wisket rats showed only a tendency to increase power by stages, substages, and light-dark phases (Table 3).

Factorial ANOVA demonstrated that the whole relative power, independently from the stages in the delta band (0.5−4 Hz), was significantly affected by frequency ($F_{(28,406)} = 157.82$; $P < 0.001$) and group and frequency interaction ($F_{(28,406)} = 1.52$; $P < 0.05$). The post hoc comparison revealed that the Wisket rats had significantly higher total relative power than control rats in the range of 1.38−1.75 Hz (Fig. 7a).

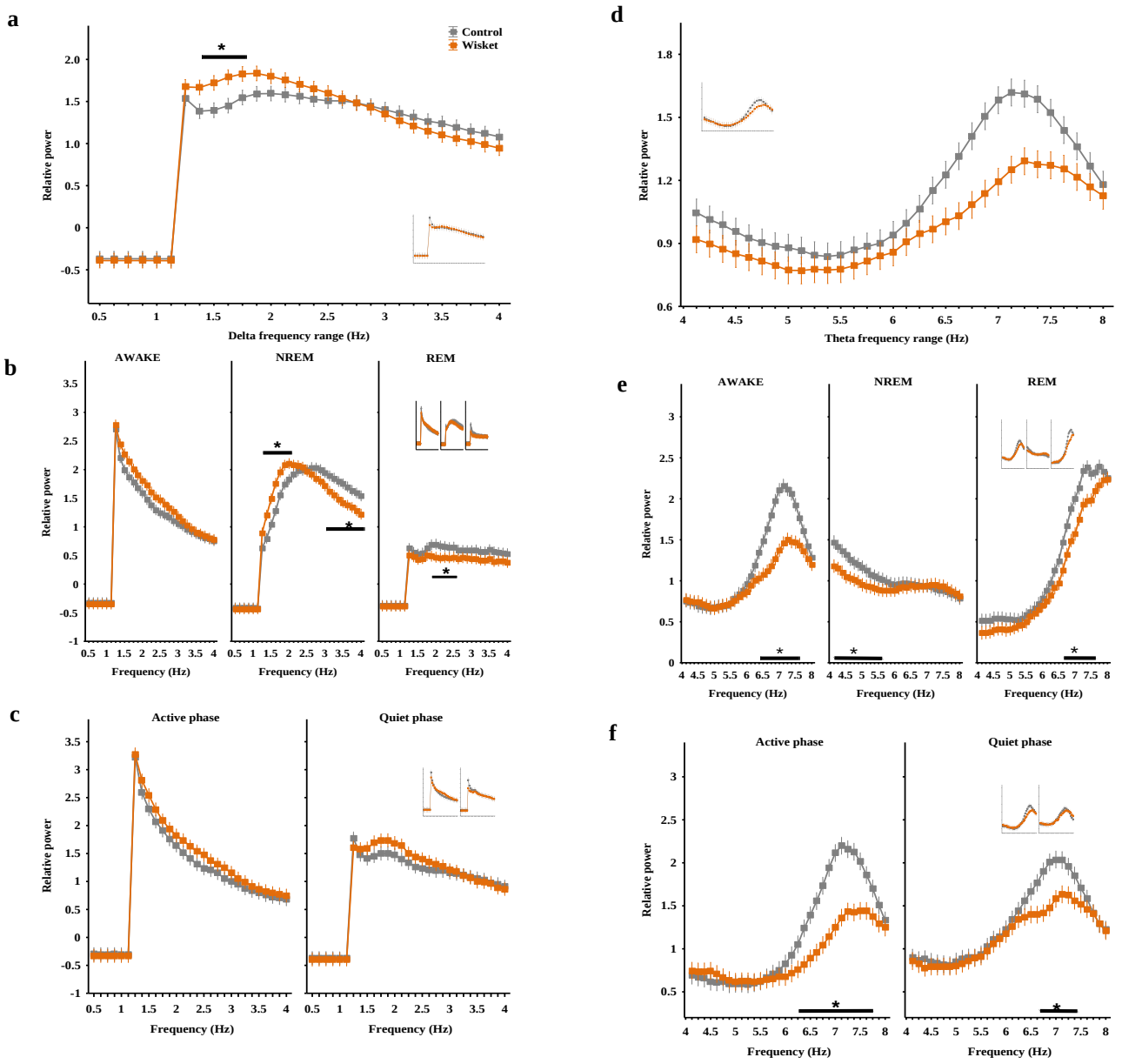

Figure 7 *Relative EEG power differences between the two groups at the delta and theta frequency bands.* ***(a)*** *Total relative delta power (0.5–4 Hz).* ***(b)*** *Delta power in different stages.* ***(c)*** *Delta power in active and quiet awake substages.* ***(d)*** *Total relative theta power (4–8 Hz).* ***(e)*** *Theta power in different stages.* ***(f)*** *Theta power in active and quiet awake substages. Curves inserted in reduced size show the results obtained from unmatched control and Wisket animals. The symbol * shows significant ($P < 0.05$) differences between the two groups.*

Concerning the analysis of the delta power by stages it showed significant effects of frequency ($F_{(28,1218)} = 337.79$; $P < 0.001$), stage ($F_{(2,1218)} = 1638.03$; $P < 0.001$), group and frequency interaction ($F_{(28,4065)} = 2.41$; $P < 0.001$), group and stage interaction ($F_{(2,1218)} = 25.60$; $P < 0.001$), frequency and stage interaction ($F_{(56,1218)} = 46.25$; $P < 0.001$), and group, frequency, and stage interaction ($F_{(56,1218)} = 1.48$; $P < 0.05$). The post hoc test revealed that the delta power was the lowest during the REM stage in both animal groups, with significantly lower in its middle range

(1.88−2.5 Hz) in the Wisket animals in comparison to the control animals. Additionally, the pattern of the delta spectrum showed differences in the groups regarding the NREM stage, i.e., the Wisket rats showed higher power at the lower frequency range (1.25−2 Hz), whereas they exhibited significantly lower power at the higher frequency range (3−4 Hz) compared to control rats (Fig. 7b). The separate analysis by substages demonstrated that the delta band was significantly affected by the group ($F_{(1,812)} = 16.75$; $P < 0.001$), frequency ($F_{(28,812)} = 195.11$; $P < 0.001$), substage ($F_{(1,812)} = 40.67$; $P < 0.001$), and frequency and substage interaction ($F_{(28,812)} = 13.11$; $P < 0.001$). Therefore, the Wisket rats showed a tendency for higher delta power in both awake substages (Fig. 7c).

Factorial ANOVA displayed that the total relative power independently of the stages in the theta band (4–8 Hz) was significantly affected by group ($F_{(1,448)} = 10.58$; $P < 0.001$) and frequency ($F_{(31,448)} = 25.05$; $P < 0.001$). The post hoc comparison revealed that the Wisket rats had significantly lower power than control ones at the higher frequency theta band (6.38−7.62 Hz interval; Fig. 7d). Regarding the theta power by stages, it was significantly affected by group ($F_{(1,1344)} = 206.48$; $P < 0.001$), frequency ($F_{(31,1344)} = 113.34$; $P < 0.001$), stage ($F_{(2,1344)} = 51.38$; $P < 0.001$), group and frequency interaction ($F_{(31,1344)} = 2.64$; $P < 0.001$), group and stage interaction ($F_{(2,1344)} = 10.92$; $P < 0.001$), frequency and stage interaction ($F_{(62,1344)} = 55.54$; $P < 0.001$) and group, frequency, and stage interaction ($F_{(62,1344)} = 1.48$; $P < 0.001$). Therefore, there were differences in the pattern of the theta spectrum between the stages, even a decrease in power as the frequency enhanced during NREM sleeping (Fig. 7e). The theta power at the lower frequency band (4.12−5.62 Hz) was also significantly lower during NREM sleep, whereas the higher frequency bands (6.38−7.62 Hz and 6.5−7.5 Hz, respectively) were lower in the Wisket rats in comparison to the control rats throughout awake and REM stages.

The separate analysis by substages disclosed that the theta band was significantly affected by group ($F_{(1,896)} = 123.00$; $P < 0.001$), frequency ($F_{(31,896)} = 63.92$; $P < 0.001$), substage ($F_{(1,896)} = 120.98$; $P < 0.001$), group and frequency interaction ($F_{(31,896)} = 5.71$; $P < 0.001$), group and substage interaction ($F_{(1,896)} = 9.44$; $P < 0.005$) and frequency and substage interaction ($F_{(31,896)} = 3.64$; $P < 0.001$). Therefore, the Wisket rats exhibited significantly reduced power (6.25−7.75 and 6.62−7.38 Hz, respectively, Fig. 7f) during both active and quiet substages.

Factorial ANOVA demonstrated that the total relative power in the alpha band (8−12 Hz) was significantly affected by group ($F_{(1,448)} = 52.00$; $P < 0.001$) and frequency ($F_{(31,448)} = 170.77$; $P < 0.001$). The Wisket rats showed significantly higher relative power than control rats at the lower interval of the alpha band (8.5−9.38 Hz range; Fig. 8a).

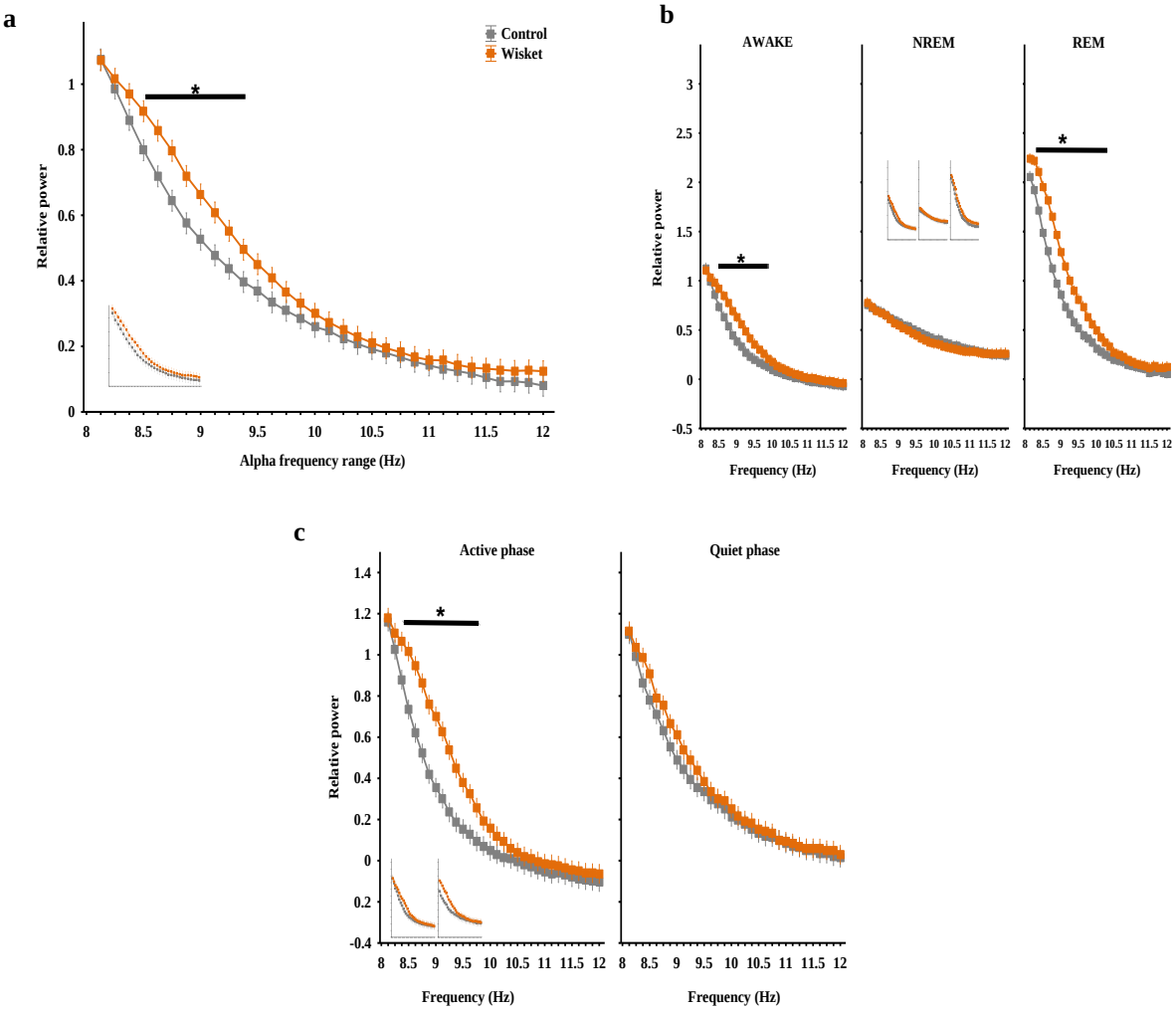

Figure 8 *Relative EEG power differences between the two groups at the alpha frequency band.* ***(a)*** *Total relative alpha power (8–12 Hz).* ***(b)*** *Alpha power in different stages panel.* ***(c)*** *Alpha power in active and quiet awake substages. Curves inserted in reduced size show the results obtained from unmatched control and Wisket animals. The symbol * shows significant (P < 0.05) differences between the two groups.*

The alpha power analyzed at stage level, showed significant effects of group ($F_{(1,1344)} = 167.26$; $P < 0.001$), frequency ($F_{(31,1344)} = 354.09$; $P < 0.001$), stage ($F_{(2,1344)} = 980.07$; $P < 0.001$), group and frequency interaction ($F_{(31,1344)} = 3.94$; $P < 0.001$), group and stage interaction ($F_{(2,1344)} = 81.04$; $P < 0.001$), frequency and stage interaction ($F_{(62,1344)} = 48.09$; $P < 0.001$) and

group, frequency, and stage interaction ($F_{(62,1344)} = 1.63$; $P < 0.005$). There were differences in the pattern of the alpha spectrum between the stages with the lowest steepness of the curve during NREM sleeping, and the Wisket rats showed higher relative alpha power in awake and REM stages in the lower frequency bands (8.38−9.75 Hz and 8.25−10 Hz, respectively) in comparison to the control rats (Fig. 8b).

The separate analysis disclosed that the alpha band by substages were significantly affected by group ($F_{(1,896)} = 126.58$; $P < 0.001$), frequency ($F_{(31,896)} = 230.56$; $P < 0.001$), substage ($F_{(1,896)} = 92.76$; $P < 0.001$), group and frequency interaction ($F_{(31,896)} = 2.90$; $P < 0.001$), group and substage interaction ($F_{(1,896)} = 30.94$; $P < 0.001$), and frequency and substage interaction ($F_{(31,896)} = 2.02$; $P < 0.001$). According to the post hoc analysis, the Wisket rats exhibited significantly higher relative power at the lower frequency alpha band (8.38−9.75 Hz) than the control ones during the active awake substage (Fig. 8c).

Factorial ANOVA revealed that the relative power during the entire investigated period in the beta band (12–30 Hz) was significantly affected by group ($F_{(1,2016)} = 344.18$; $P < 0.001$), frequency ($F_{(143,2016)} = 235.91$; $P < 0.001$), and group and frequency interaction ($F_{(143,2016)} = 4.18$; $P < 0.001$). Thus, the Wisket rats showed significantly higher total relative power than control animals at the lower frequency beta band (12.12−18.38 Hz range; Fig. 9a).

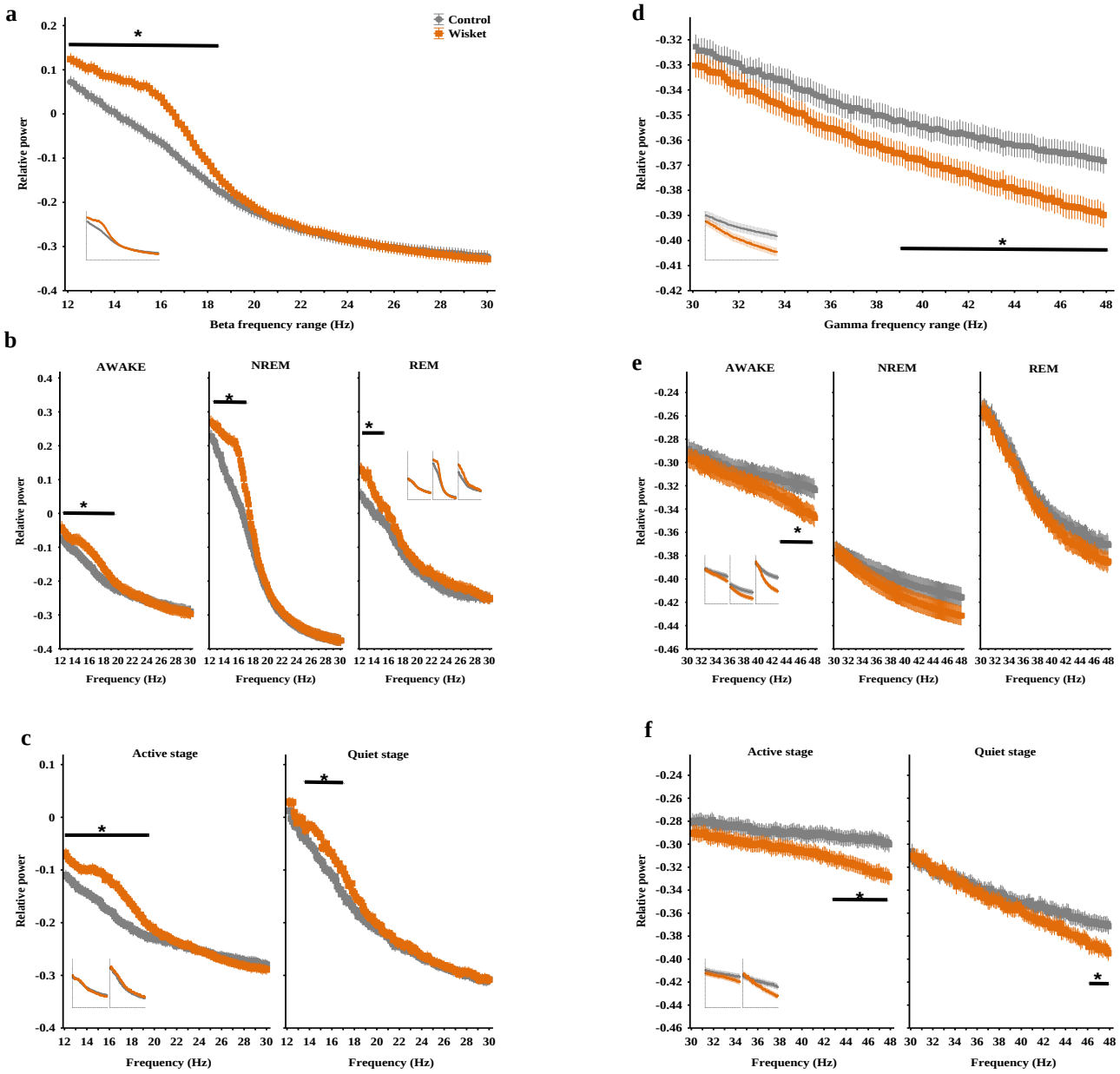

Figure 9 *Relative EEG power differences between the two groups at the beta and gamma frequency bands.* ***(a)*** *Total relative beta power (12–30 Hz).* ***(b)*** *Beta power in different stages.* ***(c)*** *Beta power in active and quiet awake substages.* ***(d)*** *Total relative gamma power (30–48 Hz).* ***(e)*** *Gamma power in different stages.* ***(f)*** *Gamma power in active and quiet awake substages. Curves inserted in reduced size show the results obtained from unmatched control and Wisket animals. The symbol * shows significant ($P < 0.05$) differences between the two groups.*

Regarding the beta power by stages revealed significant effects of group ($F_{(1,6048)} = 625.89$; $P < 0.001$), frequency ($F_{(143,6048)} = 390.36$; $P < 0.001$), stage ($F_{(2,6048)} = 1415.83$; $P < 0.001$), group and frequency interaction ($F_{(143,6048)} = 4.24$; $P < 0.001$), group and stage interaction

($F_{(2,6048)} = 35.80$; $P < 0.001$), and frequency and stage interaction ($F_{(286,6048)} = 44.05$; $P < 0.001$). Additionally, the pattern of the beta spectrum showed differences between the stages with the highest steepness of the curve during NREM sleeping, and the Wisket rats exhibited higher relative power in all stages at the lower beta frequency band (awake: 12.12−19.38 Hz, NREM: 12.88 − 17.5 Hz, REM: 12.12−15.75 Hz; Fig. 9b). The separate analysis by substages demonstrated that the beta band was significantly affected by group ($F_{(1,4032)} = 541.50$; $P < 0.001$), frequency ($F_{(143,4032)} = 312.70$; $P < 0.001$), substage ($F_{(1,4032)} = 649.00$; $P < 0.001$), group and frequency interaction ($F_{(143,4032)} = 5.00$; $P < 0.001$), and frequency and substage interaction ($F_{(143,4032)} = 20.90$; $P < 0.001$). Therefore, the Wisket rats showed significantly higher relative power at the lower frequency beta band than the control ones primarily throughout the active awake substage (active: 12.12−19.38 Hz, quiet: 12.38−12.5 Hz; Fig. 9c).

Factorial ANOVA exhibited that the relative power during the whole investigated period in the gamma band (30−48 Hz) was significantly affected by group ($F_{(1,2002)} = 588.00$; $P < 0.001$) and frequency ($F_{(142,2002)} = 20.00$; $P < 0.001$). According to the post hoc analysis, the Wisket rats showed significantly lower whole relative power than the control animals at the higher gamma frequency band (39 −48 Hz range; Fig. 9d). As regards the data analyses, gamma power by stages disclosed significant effects of group ($F_{(1,6006)} = 332.00$; $P < 0.001$), frequency ($F_{(142,6006)} = 39.00$; $P < 0.001$), stage ($F_{(2,6006)} = 9538.00$; $P < 0.0019$, and frequency and stage interaction ($F_{(284,6006)} = 7.00$; $P < 0.001$). The post hoc comparison demonstrated that there were differences in the pattern of the gamma spectrum between the stages with the lowest steepness of curve values during NREM, and the Wisket rats showed lower relative gamma power, mainly during the awake stage at the higher frequency interval (44.25−48 Hz; Fig. 9e).

The separate analysis of the gamma band by substages disclosed significant effects of group ($F_{(1,4004)} = 478.00$; $P < 0.001$), frequency ($F_{(142,4004)} = 17.00$; $P < 0.001$), substage ($F_{(1,4004)} = 8230.00$; $P < 0.001$), group and substage interaction ($F_{(1,4004)} = 54.00$; $P < 0.001$), and frequency and substage interaction ($F_{(142,4004)} = 3.00$; $P < 0.001$). Therefore, the Wisket rats showed significantly lower relative power at the higher frequency gamma band than control ones, mainly during the active awake substage (active: 42.62−48 Hz, quiet: 46.16−48 Hz; Fig. 9f).

As regards the results of the unmatched series (the curves were added to the figures in a smaller size, Fig. 7–9), similar alterations were detected as in the matched Wisket group.

5 DISCUSSION

To replicate the symptoms of a complex psychiatric disorder such as schizophrenia is very challenging, but there are few preclinical models available that combine both genetic and environmental factors. Our previous findings demonstrated several altered functions as was mentioned in the introduction section. The presented data of Series I. of the experiments revealed that antipsychotic treatment (CZP) alone induced further behavioral abnormalities, but in combination with MTF was able to improve these parameters. Other alterations were observed regarding the DA-ergic system, which was manifested in the reduced level of D_1R binding, signaling, and gene expression in the CTX of Wisket rats. In Series II. Wisket rats exhibited only a tendency of longer NREM sleep duration and shorter REM sleep phases. Complex abnormalities of the EEG oscillations were also revealed: the lower frequency ranges of the delta, alpha, and beta waves, and decreased power in the higher frequency bands of theta and gamma frequencies in most stages without changes in the circadian rhythm of our multiple-hit schizophrenia rat model. In summary, our Wisket rats generated by selective breeding can satisfy constructive and face validities.

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

Treating cognitive abnormalities of schizophrenia remains an unresolved issue [244–246]. The conventional treatment contains antipsychotic applications, like CZP [152], however, these drugs may cause metabolic syndromes and/or cognitive decline [148,247]. Some evidence suggested that adjuvant therapy, including MTF, might have procognitive effects, furthermore, the combination with atypical antipsychotics might have beneficial effects on cognitive impairments [149,150].

Our data revealed that the behavioral profile of the control animals was not significantly affected by the different drugs and combined treatments. In contrast, CZP significantly reduced the exploratory activity of the Wisket animals, indicating attenuated motivation. Along with many other psychoactive medications, CZP also has sedative effects that may contribute to some of the cognitive deficiencies, thus, these effects may be the explanation for the reduction of CZP in activity and operant response performance for food reward [149,150].

MTF had no significant influence on cognitive and exploratory parameters by itself, but it reduced the behavioral side effects of CZP. Only a few preclinical studies observed the MTF's effects on

schizophrenia-like symptoms [248,249] Wang et al. observed that a single MTF administration ameliorated the MK-801-induced PPI deficit, hyperlocomotion, recognition and spatial memory impairments, and anxiety-like symptoms in rats [248]. Contrary to these findings, MTF therapy alone had no significant impact on the cognitive functions of Wisket rats. This discrepancy may be explained by the different schizophrenia models and/or the behavioral assessments (reward- vs. punishment-based testing). In another study, MK-801 and mild stress exposure were applied for developing schizophrenia and depression-like symptoms in mice [249]. Antidepressant, and antipsychotic medications, their combination, and MTF application were studied for their effects on different behavioral tests [249]. The results revealed that combined treatment (two antidepressants) and CZP enhanced the MK-801-induced impaired behavior; furthermore, the schizophrenia-like and depressive behaviors were significantly ameliorated by the triple-drug treatment completed with MTF [249]. However, the cognitive abilities of these animals were not examined in this study, and the combination of the four drugs has prevented the identification of the CZP and MTF interaction. Our findings demonstrated that MTF co-administration significantly reduced the cognitive deficit generated by CZP in Wisket animals.

5.2 D_1R activation, binding, and gene expression in different brain structures

D_1Rs play an important role in cognitive functions, i.e. poor D_1R performance has been associated with impaired working memory [106,250,251]. The D_1Rs are highly presented in various brain regions, including the striatum, CTX, brainstem, OB, cerebellum, diencephalon, and hippocampus [105,252–258]. In accordance with these data, the D_1Rs were present in all of the examined areas, the CTX showing the highest levels of receptor density (B_{max}), and G-protein activation (E_{max}). Neuroimaging studies have shown contradictory results about the density and/or activity of the D_1Rs in schizophrenia [117,118,252,259]. In a recent study, D_1R upregulation was demonstrated in the striatum and CTX without any changes in the thalamus, temporal cortex, and hippocampus [259]. On the other hand, 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 [117,118,252]. CZP affects various neurotransmitter systems, which are involved in attention, motivation, and/or sedation, thus, its disturbing impacts may arise from the combined effects on the different neurotransmitters resulting in decreased spontaneous activity, reduced reward functions, and cognitive abilities [106,260]. Few studies have revealed a link between MTF and the dopaminergic

system; MTF prevented the nigrostriatal DA degradation and reduced the onset of dyskinesia in models of Parkinson's disease, but it had no effect on the downstream mediators of D_1R hyperactivation in the striatum [224,225].

The present data revealed that the CTX had obvious alterations in Wisket rats. They exhibited significantly decreased gene expression, binding capacity, and G-protein activation, indicating impairments in the CTX D_1R function in this schizophrenia model. 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 had significant effects on these parameters [253]. The E_{max} and B_{max} values were significantly improved by the combination therapy in this region of the brain, which may have contributed to the treatment's positive impacts on the behavior of Wisket rats. Considering region specificity of mRNA expression of D_1Rs, 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 [250,261]. 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 [262]. 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 [263]. 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 this region.

Both GABA and DA-containing neurons are abundant in the OB, and D_1Rs are found there as well, this area is also important in cognitive functions, which may be contributed, at least in part, by D_1Rs [253,264,265]. Wisket animals had higher levels of D_1R binding and G-protein-mediated transmembrane 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 [255,266]. In the brainstem, no alterations were found, whereas the diencephalon showed a higher ligand binding potency in Wisket rats (lower pEC_{50} 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, regarding the D_1R mRNA expression in the striatum and hippocampus, no significant alterations were demonstrated between the two groups [252,254,261]. Furthermore, chronic CZP treatment did not change this parameter in rodents [252,254,261]. 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 the Wisket and control groups. The cerebellum has an important role in motor coordination and cognition too, changes in cerebellar function have been linked to schizophrenia [267,268]. Thus, the reduced D_1R mRNA expression of Wisket rats might also contribute to their behavioral deficiencies.

In conclusion, in vitro data showed that the D_1R system was impaired in Wisket rats CZP treatment alone caused only moderate effects in D_1R function in both groups. In contrast, combination treatment significantly affected various brain structures regarding signaling and binding processes. Thus, the combined therapy significantly increased the maximal G-protein activation and maximal D_1R binding in the CTX in both groups. It cannot be ruled out that the improvement in D_1R function shown in the Wisket rats was associated with the positive effects of the drug combination. The two medications can affect numerous systems involved in cognitive functioning, as was mentioned above. As a result, the CZP and MTF interaction between the various transmitter systems may have contributed to the positive effects of the combination seen in the Wisket animals.

5.3 Sleep-wake rhythm

A normal wake-sleep cycle is an essential physiological mechanism supporting the maintenance of physical, mental, and emotional health. Disruptive sleeping as an important sign of schizophrenia, is accompanied by cognitive and affective symptoms of schizophrenia [26,28,269,270]. However, it is challenging to directly compare clinical and preclinical results, in part because rats, unlike humans, sleep in polyphasics [271]. The Wisket animals displayed almost normal diurnal fluctuation in their sleep-wake pattern. Different schizophrenia models also (including DISC1

gene mutant mice and rats with prenatal intervention or neonatal hippocampal lesion) demonstrated normal diurnal rhythm similar to Wisket rats [272–276]. 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. Some preclinical data are opposite to these results i.e., stable tubule only polypeptide (STOP) mutant mice and methylazoxymethanol acetate exposure on embryonic day 17 mutant rats showed that sleeping duration was reduced, sleep-wake periods of the animals was fragmented, and they exhibited reduced NREM and increased awake time [275,277]. In mGLUR5 (metabotropic glutamate receptor 5) mutant mice, studies revealed a similarly reduced frequency of REM sleep episodes along with increased NREM length [278,279].

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 [280]. Therefore, the poor cognitive functions observed in Wisket animals may be related to the less consecutive transitions of NREM-REM sleep cycles [213,214]. In contrast to prior telemetry studies, which showed that the motor activity of Wisket rats was reduced and had a fragmented pattern, the present study revealed unchanged fragmentation of the episodes [217]. Telemetry is suitable for tracking large motor activity but it cannot distinguish between different activity stages or disclose tiny movements [217]. Additionally, the micro-arousals (shorter than 8 s long fragments of the awake stage) were excluded from the EEG analysis, which may have changed the outcomes [281]. Overall, the wake-sleep phenotype of Wisket rats imitates a modest impairment seen in some of the schizophrenia patients [282].

5.4 Oscillatory pattern

The delta wave arises from the reticular nucleus of the thalamus, controlling relay neuron activity, which has projections to the PFC by tonically activating GABA-ergic neurons throughout the NMDARs [283]. Delta oscillation plays a role in cognitive functions including memory consolidation, and delta waves are related to the restorative benefits of sleep on cognition during NREM sleep [284–286]. In the present study, 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. In agreement with human studies, enhanced low-frequency oscillations were noted in untreated, first-episode, and chronic schizophrenia patients [42,46,48,52,58,287]. Our results may be derived from the increased sleep requirements in schizophrenia [285]. Consistent findings with Wisket rats, the neonatal hippocampal lesion schizophrenia model exhibited a trend of increased delta power during awake and NREM sleeping [272]. Similarly, in STOP mutant mice, the relative power was likewise elevated during NREM sleep [275]. Furthermore, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor mutant mice also exhibited higher power in a lower frequency range (0.75–1.5 Hz) of delta wave [279]. In contrast, mGLUR5 receptor mutant mice showed decreased power in the higher frequency range of the delta band during NREM sleeping [278].

Theta rhythm originated from cortico-hippocampal circuits and may be responsible for cognition and memory formation [61,288,289]. In awake rats, theta waves primarily evolve during motor activity or when the rats are motionless, but alert [288]. Regarding sleeping, theta oscillation is dominant during the REM phase in rodents, as it was revealed in both groups of rats, which suggests activation of the hippocampus [290]. Because theta power is generated by GABA neurons in the medial septum that connects to the hippocampus, its reduction during REM sleep is associated with deficits in memory consolidation [290]. In our animal model, 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. Preclinical data showed that optogenically inhibited REM theta power disrupts sleep-dependent memory consolidation [290]. Therefore, the previously observed memory deficits in Wisket animals may be explained by the reduced theta power during REM [213,214]. Conflicting results are available regarding human and rodent models. Based on the leading symptoms, the theta oscillatory pattern remains unchanged or higher in patients with schizophrenia [49,52–56]. In neonatal hippocampal lesioned rats increased theta power was shown during awake and NREM [272]. 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 [275,279,291].

Alpha-band oscillation is largely associated with the thalamus, therefore, changes in this frequency band may indicate impairment with the inhibitory input of thalamic neurons [52,292]. 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 [48,52,53]. 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 [272]. In contrast, AMPA or STOP mutant mice had reduced alpha power in REM sleeping [275,279].

The generation of beta waves occurs in several different neocortical regions, each of which has a particular purpose, such as regulating alertness and attention [57,59]. 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 [52,58]. These alterations may be involved in the disturbed cognitive functions previously shown in Wisket rats [243,293]. Regarding the preclinical data, STOP, AMPA, and voltage-gated calcium channel mutant mice exhibited decreased beta power in NREM and/or REM sleeping [275,279,294]. AMPA knock-out animals showed enhanced beta power in awake phase, but calcium channel knock-out animals demonstrated decreased power [279,294]. However, there were no alterations in this frequency range following the hippocampus lesion [272].

Gamma waves are involved in various cognitive abilities, and alert or attentive wakefulness [59,295]. In schizophrenia, impaired gamma-band oscillations are linked to positive, negative, and cognitive symptoms [57,59,296]. 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 [293,297]. Similar results were observed in patients with schizophrenia and it may be associated with negative symptoms [45,59,63–65]. 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 [294,298–300], but mGLUR5 mutant mice showed enhanced gamma activity during sleep [301].

In conclusion, 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 [282]. Data imply that schizophrenia is characterized by inadequate inhibitory modulation of sensory processing and the oscillatory pattern may be influenced by the strength and kinetics of synaptic connections (excitatory or inhibitory) [59,61,302].

Patients have altered oscillatory activity in the various EEG frequency bands, which may be responsible for their irregular sensory and cognitive function [41–49,51,59]. Conflicting results are available and they might be dependent on the subtypes or stages of schizophrenia [43,59,67,303,304]. Likewise in previous studies, the oscillation patterns were not influenced by the dark or light phases in none of the groups [275,301,305]. However, numerous differences were observed regarding the EEG power spectra during the three stages in both groups, at different frequency bands, which data were partially in agreement with other preclinical schizophrenia models [272,275,279,291,294,298–300]. There might be various underlying causes of oscillations in various frequency ranges, each of which might serve a unique purpose. Therefore, complicated abnormalities in the Wisket animals may be indicated by the variations in cortical oscillations.

5.5 Limitations

There are some limitations of the presented experiments. In series I., compared to our previous studies, ketamine treatment was performed over a shorter period of time, because of the side effects (e.g., diarrhea) caused by the extended treatment time (5 vs. 15 days) [211,213]. The significant differences between the Wistar and Wisket rats in behavioral tests indicate that the model rats with shorter ketamine treatment duration also exhibit a schizophrenia-like phenotype. To reduce the stress of social isolation, the rats were housed in pairs during the treatment as in previous experiments, making it impossible to precisely estimate the food and fluid consumption as well as the drug doses in the individual animals [225,226]. However, the body weight of the animals was not affected by any of the treatments. It appeared that the MTF_CZP combination was not tasty for the animals, since it was observed a tendency their lower fluid intake. This impact was more pronounced in control rats, but the lower fluid intake was not accompanied by any behavioral impairments. The Wisket animals showed a similar tendency, but it was not statistically significant, and the treatment had no effect on either food intake or body weight. Additionally, the post hoc analysis did not show significant differences regarding the fluid intake between the Wisket animals with CZP alone or in combination at any of the examined periods. Despite the reduced fluid consumption found in the combined treatment groups, earlier studies were in agreement with the dosages of the CZP and MTF administration [148,224]. Therefore, even the low dosages of CZP (1–10 mg/kg/day) or MTF (50 mg/kg) affected the cognitive abilities, as a result, the study suggested that the positive behavioral effects of combined treatment arose from MTF

rather than the moderately reduced amount of CZP intake [148,149]. 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 [306]. 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 [307]. In the future, additional investigation is required to understand the glucose metabolism in Wisket rats among this experimental conditions to examine the antipsychotics-generated metabolic syndrome and how an anti-diabetic treatment can ameliorate it.

An investigation of the association between the behavior and the D_1R system would have yielded useful information about the connection between them. The experimental animals could be tested separately during the behavioral experiments. However, 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 [308]. The preparation method used here enabled the reasonably precise estimation of each measurement parameter. This method was chosen for two main reasons: (1) several test samples were required to determine the concentration dependence of the ligand binding parameters across a broad range, and (2) the need for protein content of the cell membrane fractions used in the receptor binding assays to include at least 100 µg in each reaction tube

In series II., our recording technique has many restrictions, including the absence of deep hippocampus electrodes and a constrained number of channels. Additionally, since we employed a tethered option instead of wireless recording, the rats were kept in cages alone to offer the proper conditions for a full recovery following surgery and prevent damage to the EEG equipment and cables. Several data revealed that the extended duration of individual housing (more than a week), particularly in young animals, involves various biological characteristics [309–311]. The animals were socially isolated for 1 week in adulthood but were kept in visual, auditory, and olfactory contact with other rats throughout the entire experimental period in this investigation [240]. 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.

6 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 in Wisket rats plays a role in the development of behavioral symptoms resembling schizophrenia [212,216,219,220,298,312]. 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.

6.1 Clinical relevance

It is a well-known phenomenon that antipsychotic agents have serious, or even fatal adverse effects [108]. Metabolic syndrome in schizophrenic patients is a really common consequence of antipsychotic administration [148]. Consequently, adjuvant therapies may be beneficial in the course of treating schizophrenia [152–154]. MTF is a plant-based medication that is commonly used to treat diabetes, and growing evidence indicates that it may also have positive effects on cognitive processes [149,150]. 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 [23–25,41–51]. Wisket animals exhibited numerous abnormalities in the different oscillatory bands, 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.

7 SUMMARY

The presented studies aimed to characterize our chronic schizophrenia Wisket rat model 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 CTX 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 was 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 triple-hit schizophrenia rat model mimics several schizophrenia-related abnormalities, which may contribute to the validation of this animal model.

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