Development and characterization of chronic animal models of schizophrenia

Ph.D. Thesis

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Contents

Publications

Full papers related to the Thesis

I. Petrovszki Z, Adam G, Kekesi G, Tuboly G, Morvay Z, Nagy E, Benedek G, Horvath G. The effects of juvenile capsaicin desensitization in rats: Behavioral impairments. *PHYSIOLOGY AND BEHAVIOR* 125: *pp. 38-44*. (2014) IF: 3.033

II. Petrovszki Z, Adam G, Tuboly G, Kekesi G, Benedek G, Keri S, Horvath G. Characterization of gene-environment interactions by behavioral profiling of selectively bred rats: The effect of NMDA receptor inhibition and social isolation. *BEHAVIOURAL BRAIN RESEARCH* 240:(1) *pp. 134-145*. (2013) IF: 3.391

Full papers, not involved in the Thesis

Petrovszki Z, Kovacs G, Tomboly C, Benedek G, Horvath G. The Effects of Peptide and Lipid Endocannabinoids on Arthritic Pain at the Spinal Level. *ANESTHESIA AND ANALGESIA* 114:(6) pp. 1346-1352. (2012) IF: 3.3

Kovacs G, Petrovszki Z, Mallareddy JR, Tóth G, Benedek G, Horvath G. Characterization of antinociceptive potency of endomorphin-2 derivatives with unnatural amino acids in rats. *ACTA PHYSIOLOGICA HUNGARICA* 99:(3) pp. 353-363. (2012) IF: 0.882

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Abstracts related to the Thesis

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Petrovszki Z, Kekesi G, Benedek G, Horvath G. Chronic pain threshold changes in a new complex schizophrenia model. (MITT 2013.)

Horvath G, Adam G, Petrovszki Z, Benedek G. Long-lasting effects of social isolation and NMDA-antagonist treatment on thermoregulation and motor activity. *FRONTIERS IN NEUROSCIENCE* Online: *Paper P6.10.* (2011)

Petrovszki Z, Gombkötő P, Nagy A, Benedek G, Tuboly G, Horváth G. Long-lasting change of auditory evoked potentials in a complex animal model of schizophrenia. (MÉT 2011.)

Abbreviations

CNS = central nervous system
DA = dopamine
DI = discrimination index
DISC1 = disrupted in schizophrenia 1
DSM = Diagnostic Statistical Manual
F = familiar object
GABA = γ-aminobutyric acid
GLU = glutamate
KET = ketamine treatment
KO = knockout
LTD = long term depression
LTP = long term potentiation
N = novel object
NAcc = nucleus accumbens
NaNo = naive socialized rats without treatments
NaTr = naive animals with social isolation and ketamine treatment
NMDA = N-methyl-D-aspartate
NOR = novel object recognition test
PA = pulse alone
PolyI:C = polyinosinic-polycytidylic acid
PP = prepulse-pulse pair
PPA = prepulse alone
PPI = prepulse inhibition
PWD = paw-withdrawal test
RV = relative volume
S = sample object
S.C. = subcutaneously
SelNo = selectively bred animals without any treatment
SelTr = selectively bred rats with social isolation and ketamine treatment
SI = social isolation
TF = tail-flick test
TRPV1 = transient receptor potential vanilloid1
VF = von Frey test

1. Introduction

Schizophrenia is a devastating psychiatric disorder that impairs mental and social functions. Although loose descriptions resembling schizophrenia are obtained in texts dating back more than two thousand years, the first comprehensive and scientific definition was described by the late 1800s as *dementia precox* by Emil Kraepelin. In 1911 Eugene Bleuler firstly applied the schizophrenia name, as a mental illness with chronic psychotic symptoms, but not a dementia [1]. After a lot of emerging approaches it became necessary to develop more stringent diagnostic criteria which can be described by two major classification systems, Diagnostic Statistical Manual (DSM I-V) and International Classification of Disease.

1.1 Symptoms of schizophrenia

The disease manifests itself with positive symptoms (hallucinations, delusions and thought disorder), negative symptoms (deficits in social interaction, emotional expression, motivation, speech difficulties and abnormalities) and cognitive dysfunctions (impaired attention, information processing, problem-solving, verbal and visual learning and memory); therefore, schizophrenia is considered as a complex, multifactorial disease. The negative and cognitive symptoms are more persistent and chronic, while the psychotic symptoms follow a certain episodic pattern. Onset of behavioral symptoms appears from adolescence until the age of 40 for most patients, in men usually occurs earlier than in women. The development, course and outcome of the disease has high inter individual variability. Several other, non-specific sings, can also be observed in schizophrenia; such as sensory gating disturbance, motor behavioral changes and decreased pain sensitivity.

Sensory gating, as measured by prepulse inhibition of the acoustic startle reflex (PPI), describes neurological processes of filtering out redundant or unnecessary stimuli in the brain and it prevents an overload of irrelevant information in the higher cortical centers of the brain [2]. Sensory gating, as a non specific sign, is reduced in a variety of neuropsychiatric disorders (e.g. Huntingtons's disease, Tourette's syndrome, autism, bipolar or panic disorder) including schizophrenia [3-5].

Schizophrenic patients frequently manifest abnormalities in both the extent and nature of motor activity [6]. Slowing of motor activity is common in schizophrenia, and it is variably associated with negative and depressive symptom clusters. Excessive motor

activity, often apparently purposeless, is more often associated with exacerbations of positive symptoms.

Various clinical case studies have described that both prevalence and intensity of pain appear to be diminished, for example, appendicitis, fractures and abdominal surgical emergencies seems to be reduced in persons with schizophrenia [7]. Thermal, electrical, cold and tactile stimulation have been utilized to assess pain threshold and pain tolerance in schizophrenia [8-11]. A significant part of these experimental pain studies concluded that persons with schizophrenia appear to be less sensitive to pain compared to healthy controls.

1.2 Epidemiology and etiology

Schizophrenia affects approximately 1% of the population worldwide [12, 13]. Despite the fact that more than one hundred thousand schizophrenia-related publications can be found in Pubmed, exact etiology and pathomechanism of schizophrenia is not known until now. Many studies revealed relationship between the degree of urbanicity and risk of schizophrenia, strongly supporting the proposition that some factors associated with urbanicity may be causally related to schizophrenia [1]. Such effect may be: urban-rural differences in rates of cannabis and other substance use, degree of social stress and social connectedness, poverty, environmental toxins or vitamin D deficiency.

The gender differences in the clinical expression and outcome of schizophrenia have long been recognized [1], and meta-analyses also revealed that males have a higher lifetime risk of developing schizophrenia with a male-female relative risk of about 1.4 [1].

There are also some other factors which are present in the unfolding of schizophrenia. It has long been known that patients, who subsequently received a diagnosis of schizophrenia, are often born in winter. One possible explanation might be that the second trimester coincides with the flu seasons, which may increase the risk of schizophrenia. Several studies have revealed that those children, whose mother suffered from infections (flu, toxoplasma, rubella) during pregnancy, are more likely to develop schizophrenia [1]. Older paternal age at conception has been linked to an approximate doubling of the risk for developing schizophrenia, impaired spermatogenesis leading to an increased likelihood of *de novo* mutation and aberrant epigenetic regulation has been advanced as explanations.

Genetics play a major role in the etiology of schizophrenia, however, none of the identified risk genes are specific to schizophrenia, but rather indicate a general vulnerability to mental health disorders [14]. The chance of developing schizophrenia in a person is about 9-16% if one parent is affected and 40-68% if both are ill. Siblings and dizygotic twins share 50% of their genetic material, and if either of them has schizophrenia, then the risk of the other developing the illness is 10–15% [1]. However, monozygotic twins share 100% of their genetic material, and if one twin has schizophrenia, the risk of schizophrenia in the other is about 40–50%. In the largest published familial schizophrenia cohort, Toulopoulou et al. demonstrated that a major portion of phenotypic correlation between schizophrenia patients in certain cognitive measures could be explained by shared multiple genes [2].

Several "structural" chromosomal abnormalities have been described in schizophrenia, which might harbor a risk gene or genes for schizophrenia. Genes or gene products identified in the pathogenetic background of schizophrenia include neuroregulin 1 (which play an important role in the development of the nervous system), dysbindin (affecting hippocampal based cognitive processes during juvenile brain development), disrupted in schizophrenia 1 (DISC1, having a role in both neural signaling and development), dopamine receptors D1–D4; catechol-O-methyl-transferase (is critically involved in dopamine (DA) metabolism) and metabotropic glutamate receptors [15, 16].

1.3 Neuroanatomical changes

Several abnormalities in the brain structure were detected in schizophrenia [4, 17, 18]. Enlarged brain ventricles are seen in some schizophrenics, indicating a deficit in the volume of brain tissue. Advances in the *in vivo* magnetic resonance imaging technology have led to the identification of reductions in temporal lobe structures, in particular the hippocampus, amygdala, the superior temporal gyrus, furthermore the prefrontal cortex, the thalamus, anterior cingulate cortex and corpus callosum [17]. Abnormalities in the superior temporal gyrus correlate with positive symptoms, while the gray matter density reductions in medial temporal lobes correlate with memory impairments. Reduced white matter structures such as corpus callosum and other fiber tracts have also been reported in schizophrenia, and these alterations appear to be correlated with cognitive impairments [17].

1.4 Pathophysiological changes

Several neurotransmitter abnormalities such as the dopaminergic, glutamatergic (GLU), GABAergic (γ-aminobutyric acid) deficits play important roles in schizophrenia [19, 20].

The dopamine hypothesis of schizophrenia originates from the 1960s from the observations that psychostimulant drugs (e.g.: amphetamine), which increase neuronal dopamine levels, can lead to psychotic symptoms that are almost indistinguishable from schizophrenia [20]. It has been shown that the subcortical hyper-dopaminergic state is associated with hypo-dopaminergic state of the frontal cortex [21]. This theory suggests that normal, baseline mesolimbic dopamine output yields normal psychiatric functioning and the positive symptoms of schizophrenia are a direct result of too much DA neuronal firing originating in the midbrain and allowing excessive DA release and activity in limbic structures**.** The excess DA might actually be derived from the abnormal GLU neurocircuitry system [20, 21]. Descending glutamate pathway, which starts in the frontal cortex regulates the mesolimbic DA neurons by excitatory ionotropic N-methyl-D-aspartate (NMDA) glutamate receptors. This sequence of neurons consists of several stops (Fig.1.). Normally, a fully functioning primary glutamate neuron (pyramidal neuron) fires upon a smaller GABA interneuron which releases inhibitory GABA onto a secondary GLU pyramidal neuron (which now is the third neuron in this cascade) causing it to lower its firing rate. This low GLU tone at this second GLU neuron is normal. The fourth neuron is dopaminergic in the midbrain and fires at a normal rate. The NMDA receptor hypo-functioning hypothesis is a newer hypothesis, formulated roughly two decades ago, suggests that NMDA receptors attached to the GABA interneurons situated between the primary and secondary GLU cortical neurons are responsible for the symptoms. The loss of GABA activity will cause an abnormal graduation of the secondary GLU neuron‘s firing rate. This excessive GLU tone has massive impact on the DA mesolimbic pathway causing increased DA neuronal activity (the dopamine hypothesis) thereby resulting psychotic symptoms [21].

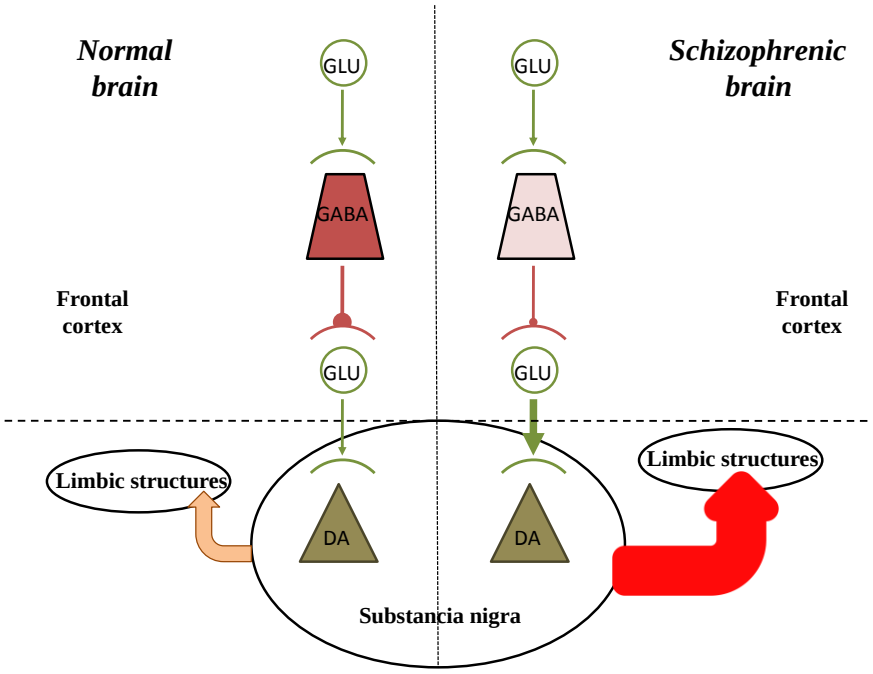


Figure 1. Normal GLU-GABA-GLU-DA neurocircuit loop with non-psychotic state (left side). Abnormal GLU-GABA-GLU-DA neurocircuit loop generates psychotic positive symptoms of schizophrenia (right side).

Different DA pathways play a role in the development of negative and cognitive symptoms, which suggests that normal psychiatric functioning occurs as a result of baseline or normal dopamine output reaching the frontal cortex, but the negative symptoms of schizophrenia are a direct result of too little DA neuronal firing originating in the midbrain and allowing poor DA release and activity of the frontal cortex [17, 21] (Fig.2.). A similar relationship exists in the first three steps with a primary GLU neuron, a GABA interneuron, and a secondary GLU neuron interacting. Next, however, is a synapse further down in the midbrain with yet another GABA interneuron which impinges upon DA neuronal projections that proceed back to the frontal cortex. The dysfunctional NMDA receptors cause lost tone of GABA, so the secondary GLU neuron will be again hyperactive and this neuron causes much higher GABA concentrations, which inhibits dopamine neurons. This type of circuit can become defective due to abnormal GLU NMDA receptors located on GABA interneurons, or any other impingement on the circuit that would result in final downstream of DA common pathway.

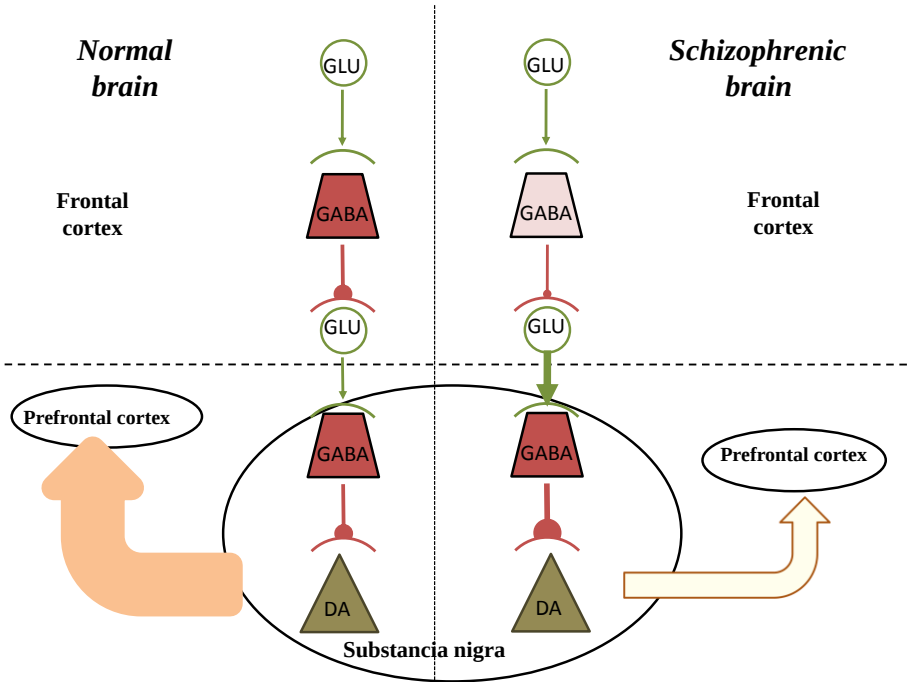


Figure 2. Normal GLU-GABA-GLU-GABA-DA neuronal circuitry (left side). Abnormally GLU-GABA-GLU-GABA-DA neuronal circuitry may cause negative symptoms (right side).

1.5 Animal models

In order to understand the biological mechanisms underlying a complex disorder and in search of novel drug targets, valid animal models are necessary.

Nowadays, there are many animal models of schizophrenia, which to a smaller or greater extent (showing one or several symptoms) reflect the key symptoms observed in human patients suffering from this disease. Rodent models of schizophrenia mostly display symptoms analogous to the negative and cognitive signs. Social withdrawal and disturbed sensory motor gating are identified as the equivalent of negative symptoms [22-26]. Positive symptoms (e.g. hallucinations, delusions) observed in patients diagnosed with schizophrenia are of particular importance; however because of the subjective nature of these symptoms they cannot be convincingly ascertained in animals [27].

Several studies monitored the effects of acute administration of psychotic drugs (single injection), but nowadays it is accepted, that appropriate chronic models should generate animals with schizophrenic phenotype that mimics several aspect of the human psychiatric disorder with a high constructive, face and predictive validity. These models may be appropriate to examine the effects of chronic antipsychotic drug treatments, according to the human practice [28].

There are four main groups of chronic animal models for schizophrenia: pharmacological-, lesion-, environmental- and genetic models. Recently it is supported that single animal models cannot recreate the diversity and complexity of schizophrenia, but the combination of these different procedures may help to produce a more reliable animal model [18, 20].

1.5.1 Environmental models

There are many developmental models, which cause disorders of different brain structures. It seems that underlying process of the disease occurs in the early stages of neurodevelopment and manifests only later, during the developmental restructuring of the central nervous system. Prenatal immune challenge by a number of external factors (such as bacteria, viruses and other pathogens), prenatal protein deprivation, maternal malnutrition or stress during pregnancy related with the increased glucocorticosteroid levels during fetal development result in disturbed development of brain structures, and cause a number of behavioral changes related to schizophrenia, *i.e.* reduced social interaction, impaired novel object recognition and disrupted PPI [29, 30].

The profound neurobiological effects of stress at postnatal life are believed to be the basis of many neuropsychiatric changes too. Since Hatch and colleagues first reported behavioral abnormalities in socially isolated rats [31], a large body of evidence has accumulated to suggest that postweaning social isolation has profound, long-term effects on rodent brain and behavior [32-37] [30]. Therefore, social isolation is an alternative, non-pharmacological model of schizophrenia that produces a number of behavioral consequences in adulthood that are similar to schizophrenia symptoms, including deficits in sensorimotor gating, pain sensitivity, motor activity, impaired novel object recognition and enhanced sensitivity to psychoactive drugs. This intervention causes reduced size and deficits in the prefrontal cortex, significant neurotransmission abnormalities including enhanced dopamine functions in the basal ganglia [4, 38, 39], and may generate a number of structural changes in the hippocampus, such as reduced length and density of dendrites in pyramidal cells and reduced number of newly formed neurons [30].

1.5.2 Genetic models

As mentioned above, many genes are implicated in schizophrenia, therefore it is difficult to generate animal models that have similarly complex genotype. Since about

two decade, researchers have tried to create various gene knockout (KO) mice, which simulate some symptoms of schizophrenia. The dopamine transporter knockout mutant mouse fits with the DA hypothesis of schizophrenia, having deficits in sensorimotor gating and spatial cognitive function [16, 18]. The neuregulin-1 knockdown mouse is deficient in NMDA receptor expression showing several behavioral abnormalities such as hyperlocomotion, increased stereotype and impaired social interactions [18, 40]. In brains of schizophrenic patients reduced level of reelin was observed, which is a glycoprotein, that helps to regulate processes of neuronal migration and positioning in the developing brain by controlling cell–cell interactions, therefore it may play a role in the pathogenesis of the illness [40]. Deletion of its gene in mice results in widespread abnormalities throughout the brain, which are similar to the observed cytoarchitectural abnormalities in schizophrenia [16, 18]. However, knocking out of one gene does not lead to the ideal model, because it usually cannot reproduce all of the symptoms of the disease by itself.

Animal models generated by artificial selection may also be important tools to gain a better understanding of the genetic makeup behind the complex symptomatology of different syndromes including schizophrenia [41-43]. Selective breeding is a method in which the experimenter selects a specific sign of a special disease and breeds only those animals that exhibit high degree of that trait. Therefore, selective breeding can produce animals that are of a higher risk of certain disorders with genetic background. Several studies used the selective-breeding procedures to develop new lines (or strains) of rats in psychiatric disorders such as epilepsy, depression or schizophrenia. Schwabe et al. have shown that a PPI-deficit can be selectively bred in Wistar rats and is already stable in the second filial generation [5]. Cognitive abilities also show a significant inheritance, as indicated by earlier experiments in selectively bred rats [44].

1.5.3 Lesion induced models

Lesion models may also contribute to understanding the pathophysiology and neurodevelopmental functions of various brain regions in relation with schizophrenia. Lesions in the hippocampus, frontal cortex, dorsolateral prefrontal cortex or medial prefrontal cortex have been used to create structural models of psychosis in animals [16, 18].

1.5.4 Pharmacological interventions

A huge amount of drugs acting on different receptor systems (e.g. DA agonists, DA reuptake inhibitors, serotonin agonist) are used to develop a pharmacology model of schizophrenia [45].

1.5.4.1 NMDA receptor antagonists

As discussed above, the NMDA receptor is a key factor in promoting glutamatergic neuronal activity throughout and plays a crucial role in brain plasticity especially during early development by affecting on several transmitter systems in the cortico-limbic-striatal network [46, 47]. Therefore, the developing brain is highly susceptible to a chronic, low-dose blockade of NMDA receptors: it causes synaptic weakening and elimination in several brain regions, including the prefrontal cortex and the hippocampus [48-54]. Thus NMDA receptor antagonists (e.g. ketamine, phencyclidine, MK-801) worsen symptoms in schizophrenia or can induce schizophrenia-like symptoms in normal individuals [45, 55, 56]. Ketamine, a phencyclidine hydrochloride derivative and a noncompetitive NMDA receptor antagonist, is able to induce positive and negative symptoms in healthy humans similar to those associated with schizophrenia, including illusions, thought disorder and delusions, blunted emotional responses, emotional detachment, and psychomotor retardation [46, 57]. Animals treated with NMDA receptor antagonists also exhibit a number of changes related to schizophrenia, including deficits in cognitive behavioral tasks, in prepulse inhibition, as well as hyper-responsiveness to stimulants such as amphetamine [18, 20, 46, 58, 59].

1.5.4.2 Drugs acting on transient receptor potential vanilloid 1 receptors

Some evidence suggest that capsaicin desensitization or the lack of transient receptor potential vanilloid 1 (TRPV1) receptors can lead to different behavioral changes, some of which are related to schizophrenia, but investigations into these effects have been scarce [60-62]. TRPV1 receptor was discovered in 1997 and its presence has been indicated throughout the central nervous system (CNS) [63-69]. TRPV1-mediated activity was detected in different brain structures including hypothalamus, hippocampus, basal ganglia and cerebral cortex, albeit at much lower levels than in the dorsal root ganglia [70-75]. In addition to neurons, TRPV1 is expressed in the microglia, astrocytes and pericytes, too [65]. Capsaicin, the major pungent ingredient of hot peppers, derived from the plant genus *Capsicum*, and resiniferatoxin (RTX), derived

from *Euphorbia resinifera*, are known to activate TRPV1 receptors selectively, and their administration in high doses leads to the extensive degeneration in the areas innervated by primary sensory neurons (mainly the non-myelinated C-fibers) [76-78]. However, neonatal capsaicin administration did not change the density of TRPVR1 receptors in most brain areas, which might explain why vanilloid-responsive cells in the CNS remained largely overlooked [64]. Yet, the extensive distribution of TRPV1 receptors in the brain suggested that this receptor could play a significant role in the CNS, too. It is proposed that TRPV1 receptors may take part in the pathogenesis of a range of disorders as diverse as Parkinson's and Alzheimer's disease, depression, anxiety and schizophrenia [22, 23, 61, 79-82]. Of particular relevance to schizophrenia was the observation that many dopaminergic cells in the mesencephalon are TRPV1-immunopositive [64, 83]. Intranigral injection of capsaicin or treatment of rat mesencephalic cultures with capsaicin resulted in cell death of dopaminergic neurons [72], while the activation of TRPV1 receptors in the midbrain ventral tegmentum transiently increased dopamine release in the nucleus accumbens (NAcc) [84]. The data suggest a tonic facilitation of glutamate release exerted through TRPV1 activation by endovanilloids, including anandamide in the substantia nigra [85]. Only a few studies have investigated capsaicin desensitization as a method for schizophrenic models [60-62]. These studies suggest that neonatal capsaicin treatment of rats produces hyperactivity and several brain changes (such as smaller cross-sectional areas, larger ventricles and aqueduct, smaller hippocampal area and reduced corpus callosum thickness) which are similar to those found in brains of schizophrenic patients [61]. In contrast, the capsaicin treatment of adult rats caused decreased locomotion and increased social interaction [62].

1.6 Complex models

A few studies applied "double hit" method, *i.e.* certain combinations of the above mentioned models to investigate the hypothesis that these manipulations can enhance the reliability of the schizophrenia model [16, 86-88]. One of the most popular methods is the gene-enviromental interactions, including ventral hippocampal lesion with social isolation which produced an additive effect on locomotor activity and morphological changes in the prefrontal cortex and NAcc [89, 90]. Neonatal immune activator, polyinosinic-polycytidylic acid (polyI: C), treatment as an environmental factor in DISC1 transgenic mice results in a synergistic effect in the deficits of short-term

memory after puberty, although polyI: C treatment or DN-DISC1 expression by itself has little influence on wild type mice [91]. The subchronic NMDA-receptor antagonist treatment together with social isolation in adult or juvenile rats induced several behavioral abnormalities, such as hyperresponsiveness to different stress situations, drugs and altered pain sensitivity [37, 88, 92-94]. Importantly, combining the two manipulations did not produce detectable additive or synergistic effects on behavior, however, these animals showed more schizophrenia-like signs.

2. Aims of study

1. Gene-environment interactions have important role in the development of psychiatric disorders. The first goal of the thesis was to generate a new substrain of rats with signs related to schizophrenia by combining three factors *i.e.* selective breeding after postweaning, social isolation and chronic ketamine treatment through several generations.
2. The second aim was to characterize behavioral profiles (sensory gating, pain sensitivity, memory function and motor activity) of four experimental groups to reveal whether the selective breeding or the complex treatment plays a major role in the observed changes: naive socialized rats without any treatment (NaNo), or with isolation and ketamine treatment (NaTr) and the 15^{th} generation of selectively bred animals without any treatment (SelNo), or with isolation and ketamine treatment (SelTr).
3. Given that there is some evidence to suggest that schizophrenia might be connected with TRPV1 receptor disturbances, we assumed that juvenile capsaicin desensitization might produce significant changes in behavioral profiles related to schizophrenia. Thus, the third aim of the thesis was to investigate the effects of juvenile desensitization on behavioral parameters impaired in schizophrenia, such as sensory-motor gating, motor activity and memory function, besides demonstrating the effects on functions proven to be affected by TRPV1 receptor systems, *i.e.* pain sensitivity and urinary bladder function.

3. Materials and Methods

3.1 Animals

After institutional ethical approval was obtained from the Animal Care Committee of the University of Szeged, Wistar rats were used for the experiments. The animal housing rooms, as well as the experimental rooms, were kept under standard laboratory conditions (light-dark circle: 12:12 h; light on at 06:00 h; temperature 22 ± 1 ^{o}C; relative humidity: 55 ± 10 %). Inside the cages wood shavings were placed as bedding and nesting material, commercial rat diet and bottled tap water were available *ad libitum*. The cages were placed in shared racks so that auditory and olfactory contacts were maintained. The body weight of the animals was determined weekly during the whole study.

3.2 Drugs

The drugs employed were capsaicin (Plantakem Kft, Sándorfalva, Hungary), ketamine hydrochloride (Calypsol, Richter Gedeon Rt., Budapest, Hungary), xylazine hydrochloride (Rompun, Bayer, Leverkusen, Germany), gentamycin (Sanofi-Aventis, Budapest, Hungary), dexmedetomidine hydrochloride (Orion-Pharmos Pharmaceuticals Turku, Finland), λ-carrageenan (Sigma-Aldrich Kft., Budapest, Hungary) and morphine hydrochloride (Teva Zrt, Debrecen, Hungary). Capsaicin was dissolved in 10% Tween and 10% ethanol and further diluted with saline. All the other drugs were dissolved in saline. For desensitization the volume for subcutaneous (s.c.) injection was 0.2 ml/100 g body weight (except on day 4, 0.4 ml/100 g).

3.3 Selective breeding process

Starting from a population of outbred Wistar rats (‘parental generation’: 10 males and 10 females), a breeding line was established by selective breeding according to the rats sensitivity to acute heat pain after social isolation and ketamine treatment. In further generations, the parental generation consisted of between 13-16 animals of each sex. The paradigm for selective breeding through several generations was as follows: rats, after weaning at 3 weeks of age (21–23 days) were tested by measuring the pain threshold (tail-flick test, TF) and then housed individually for 28 days (between 4-7 weeks of age), to develop social isolation, in cages of 42 × 15 × 18 cm (l × w × h). The animals were treated with ketamine 30 mg/kg intraperitoneally, 4 ml/1000 g body

weight, daily, 5 times/week, 15 injections in total) from 5 to 7 weeks of age. Duration of ketamine treatment and isolation parameters were adapted from earlier studies [88, 94, 95]. At the end of the treatment, animals were re-housed in a group setting (4-5 rats per cage) and had 1 week of recovery, with no treatment afterwards. Behavioral assessment started at the age of 9 weeks with the TF test. Five rats of both sexes, that showed the highest pain threshold, as indicated by the TF latencies, were selected for the next breeding generation. Their offspring (1^{st} generation) and the subsequent 2^{nd} generation were also tested only in the TF test, and again 5 rats of each sex with the highest pain thresholds were chosen as parents of the next generation of the breeding line. From the 3^{rd} generation we also investigated sensory gating by prepulse inhibition (PPI) test (at the age of 10 weeks), and the animals showing a high pain threshold, along with a low PPI were selected for a further breeding line. From the 6^{th} generation, recall memory functions and motor activity by applying the novel object recognition test (NOR) were also observed (at the age of 11 weeks). Thus, animals with impaired pain sensitivity, sensory gating and memory were selected for the further breeding lines. From the second generation, 5-7 animals of both sexes were selected for breeding. Sibling mating was avoided by paying close attention to the litter of origin, and the litter size was reduced to a maximum of 6-8 pups (the number of males and females was approximately equal), ensuring that each family contributed equally to the next generation. We found no signs of inbreeding depression (*i.e.* reduction of fertility, deformed offspring, small litters, poor mothering ability) in the selected line. Male rats of the 15^{th} generation were involved in the present experiment.

3.4 Capsaicin desensitization process

In four series of experiments after weaning (designated as the first day of the experiment) male Wistar rats were treated on four consecutive days with increasing doses of capsaicin (10, 20, 50 and 100 mg/kg s.c.) or its vehicle. Since desensitizing doses of capsaicin cause excessive pain and discomfort, administration was done under ketamine-xylazine (72 and 8 mg/kg intraperitoneally, respectively) anesthesia. The age of the animals used for behavioral experiments was between 2 and 3 months.

3.5 Behavioral tests

3.5.1 Wiping test

Ocular application of capsaicin (1 drop 0.001 % capsaicin) into one of the eyes was done with a pipette, and the animals were observed for the number of front paw eye wipes and blepharospasm for 30 sec. The test was performed at least 5 weeks after the capsaicin desensitization.

3.5.2 Ultrasound examination of the urinary bladder

The method was based on our earlier study [96]. The rats were anesthetized with dexmedetomidine (150 μg/kg s.c.), which has long-lasting hypnotic anesthetic effects; furthermore, it produces diuresis and overflow incontinence which allows the ultrasound examination of the urinary bladder. We used sonography (7.5 MHz linear passed array transducer; Hitachi EUB 405), and the bladder volume was estimated by substituting the diameters into the ellipsoid equation formula: $V = a \times b \times c \times \pi/6$, where *a*, *b* and *c* are the lengths of each major axis. Since bladder volume increases significantly with body weight [96], we corrected the volume (ml) for 100 g body weight accordingly: *relative bladder volume (RV)* = (*bladder volume* x 100)/(*body weight*). Bladder volume was assessed when the first urine drop appeared, and two more times with 30 minute intervals.

3.5.3 Acute heat pain sensitivity

Acute nociceptive threshold was assessed by the TF test. During the test, the rats were wrapped in a towel and held firmly to prevent too much movement, but gently enough to minimize stress. The reaction time was determined by immersing the distal 5 cm portion of the tail in hot water (46, 48 and 52 °C in capsaicin model and 48 °C in the complex model) until a tail-withdrawal response was observed (cut-off time: 40, 20 and 10 s respectively). TF latencies were obtained three times in the capsaicin and four times in the complex model at 0, 30, 60, and 90 min and, were averaged to establish the pain threshold for each group.

3.5.4 Assessment of mechanical and thermal sensitivity in inflammatory pain model

Mechanical and thermal sensitivities were recorded in the same testing box (11 x 17 x 20 cm).

Mechanical sensitivity was assessed with a Dynamic Plantar Aesthesiometer (automatic von Frey test (VF); Ugo Basile, Italy). Measurements were done with a straight metal filament that exerts an increasing upward force at a constant rate (4.25 g/s) with a maximum cut-off force of 50 g (cut off time 8 s). The filament was placed under the plantar surface of the hind paw and used through a mesh base. Measurement was stopped when the paw was withdrawn, and results were expressed as paw withdrawal thresholds in grams.

To determine the heat pain threshold, the paw-withdrawal test (PWD) was used [97]. Heat stimulation was applied to plantar surface of the hind paw. The time until the withdrawal of the tested paw was measured and cut-off time was set at 20 s to avoid tissue damage.

After a habituation period (at least 20 min) the baseline pain thresholds were detected (-180 min) and then unilateral inflammation was induced by intraarticular injection of carrageenan (300 µg / 30 µl) into the right ankle joint (0 min) [98]. Measurements were repeated 3 hours after the carrageenan injection, then the animals were treated with 3 mg/kg morphine s.c., and the mechanical and thermal nociceptive thresholds were determined at 30-min intervals for 90 min.

3.5.5 Prepulse inhibition test

PPI of the acoustic startle response was measured in a plexiglas startle chamber, which was divided into four identical compartments (12 x 17 x 15.3 cm) in a sound-attenuated room. Noise bursts were applied through a speaker mounted close to the backside of the chamber. Under the cage, a piezoelectric accelerometer (*i.e.* force transducer) sensitive to rat startle-like movements produced an electrical signal that was amplified by a signal conditioner and visualized on a computer screen. Rats were allowed to habituate to the background noise (70 dB) for 10 minutes, immediately thereafter they were exposed to three different trial types: a PULSE ALONE (PA) in which a 40 ms 95 dB white noise burst was presented; PREPULSE ALONE (PPA), 20 ms 76 dB; and the PREPULSE-PULSE PAIR (PP) in which prepulse stimuli were followed by the acoustic startle stimulus with a latency of 150 ms. All types were presented 10 times. The interstimulus intervals ranged from 7 s to 13 s, and there was a 10 minute resting period between each trial. %PPI values were calculated as percentages using the following formula: $\%PPI = [1 - (\textit{startle response for PP trial}) / (\textit{startle response for PA trial})] \times 100$.

Since the startle reaction increases significantly with body weight, we normalized the reaction to body weight, accordingly:
Relative startle reaction: (*startle reaction* x 100)/(*body weight* (g)).

3.5.6 Novel object recognition test

NOR test is used to evaluate cognition, particularly recall memory, in both animal models. It was conducted in a plexiglas box (60 x 34 x 33 cm) without bedding. Toy brick towers (Lego Group, Billund, Denmark) with similar size (8 x 2 x 3 cm) were used as test objects. They were affixed to the floor of the box with play-doh to prevent the objects from being displaced during testing. The objects had no natural significance for the rats, and previous pilot work showed no preference for either object used. The objects were placed 13 cm from the opposing corners in the arena, and the rat was consistently placed in the middle of the arena to be equidistant from both objects. The rats were habituated to the testing room for 60 minutes prior to the start of experiments. Between the testing of different animals, the arena and the objects were cleaned with 70% ethanol.

Habituation phase: During a single 10 minute session, each rat was placed in the center of the chamber and allowed to explore the open field without any objects.

Sample phase: One minute after the habituation session, the sample phase began. Two objects with the same size and shape (S1 and S2) were mounted in the open field. Rats were placed again into the center of the open field, and allowed to explore the two objects for 5 minutes.

Test phase: At the end of the sample phase, each rat returned to their home cage for an hour interphase interval, while one of the objects was replaced with another, visually non-identical one (N: novel). The other object (F: familiar) was the same as in the sample phase. Afterwards, a 5-minute test phase followed.

Behavior was scored online without video device in the capsaicin model, but in the complex model we used an infrared video device (WCM-21VF, CNB, China). The following parameters were scored in each phase: duration or frequency of occurrence of stereotypic behaviors (rearing, self-grooming), the time of exploratory activity, walking and inactivity. Object exploration was defined as licking, sniffing or touching the object with the forepaws, but not leaning against, turning around, standing on or sitting on the object. Since the habituation phase lasted twice as long as the other phases, behavioral activity was divided into and scored in sub-phases (0-5 and 5-10 min) for analysis.

The discrimination index (DI) was calculated for both the sample and test phases as follows: DI: (*time spent exploring N vs S1 obj*ect − *time spent exploring F vs S2 object*) / (*total time spent exploring both the objects* [*S1+S2*] *vs* [*N+F*]). If the animals did not explore the objects during the sample or test phases, they were considered as non-responders and data from these animals were not included in the final analysis (altogether one animal from the SelTr group was excluded on such grounds). The scoring of the different behaviors was carried out by investigators blind to the applied treatment.

3.5.7 Telemetry

This method is appropriate to monitor gross locomotor acitivity in freely moving animals (Respironics, Mini Mitter, Vitalview, Oregon, USA). Animals were peritoneally implanted with Minimitter transmitters (E-Mitter, Philips Respironics®) under ketamine-xylazine anesthesia. A small lower abdominal incision was made and a sterilized probe was inserted into the peritoneal cavity. Following the surgery the animals received antibiotic treatment (13 mg/kg gentamycin, s.c.) to prevent infection. After a one-week recovery period the animals were housed individually in cages measuring 42 x 30 x 18 cm, and standard laboratory chow and tap water were available *ad libitum*. The cages were placed on receiver platforms (ER-4000 Energizer Receiver) in an isolated room maintained under standard laboratory conditions. Motor activity was monitored continuously for 5 days without any disturbance.

3.6 Protocols

3.6.1 Experimental paradigm of the complex model

Four experimental groups of male rats were compared: naive socialized rats without any treatment (NaNo), or with isolation and ketamine treatment (NaTr) and 15th generation selectively bred animals without any treatment (SelNo), or with isolation and ketamine treatment (SelTr). Groups were matched according to body weight (50 ± 1.7 g) and their TF values at the age of 3 weeks. The testing schedule is presented in Table 1.

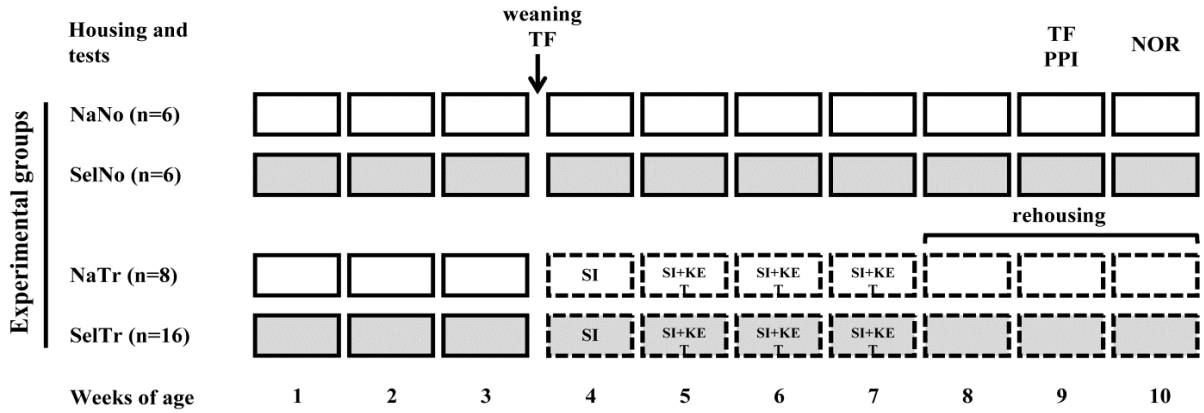


Table 1. Experimental paradigm of complex model TF: tail-flick test; PPI: prepulse inhibition test; NOR: novel object recognition test; NaNo: naive animals without treatments; NaTr: naive animals with social isolation and ketamine treatment; SelNo: selectively bred rats without treatments; SelTr: selectively bred rats with social isolation and ketamine treatment; SI: social isolation; KET: ketamine treatment.

3.6.2 Experimental paradigm of capsaicin model

Four experimental series of rats were tested with two sub-groups within the series: control animals received vehicle and the second group were treated with capsaicin.
In the first series the TF, PWD, VF and ultrasound examination were performed, in the second the telemetry test, in the third series the NOR test was involved and in the fourth series the PPI test was carried out (Table 2.). All of the animals were involved in the wiping test to check the desensitization.

Type of tests	Treatment	Number of animals	Age at test (week)	Series
Tail-Flick	Capsaicin	11	9	1
	Vehicle	8		
Paw withdrawal	Capsaicin	11	9	1
	Vehicle	8		
von Frey	Capsaicin	11	10	1
	Vehicle	8		
Ultrasound	Capsaicin	11	12	1
	Vehicle	8		
Telemetry	Capsaicin	8	10-14	2
	Vehicle	7		
Novel object recognition	Capsaicin	5	7	3
	Vehicle	7		
Prepulse inhibition	Capsaicin	10	12	4
	Vehicle	8		

Table 2. Experimental paradigm of capsaicin model

3.7 Statistical analysis

Data are expressed as means ± SEM. Data were assessed using one- and two-way ANOVA with repeated measures and the Fisher-LSD *post hoc* test. A p-value less than 0.05 was considered significant. For the analyzes, STATISTICA 11 software (Statsoft Inc.,Tulsa, OK, USA) was used.

In the complex model the median split method was used for transforming continuous variables into categorical ones. A quartile-based scoring method was used. The values in the first (lower) quartile received 0 points, values in the third (upper) quartile received a score of 2, and the values between them received 1 point. Five aspects (TF latency at the age of 9 weeks, relative startle reaction, %PPI, DI and grooming activity) were rated from 0 (lowest risk) to 2 (highest risk), and summarized to generate the total schizophrenia score, which ranged from 0-10. Using this score, it was possible to classify animals as either low- or high-risk for schizophrenia using quartiles of the total schizophrenia score.

Sampling frequency regarding general motor activity in telemetry, data was set to 1 min throughout the experiment and one-hour average of the data was analyzed.

4. Results

4.1 Body weight

The body weight of animals in the complex model measured on the different testing days (Fig. 3.) showed a significant effect of time ($F_{3,117}$ = 3077.81, p< 0.0001) and strain ($F_{1,39}$ = 24.78, p< 0.001), and the interaction between time and strain ($F_{3,117}$ = 7.13, p< 0.001) was also significant. That is, the substrain started to exhibit a lower body weight from age of 9 weeks. The social isolation together with ketamine treatment did not result in further weight loss.

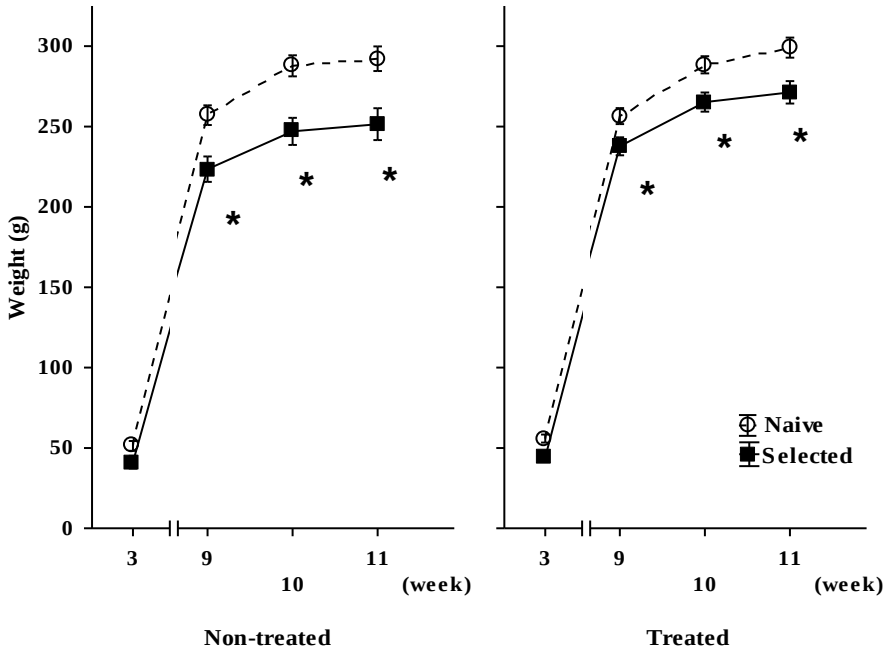


Figure 3. The body weight measured on the day of different behavioral tests in the complex model. * indicates significant differences between the naive and selected groups. Data are expressed as means ± SEM.

All of the animals survived capsaicin desensitization with this dose regimen, suggesting that this method is non-lethal for juvenile animals. The ANOVA of body weight revealed a significant effect of time, but not for treatment, thus capsaicin-treated animals had similar body weight as the control ones (Fig. 4.).

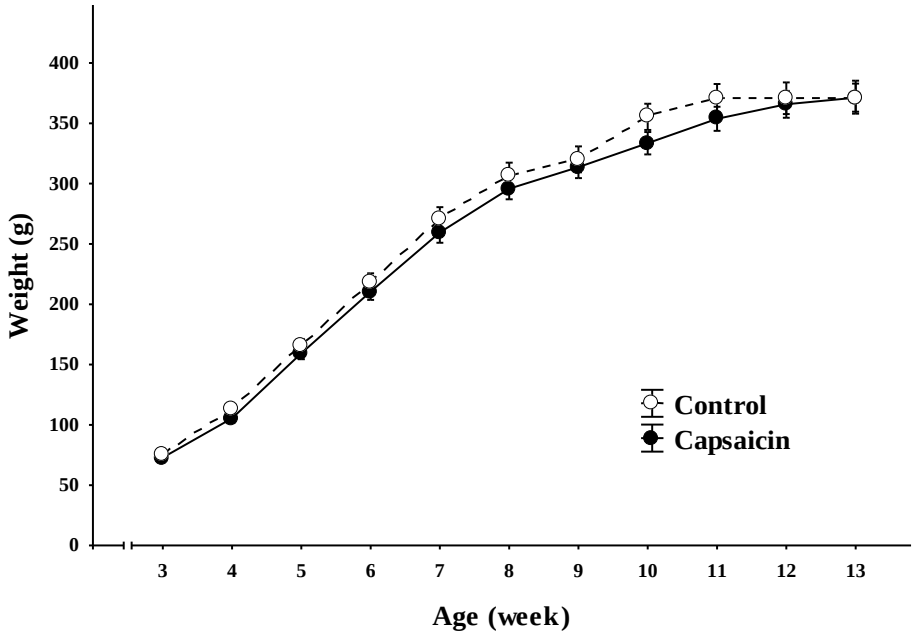


Figure 4. The body weight at capsaicin model. Data are expressed as means ± SEM.

4.2 Wiping test and urinary bladder function

We performed the wiping test and measured the urinary bladder to verify the effect of capsaicin desensitization. Regarding the effects of the capsaicin eye drop, it produced blepharospasm and violent wipes of the eye in control, but not in the capsaicin-treated animals, confirming desensitization (Fig. 5.).

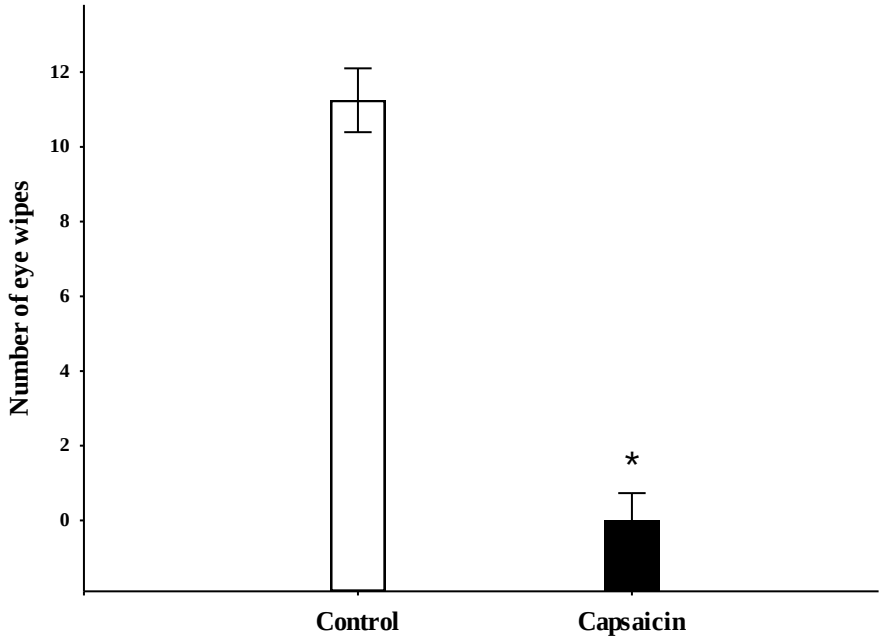


Figure 5. Wiping test. Data are expressed as means ± SEM. The symbol * denotes significant differences from capsaicin group.

The first urine drop appeared about 20 min after the dexmedetomidine administration. Urine dribbling was observed almost continuously, suggesting a continuous overfilling of the bladder. As for the effect of capsaicin desensitization on bladder capacity, ANOVA with repeated measures revealed a significant effect of treatment ($F_{1,17} = 8.8$; $p < 0.01$) and time ($F_{2,34} = 4.6$; $p < 0.05$), thus capsaicin treated animals had larger bladder volumes compared to the control group (Fig. 6.).

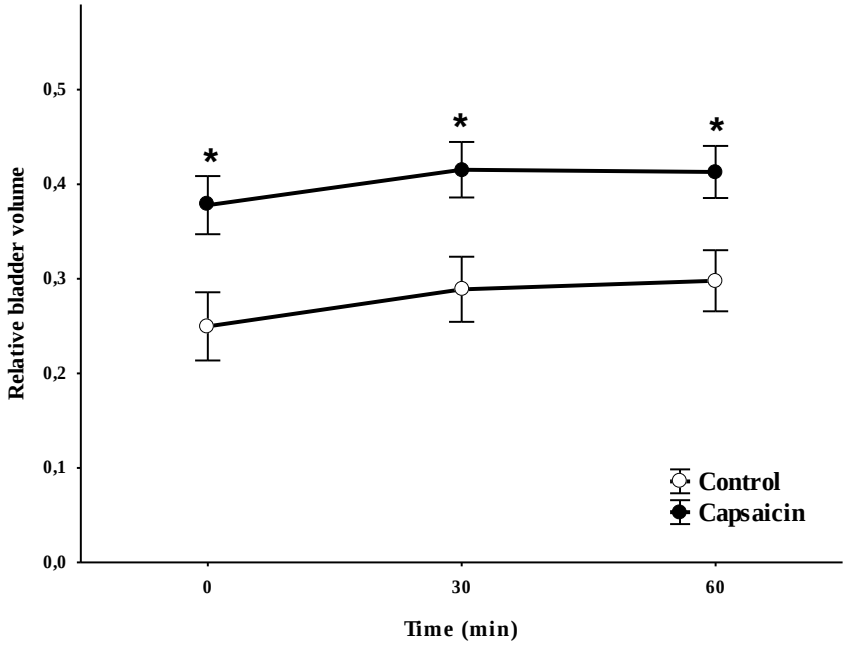


Figure 6. Relative urinary bladder volume determined by ultrasound examination at the beginning of urine dribbling (0 min), 30 and 60 min later. Data are presented as means ± SEM. The symbol * denotes significant differences from control group.

4.3 Pain sensitivity

4.3.1 Tail-flick test

Regarding the selectively bred animals, ANOVA revealed a significant effect of strain ($F_{1,39} = 4.23$, $p < 0.05$), time ($F_{1,39} = 328.15$; $p<0.001$) and significant interaction ($F_{1,39} = 4.94$; $p<0.05$) on the TF latencies measured at 3 and 9 weeks of age; thus, the TF latency significantly increased in all groups with time. *Post-hoc* comparison did not reveal differences between the groups at the age of 3 weeks, but a tendency towards TF latency increase was observed in the new substrain (naive: 4.1±0.21 s 15th generation. 4.6±0.27 s). Significant differences were observed at the age of 9 weeks between NaNo and both of the substrain groups (SelNo and SelTr), with these groups having the lowest

pain sensitivity (Fig. 7.). The latency in the NaTr group did not differ from any other group.

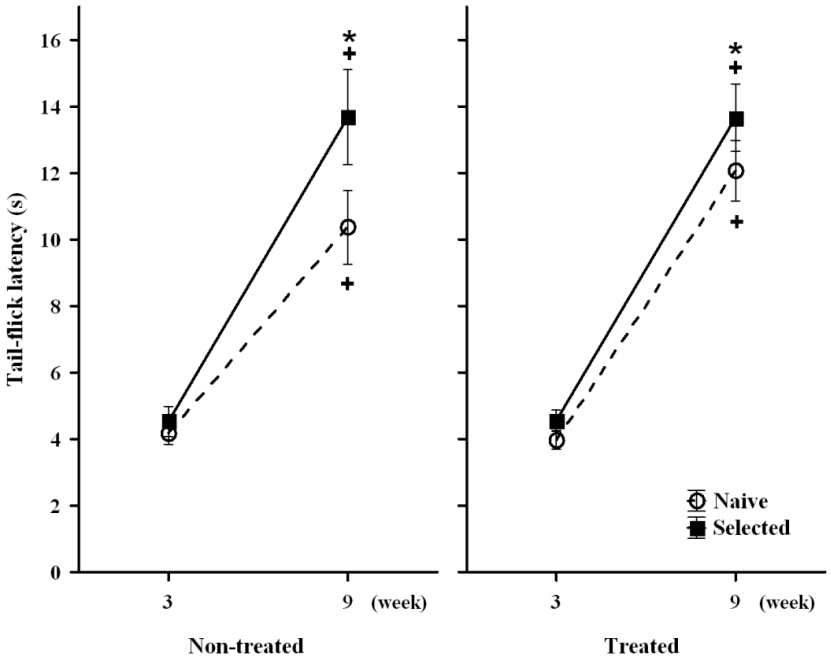


Figure 7. Tail-flick latency at the age of 3 and 9 weeks in the complex model. Data are expressed as means ± SEM. The symbols indicate significant differences between time points (+) and compared to the naive non-treated (NaNo) group (*).

As regards the capsaicin treated series, two-way ANOVA revealed a significant effect of temperature ($F_{2,34} = 103$, $p < 0.001$), but not of capsaicin treatment (Fig. 8.); thus, the acute-heat pain sensitivity increased by temperature, but was not influenced by capsaicin desensitization.

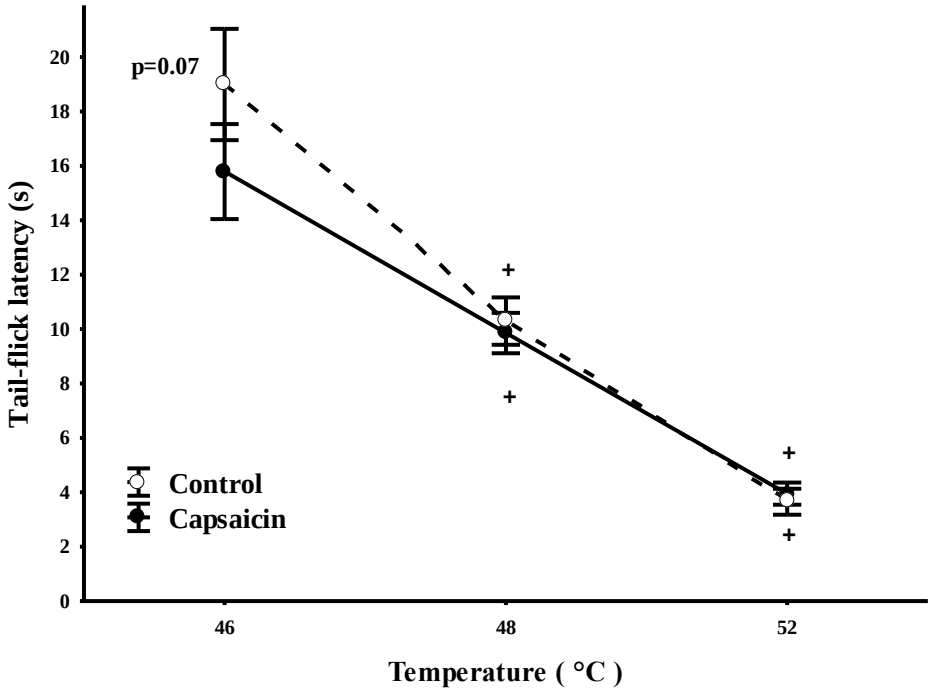


Figure 8 . Tail-flick latencies at different water temperatures in the capsaicin model. Data are presented as means ± SEM. The symbols indicate significant differences between time points (+).

4.3.2 Mechanical and thermal pain sensitivity in inflammatory pain model

The baseline of von Frey and PWD test did not differ significantly in the capsaicin and control groups on pain sensitivity (Fig. 9.).

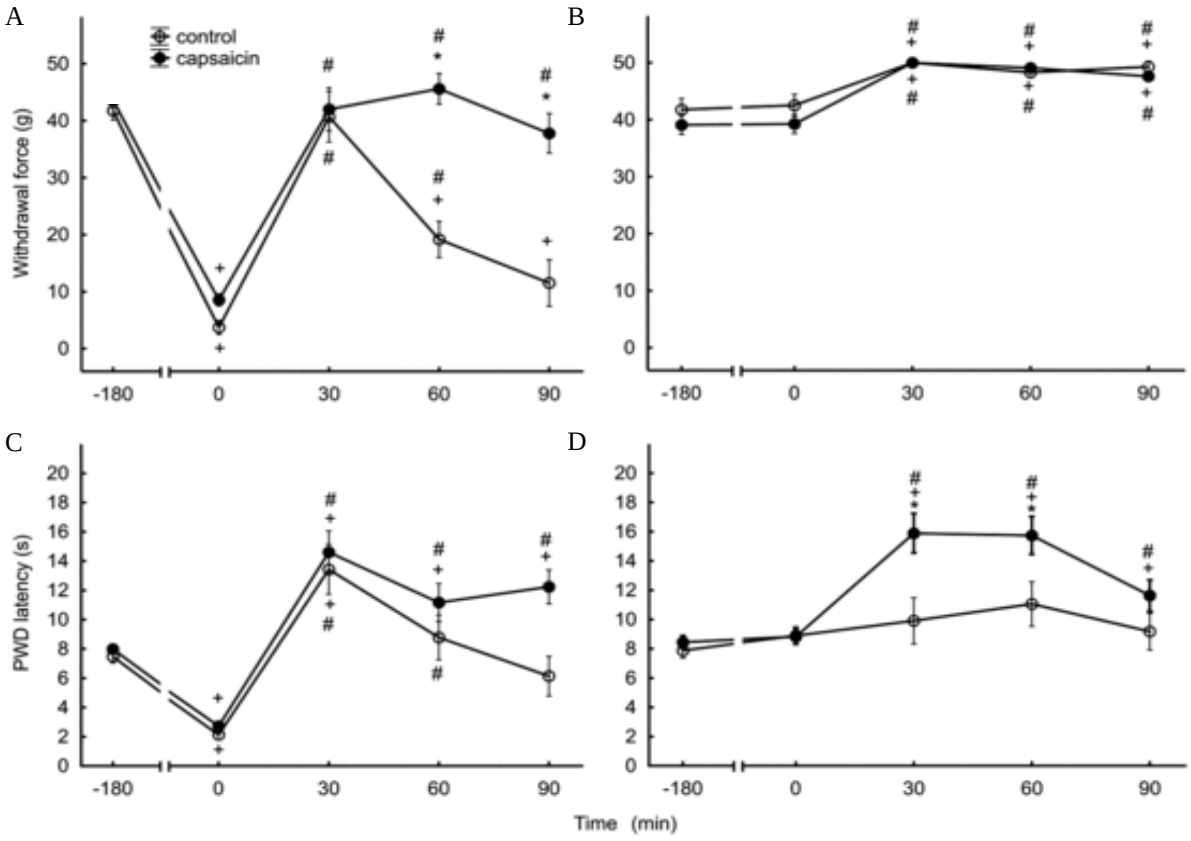


Figure 9. Mechanical (A,B) and thermal (C,D) pain thresholds before (− 180 min) and after (0 min) carrageenan administration, and the effect of morphine (3 mg/kg, s.c.) on the inflamed (A,C) and non-inflamed (B,D) sides. Data are presented as means ± SEM. The symbols denote significant differences: * from control group, +: from pre- and # from post-carrageenan values.

Regarding the threshold for mechanical allodynia, significant effects of treatment ($F_{1,35}$ = =11.0, $p < 0.005$), side ($F_{1,35} = 90.2$; $p < 0.001$), time ($F_{4,140} = 50.6$; $p < 0.001$) and their interactions were observed. *Post- hoc* comparison revealed that juvenile capsaicin desensitization resulted in a slightly decreased mechanical allodynia ($p = 0.13$), while the anti-allodynic effect of morphine was significantly prolonged in desensitized animals (Fig. 9.A). On the non-inflamed side, significant increases in the withdrawal threshold were observed in both groups after morphine administration (Fig. 9.B).

In the case of thermal hyperalgesia, significant effect of treatment ($F_{1,35}$ = =19.0, $p < 0.001$), side ($F_{1,35} = 14.8$; $p < 0.001$), time ($F_{4,130} = 31.4$; $p < 0.001$) and their interactions were observed. Carrageenan resulted in a similar degree of thermal hyperalgesia in both groups. Morphine caused a significant increase in PWD latency on the inflamed side with a more prolonged effect in the desensitized group (Fig. 9.C).

Furthermore, morphine caused a significant increase in the nociceptive threshold on the non-inflamed paw in the capsaicin-pretreated animals (Fig. 9.D).

4.4 Sensory gating

ANOVA revealed a significant effect of prepulse stimulation ($F_{1,39} = 72.47$, $p < 0.0001$), and strain ($F_{1,39} = 6.50$, $p < 0.05$) (data are not show) on the magnitude of the startle reaction in the complex model. The response significantly decreased in the case of prepulse stimulation in all groups, except the SelNo group. The *post hoc* comparison revealed significant differences between the NaNo and SelTr groups with the PA, while both of the selectively bred groups showed a significantly higher degree of relative startle reaction compared to both of the naive groups with the PP (data are not shown). Regarding the sensory gating, the effect of strain was significant ($F_{3,39}= 5.59$; $p<0.005$); thus, both groups of the substrain (SelNo and SelTr) had lower %PPI compared to the naive groups (NaNo and NaTr) (Fig. 10.).

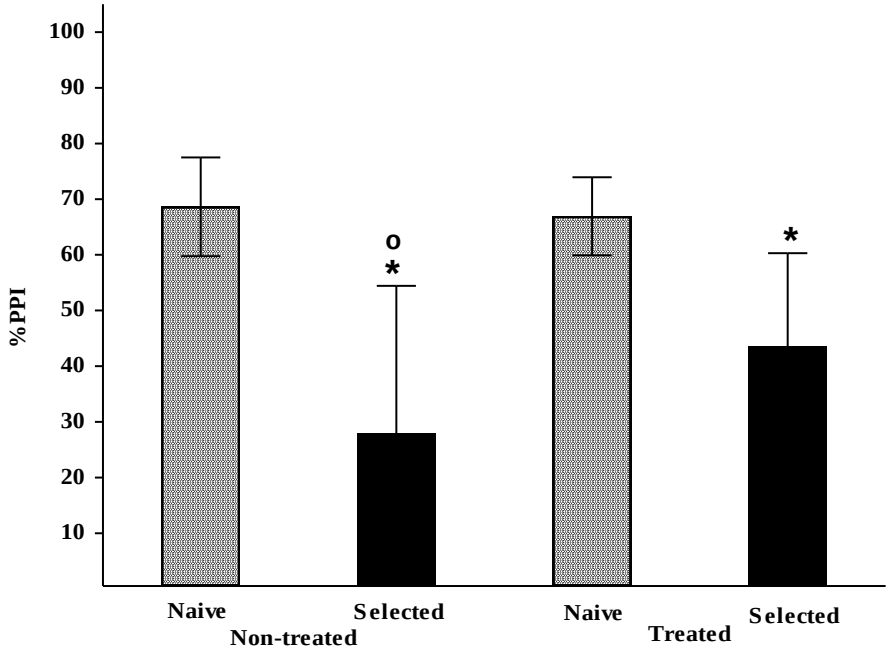


Figure 10. %PPI values in the different groups of complex model. Data are expressed as means ± SEM. * and **o** indicate significant difference compared to the naive non-treated (NaNo) and treated (NaTr) groups, respectively.

In the capsaicin animal model, repeated measures ANOVA of the relative startle reaction revealed a significant effect of prepulse stimuli ($F_{1,16} = 68.95$, $p = 0.001$), but not of treatment. Capsaicin-treated animals showed similar startle reflex amplitude

elicited by PA or PP compared to the control group; the response amplitude significantly decreased in both groups with PP (data are not show). Therefore, the %PPI did not show significant differences between the two groups (Fig.11.).

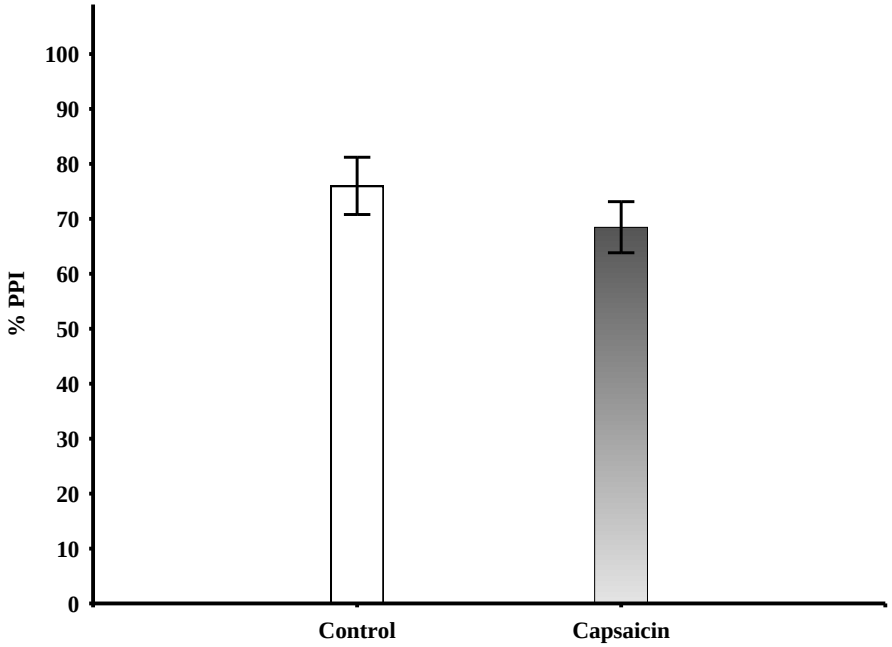


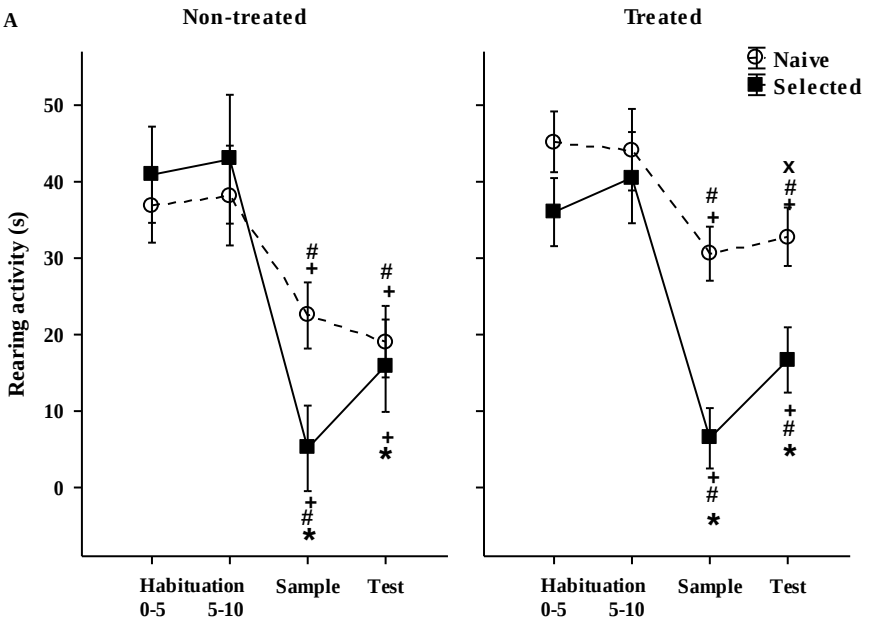
Figure 11. %PPI values in the different groups of capsaicin model. Data are presented as means ± SEM.

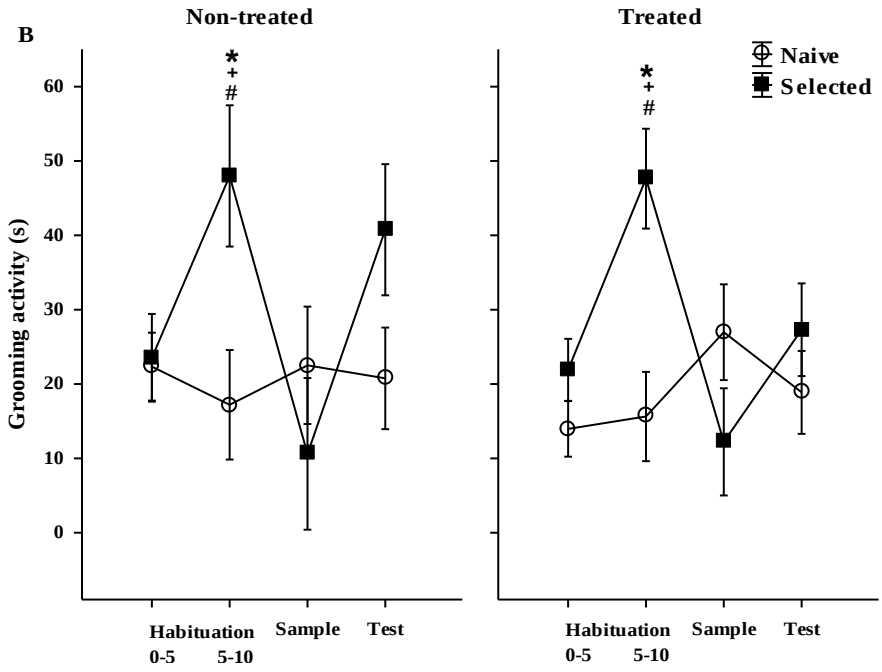
4.5 Motor behavior

Regarding the motor behavior during NOR test in the complex model ANOVA revealed a significant effect of strain ($F_{1,39}$=6.75, p<0.05), phase ($F_{3,117}$=27.35, p<=0.001), and interaction between phase and strain ($F_{3,117}$=3.69, p<0.05) on the rearing activity (Fig. 12.A). Rearing activity decreased with time (phase) in all groups, and the 15th generation showed lower rearing activity in the sample and testing phases. The NaTr group showed enhanced rearing activity in the test phase compared to all the other groups.

Strain differences were also found in the grooming behavior, *i.e.* the substrain showed increased grooming activity during the second part (5-10 min) of the habituation phase ($F_{1,39}$=4.18, p<0.05; Fig. 12.B).

Analysis of walking duration revealed a significant effect of strain ($F_{1,39}$=8.88, p<0.01), phase ($F_{3,117}$=56.61, p<0.001) and a phase-treatment interaction ($F_{3,117}$=3.39, p<0.05); thus, walking activity decreased with time (phase), and was lower in the new substrain, while the NaTr group showed enhanced activity in the sample and test phases (Fig. 12.C).


A
Non-treated
Treated
Naive
Selected
Rearing activity (s)
50
40
30
20
10
0
Habituation
0-5
5-10
Sample
Test


B
Non-treated
Treated
Naive
Selected
Grooming activity (s)
60
50
40
30
20
10
0
Habituation
0-5
5-10
Sample
Test

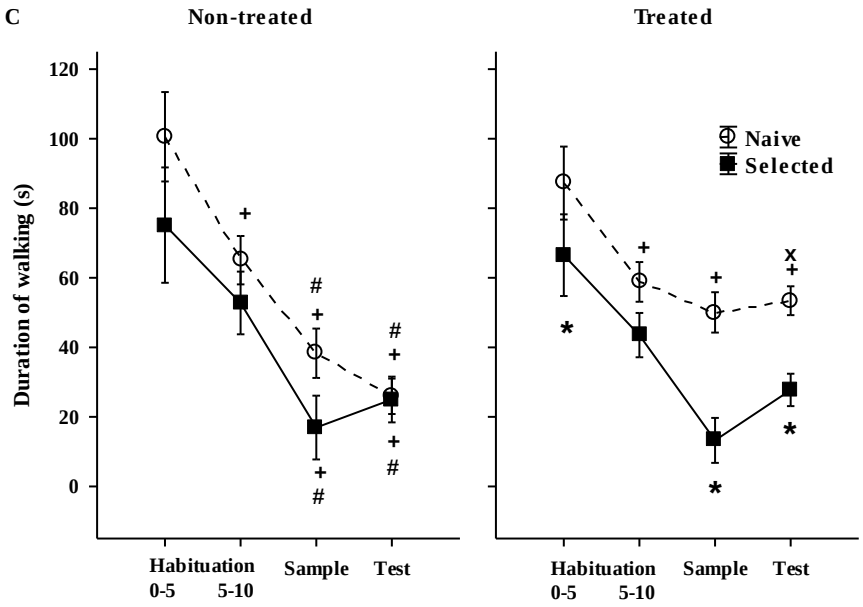


Figure 12. Rearing (A), grooming (B), and walking (C) activities in the different phases of the NOR test in the complex model. + and # indicate significant difference from the first (0-5 min) and second (5-10 min) habituation period respectively; x and * sign significant difference with treatment and strain respectively.

No significant differences were observed in any types of motor behavior (e.g. rearing, grooming) and inactivity in any phases between the two groups in the capsaicin model (data are not shown).

Regarding the analysis of the telemetric data, motor activity showed a daily rhythm with night maxima and day minima in the control and capsaicin desensitized animals. The separate analysis of dark and light phases showed a significant effect of treatment ($F_{1,62} = 5.87$, $p < 0.05$) and a close to significant effect of phase ($F_{1,62} = 3.56$, $p = 0.06$;), which is to say that the desensitized animals exhibited enhanced motor activity during the active phase compared to control rats (Fig. 13.).

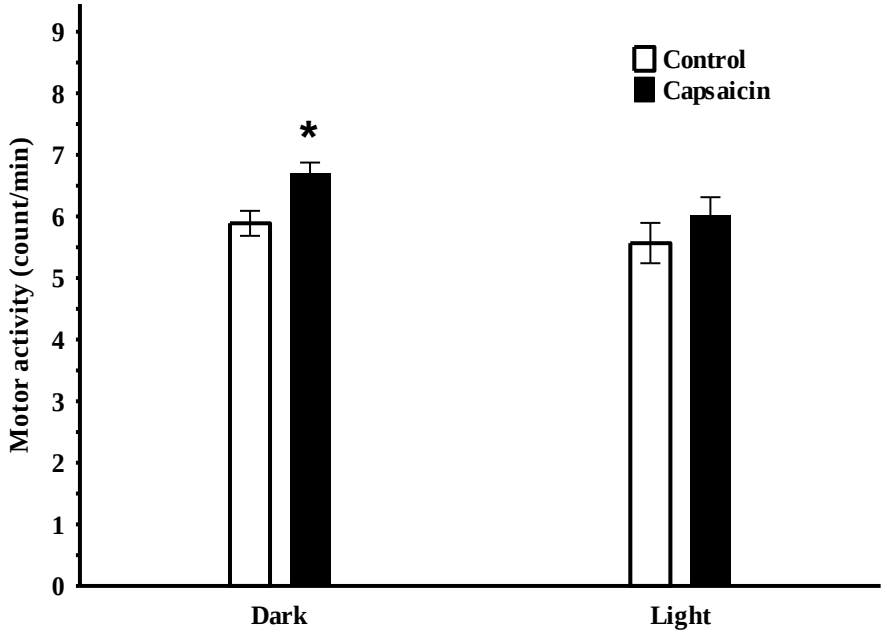


Figure 13. Mean motor activity during dark and light periods. Data are presented as means ± SEM. The symbol * indicates significant differences between the two groups.

4.6 Memory functions in novel object recognition test

Both NaTr and SelTr groups showed an increased exploring time of the objects (Fig. 14.A). As for the DI, ANOVA revealed a significant effect of phase ($F_{1,38}$=5.70, p<0.05). The *post hoc* comparison revealed that in the NaNo group DI was significantly enhanced in the presence of the new object, while this enhancement could not be observed in any other groups (Fig. 14.B)

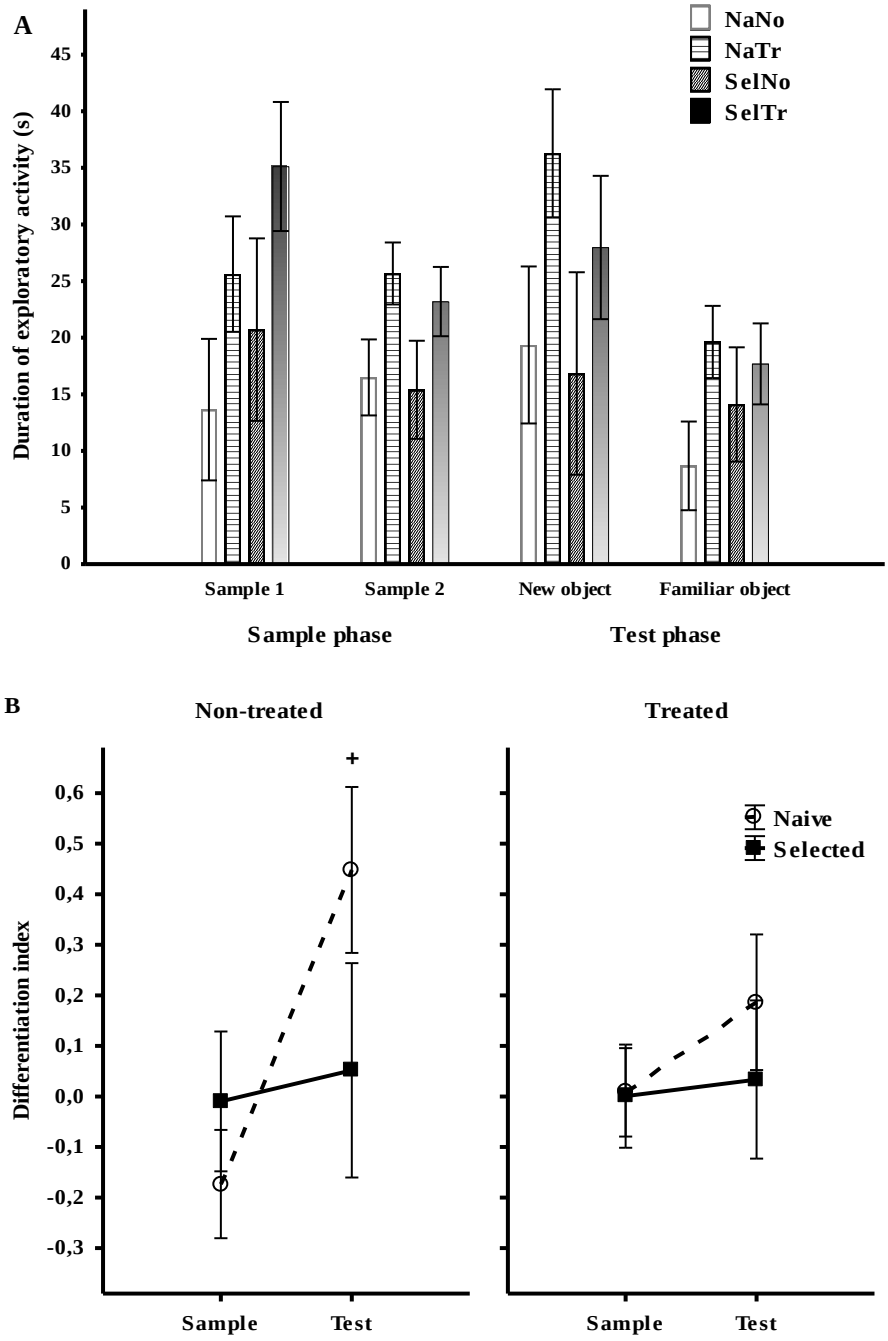


Figure 14. Exploratory (A) activities in the different phases of the NOR test. (B): Differentiation index in the sample and test phases. + indicates significant difference between the phases. Data are expressed as means ± SEM.

In the sample phase, no significant differences were observed in the time spent exploring the two identical objects between the groups in the capsaicin model (Fig.15.A.). In the test phase, the time of the novel object exploration was significantly longer than that of the familiar one in control animals ($p < 0.01$), while this difference

was not significant in the desensitized group. As regards the DI significant increase was observed in both groups of capsaicin series (Fig.15.B.).

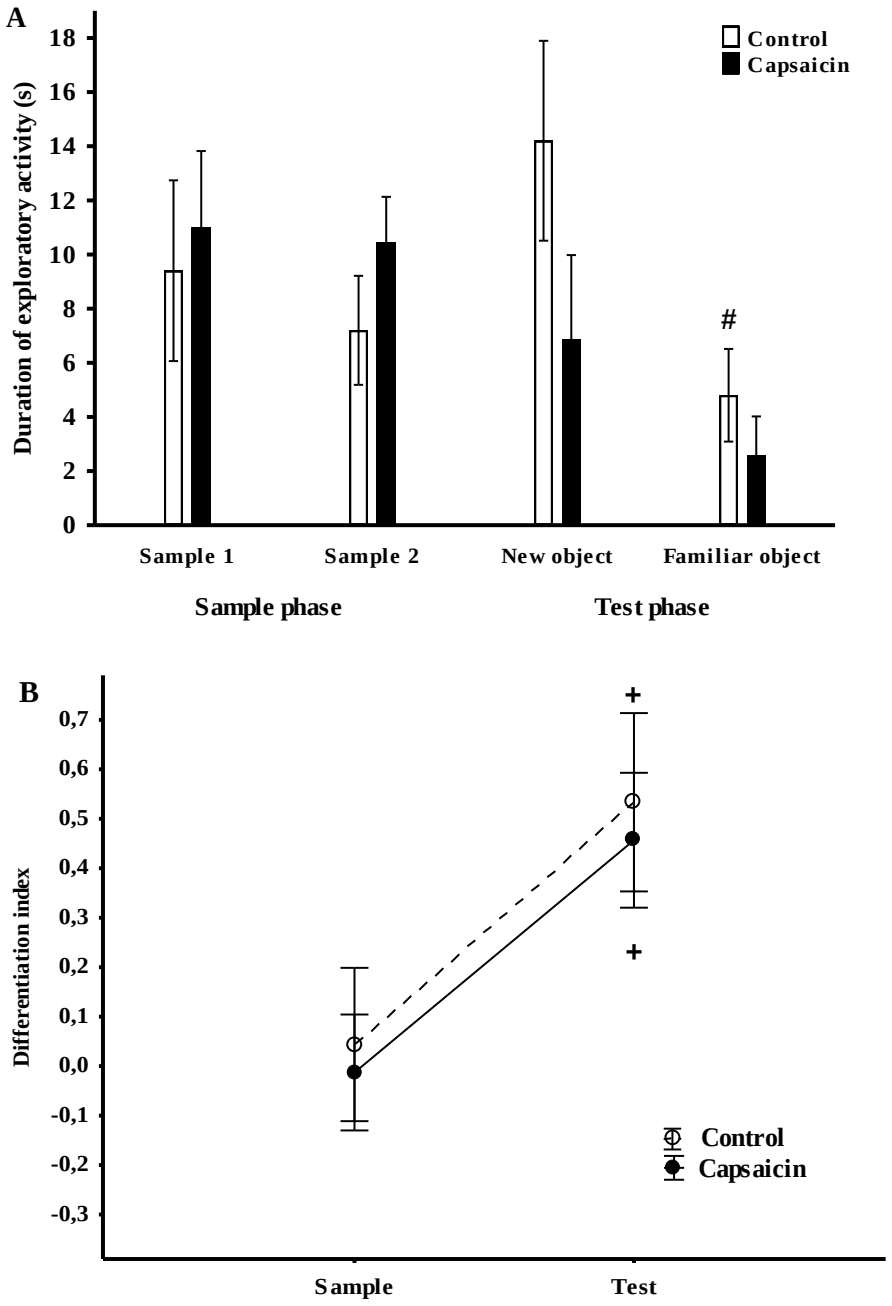


Figure 15. Object exploratory activity (A) and differentiation index (B) in the sample and test phases of NOR test. Data are presented as means ± SEM. + indicates significant difference between the phases and # indicates significant differences in the exploration time between the familiar and novel objects.

4.7 Categorization

ANOVA revealed significant differences between the four groups ($F_{3,39}$=9.47, p<0.001) in the summarized score of the different groups, *i.e.* the NaNo group had the lowest

score, while the SelTr group scored the highest (Fig. 16.A). The histogram of the summarized score shows that all NaNo animals scored lower than 6 points, while in all of the other groups there were some animals that scored higher, and the highest ratio of these was observed in the SelTr group (Fig. 16.B).

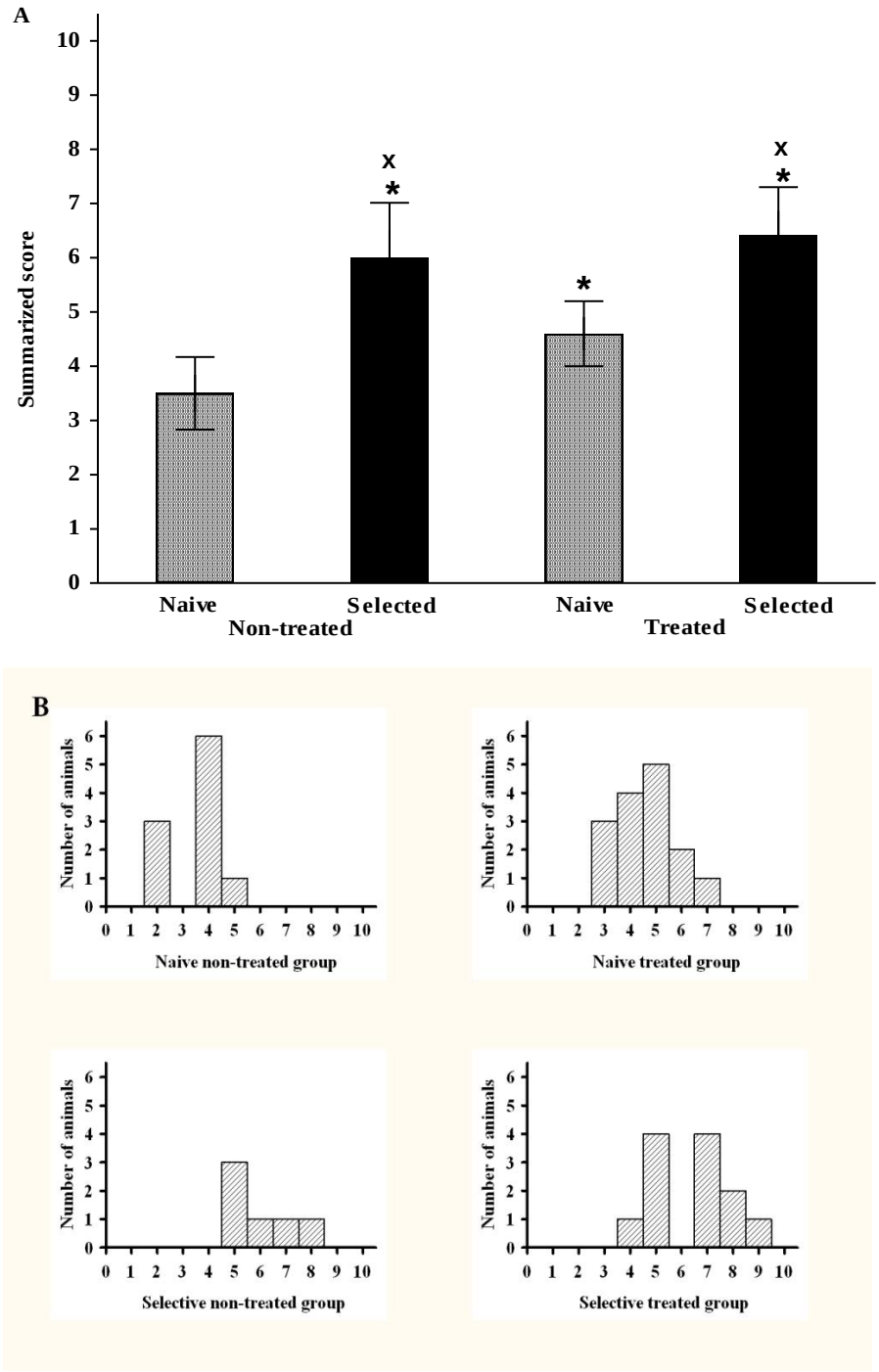


Figure 16.

Means ± SEM (A) and the distribution (B) of the summarized score within each group. The symbols * and x indicate significant differences compared to the naive non-treated (NaNo) and treated (NaTr) groups, respectively.

5. Discussion

Schizophrenia poses a challenging degree of complexity with respect to genetic and environmental factors; nonetheless, only a few studies have addressed possible gene-environment interactions in the context of schizophrenia models [91, 99, 100]. We found that combined selective breeding, postweaning social isolation and subchronic NMDA antagonist treatment caused permanent impairments in sensory gating, memory function, pain sensitivity and motor activity; the parameters that are disturbed in schizophrenia too. Our data suggest that this complex paradigm can lead to an improved model of schizophrenia; although, further breeding is required to enhance its reliability.

On the other hand juvenile capsaicin desensitization caused long-lasting disturbances in different physiological processes related to C-fiber functioning; *i.e.* wiping response, morphine sensitivity and urinary bladder capacity. It also caused significant deterioration in memory function and motor activity under freely moving conditions, but no disturbances of the sensory gating were observed, suggesting that capsaicin desensitization by itself can lead to only few disturbances that might be related to schizophrenia.

5.1 Sensory functions

Clinical reports pointed out that many patients with schizophrenia are less sensitive to pain than other individuals, and this is likely associated with increased morbidity and mortality [28, 101, 102]. Data are available to suggest that juvenile isolation induces significant changes in pain sensitivity, which might be due, at least partially, to changes in the number and activity of opioid receptors; suggesting a high importance of housing conditions in schizophrenia models [34, 40, 88, 93, 94, 103-106]. Subchronic ketamine treatment and subsequent social isolation in adult rats produces slight changes in pain sensitivity [93]. Our previous study demonstrated that juvenile isolation, but not ketamine treatment, attenuated responses evoked by acute heat stimuli; while the combination of the two manipulations did not result in a further increase in TF latency [88]. These interventions alone and in combination enhanced the antinociceptive effect of morphine. While selective breeding led to a significant increase in pain threshold, the complex treatment applied in the present study did not result in a further enhancement; suggesting that genetic factors played a larger role in this effect.

The current findings support earlier data which showed that both neonatal and adult capsaicin desensitization or KO of the TRPV1 receptors lead to an irreversible

suppression of wiping behavior in response to irritating chemical stimuli [107, 108]. The presence of persistent changes in wiping behavior of rats treated at the juvenile age provides behavioral verification of the efficiency of capsaicin treatment.

Several studies showed that both neonatal and adult capsaicin desensitization resulted in decreased mechanical and/or thermal pain responses in different inflammatory models [109-111]. We did not observe significant alterations in acute and inflammatory pain sensitivities in desensitized animals, which is in agreement with some earlier data obtained in neonatal or adult desensitized rats [60, 112]. It is assumed that alterations in capsaicin-insensitive neurons and/or reorganization of the CNS may contribute to the normal pain sensitivities in capsaicin-treated animals [112, 113]. However, the antinociceptive effect of morphine was enhanced and prolonged during joint inflammation in both mechanical and thermal tests. Only one study investigated the antinociceptive property of morphine in inflammatory pain, which found that morphine caused a greater effect in the inflamed than in the non-inflamed paw in control rats, and this difference was absent in capsaicin-treated animals [114]. We found a similar phenomenon in our model, as well as a prolonged effect of morphine. Opioid receptor binding studies showed that the number of binding sites and binding affinity in the dorsal horn remained unaltered after adult capsaicin treatment, but were decreased by neonatal capsaicin exposure [115, 116]. The paradoxical finding that desensitization enhances the effects of morphine might be due to the decreased nociceptive input to dorsal horn neurons because of the absence of TRPV1-expressing afferent fibers.

Sensory dysfunction was indicated by impairment of urinary bladder function as well. A considerable amount of evidence indicates that capsaicin-sensitive mechanisms regulate the micturition threshold by relaying information to the CNS about the volume of fluid present in the bladder [117-121]. Both neonatal and adult capsaicin treatments lead to an impairment of urinary bladder function, such as an increased threshold for micturition or a reduced frequency of micturition contractions [118, 122]. Furthermore, these interventions lead to increased bladder capacity detected by the cystometrographic method [118, 120]. Almost 20 years ago, a simple, noninvasive and reliable ultrasonographic method was described for the determination of urinary bladder capacity in anesthetized rats [96]. Our *in vivo* results revealed that the bladder capacity is significantly larger in juvenile desensitized rats compared to the control animals. Enhanced bladder volume was also observed 5 weeks after adult capsaicin desensitization in rats (RV: 0.3 ±0.016 and 0.44 ± 0.019, control and desensitized

animals, respectively) [123]. Thus, capsaicin desensitization applied at any age results in enhanced bladder capacity, suggesting that sensory transmission in the micturition reflex depends on TRPV1 receptors at all stages of development.

5.2 Sensory gating

PPI is regulated by hippocampal, prefrontal, amygdaloid and basal ganglia regions, and the potential contribution of the pathological changes in these regions to PPI deficits in schizophrenia patients has been suggested [124-126].

Previous studies revealed that repeated NMDA-antagonist treatment of neonatal or adult rats led to the disruption of PPI in some but not all the animals [127-130], and the cessation of NMDA-antagonist treatment resulted in remission, suggesting that the treatment by itself is not sufficient to produce a long-lasting PPI disturbance [59, 131-133]. Plenty of evidence suggest that long-term postweaning social isolation may also induce impaired PPI in rodents, but resocialization may lead to recovery; however, the data are somewhat inconsistent [4, 45, 134-140, 140-143]. It is assumed that changes in the prefrontal cortex after social isolation and/or imbalances between neural connections within the cortico-striato-limbic circuitry lead to the observed PPI disturbances [4]. Disturbed PPI as a result of selective breeding, was proven to be heritable, and this can be used to develop an animal model for schizophrenia [5, 144-147]. The rats with impaired PPI also exhibited deteriorated social behavior, impaired reward responses, and abnormalities in information processing; which indicates that rats with low PPI show other schizophrenia-like disturbances, as well. Regarding the combination of genetic manipulation with environmental factors, it has been shown that postweaning social isolation for 12 weeks did not impair PPI in Nurr1 wild type mice, but it was disturbed in heterozygotic animals [148].

We did not find a striking effect of social isolation and ketamine treatment on PPI after two weeks of treatment cessation in naive animals. Selective breeding was effective, but the combination of these interventions did not lead to further impairment; suggesting that genetic factors played the major role in the development of PPI disturbance.

Only a few studies investigated the role of TRPV1 receptors in sensory gating with controversial results. It has been shown that cannabidiol (is a natural component of the marijuana plant) through the activation of TRPV1 receptors reversed the NMDA-receptor antagonist-induced (MK-801) disruption of PPI in mice [149]. Neither capsazepine (TRPV1 receptor antagonist) nor acute or chronic cannabidiol affected PPI

by themselves, and capsazepine by itself did not influence the effect of MK-801 [149, 150]. In contrast, a more recent study found disrupted PPI after cannabidiol administration in rats, but it had no effect on the MK-801-induced disruption of PPI [151]. The only one piece of literature regarding the effect of neonatal capsaicin desensitization on PPI supports our present finding that desensitization has no effect on PPI [152]. The ineffectivity of capsaicin desensitization or capsazepine *per se* on PPI suggests that TRPV1 receptors do not directly interfere with normal sensorimotor gating, but further studies are required to reveal the effects of capsaicin desensitization on PPI under different conditions.

5.3 Cognitive function

Our results show that the complex treatment and the capsaicin desensitization caused significant deterioration in memory function. Cognition, including memory, is impaired in schizophrenia, and both social deprivation and repeated treatment with NMDA antagonists of juvenile animals can disrupt memory functions, which are related primarily to the prefrontal cortex [46, 153-158]. However, several studies failed to induce impairments in tests of memory with these treatments, or only modest learning disturbances were observed [46, 49, 50, 59, 124, 129, 130, 156, 157, 159-161]. Ashby et al. investigated the effects of subchronic NMDA-antagonist, MK-801, and postweaning social isolation (for 5 weeks) on hippocampal long term potentiation (LTP) after a 7 days' washout period [87]. While subchronic MK-801 treatment enhanced hippocampal LTP, postweaning social isolation did not influence it, and the combination of the two manipulations did not result in detectable additive or synergistic effects on hippocampal plasticity.

Ample data are available on the effects of activation or desensitization of TRPV1 receptors on various CNS structures and functions, with inconsistent results. Exposure of central neurons to high doses of capsaicin triggers cell death or apoptosis, and the degeneration might be due to calcium release into the cells and the induction of proteases [69, 79, 162-164]. However, the majority of the neurons may be spared by the protective effect of exogenous nerve growth factors, and also markers that are associated with CNS neurons which are unchanged after neonatal capsaicin administration [64, 165]. We applied capsaicin desensitization at a young age that would be defined as early adolescence in human terms**,** when the development of the central and peripheral nervous system has not finished yet [166-168]. Earlier results

suggested that capsaicin-induced neurodegeneration in specific brain sites declines progressively during maturation [78]. It is well-known that during postnatal development sensory experiences play critical roles in the refinement of cortical connections, therefore, degeneration of central axons and terminals of peripheral sensory neurons lead to intrinsic somatosensory deprivation and, in turn, functional and structural alterations in the CNS**.** Furthermore, capsaicin treatment causes significant changes in substance P content and the number of muscarinic-, dopamine1-, serotonin-, and cannabinoid receptor binding sites [169, 170]. It seems that TRPV1 receptors comprise a neuromodulatory system in the brain, operated by endovanilloids. Since several endogenous cannabinoid lipids (*e.g.* anandamide, N-arachidonoyl-dopamine) can activate the TRPV1 receptors, it may be assumed that at least a part of their behavioral effects are due to the activation of these receptors [171-174]. Their activation causes anxiogenic behaviors, while their pharmacological blockade leads to anxiolytic effects and changes in fighting behavior [79, 175, 176]. The extensive reduction of afferent information together with the damage of the areas involved in memory processes should consequently bring out cognitive disorders after capsaicin desensitization. Several earlier studies suggested that TRPV1 receptors might play an important role in memory functions mainly at the hippocampal level, but the results are controversial [65, 79, 81, 177-179]. TRPV1 receptor activation can modify both LTP and long term depression (LTD) at the hippocampal level, and LTP is damaged in TRPV1-deficient mice [81, 178, 180]. Since both LTP and LTD are presumed to play a part in the establishment of stable memories, the manipulation of TRPV1 receptors can influence these processes [79, 181, 182]. It is supposed that capsaicin and endogenous lipids can potentiate the GABA-dependent depression in the CA1 region of the hippocampus, by the activation of presynaptic TRPV1 receptors in GABAergic hippocampal nerve terminals [183]. Furthermore, rats treated with high dose capsaicin as neonates had reduced hippocampal volume and cortical thickness and they exhibited signs of learning impairment [81, 184]. However, microinjections of low dose capsaicin into the dorsal hippocampus prevented acute stress induced memory impairments [185]. These data suggest that TRPV1 channels may be a potential target for protecting both hippocampal synaptic plasticity and spatial memory retrieval.

The NOR task is based on the spontaneous novel object preference of rodents. A reduction in novel object recognition might be interpreted as a recall memory deficit, and the underlying process is a possible analogue of declarative memory in humans

[186-188]. Anatomically, this task is assumed to depend on the hippocampus, the nigrostriatal dopaminergic pathway and rhinal cortex [189, 190]. Both postweaning isolation and NMDA antagonist treatment can lead to impairment in the NOR test, but the results are controversial in this respect, too [48, 140, 157, 157, 191-195]. We have found impairment in the NOR test in treated animals of both the complex model and the desensitized group, *i.e.* the ability to discriminate between novel and familiar objects was disturbed; thus, we assume that both genetic, environmental and pharmacological factors play a role in the memory deficit. However, other memory test (*e.g.* T-maze or holeboard) also should be applied to characterize further memory deficits in detail.

5.4 Motor activity

Altered motor activity has been also reported in schizophrenia. Depending on the disease subtype, psychopathology and medication, excessive motor agitation, reduced motor activity, even akinetic episodes are observed [6, 196-200]. Both the dopaminergic and the glutamatergic systems in the prefrontal and subcortical areas are involved in these abnormalities [201]. Postweaning social isolation increases activity in novel environments, but data are controversial and the effect depends on the strain of rodents [136-138, 140, 192, 193, 202-206]. Most studies investigated motor activity during isolation, but social isolation by itself did not produce long-term changes in motor activity [106, 134]. Some reports suggest motor disturbances after NMDA antagonist treatment too, but the results are inconsistent, and the effect depends on the age of the animals [48, 161, 194]. Cessation of treatment in adult or juvenile rodents did not cause gross changes in motor activity, while early postnatal treatment was effective in this respect [48, 50, 130, 207]. Beninger's laboratory investigated the effects of subchronic MK-801 treatment and postweaning social isolation on motor activity [86, 87, 208]. Postweaning social isolation enhanced locomotor activity, while MK-801 treatment alone, did not alter it, but blunted the amphetamine-induced hyperlocomotion. The combination of the two manipulations did not produce detectable additive or synergistic effects on behavior.

In the present study, the complex analysis of motor activity during the NOR test revealed that the selective breeding decreased overall motor activity but increased the grooming behavior and no activity changes were observed in the capsaicin desensitized animals. Ketamine treatment + social isolation induced increased exploratory activity in both naive and selected groups. Interestingly, the complex treatment in selectively bred

animals resulted in an altered motor phenotype with decreased rearing and walking activity, accompanied by increased exploratory and grooming activities. The enhanced grooming behavior can indicate anxiety, and might present a useful strategy to investigate stress-related responses in animal models of neuropsychiatric disorders [209-211]. To clarify these results regarding the different aspects of motor behavior, further investigation of motor behavior is necessary in these animals.

During telemetry monitoring we have found that the juvenile capsaicin desensitized animals showed increased activity during active phase under freely-moving circumstances. There are studies indicating that motor activity can be suppressed by the activation of TRPV1 receptors, for instance, low doses of capsaicin, its various analogs and anandamide inhibit ambulation, stereotypic behavior and activity in the open field test in a capsazepine-reversible manner [212-214]. Furthermore, dopamine transporter KO animals showed hyperlocomotion accompanied with decreased anandamide levels and upregulated TRPV1 receptors in the brain [85]. Findings about the effect of capsaicin desensitization on motor activity are inconsistent. Some authors found no major differences between capsaicin- or vehicle-treated animals in spontaneous and novelty-induced grooming, or in open-field exploration after either neonatal or adult capsaicin desensitization [175, 184]. Others observed that neonatally desensitized rats were hyperactive in new environments, and this hyperactivity was abolished by haloperidol [61]. A recent study found that TRPV1 KO animals showed slightly increased motor activity observed with the Minimitter device [184], while an earlier study showed that the deletion of the TRPV1 gene causes no changes in locomotion in the open field [81]. Most of the studies used short observation periods, sometimes as short as 15 min. Since we did not find significant differences in the activity in NOR test paradigm during a short period either, we suppose that brief investigation in these tests can not reveal the fine disturbance in motor activity. However, the telemetric method allows the long-term investigation of motor behavior of freely moving animals in their home cages. These data, together with earlier results, suggest that capsaicin desensitization can disturb the motor behavior for a long period, and as a putative explanation it is be proposed that a tonic activation of TRPV1 channels suppresses the general locomotor activity. Thus, the desensitized animals exhibited hyperactivity, as seen in certain types of schizophrenia.

6. Conclusions

We developed a new substrain of rats by selective breeding after juvenile isolation and ketamine treatment showing several signs which resemble those found in schizophrenia. Our present results confirm that selective breeding is still one of the most fundamental and effective methods for the assessment of complex traits influenced by multiple genes.

Reduced pain sensitivity, disturbed sensory gating, altered motor activity and decreased memory function were observed in the 15^{th} generation of the substrain. The summarized score based on categorization revealed that the selectively-bred and treated animals differed most markedly from the naive, non-treated rats. This suggests that genetically pre-disposed traits together with environmental risk factors resulted in the most prominent impairment relative to naive animals with no environmental perturbation.

We firstly showed that juvenile capsaicin desensitization caused complete and long-lasting abolishment of eye-wipe response and blepharospasm, as well as the urinary bladder capacity changes, suggesting the percent disturbance of TRPV1 receptors containing axons. Juvenile capsaicin desensitization did not change significantly heat and mechanical pain sensitivity; however, morphine produced a prolonged decrease in the nociceptive response to inflammation in capsaicin treated animals. The desensitized animals showed slight learning impairments and higher levels of activity indicating that capsaicin desensitization can cause some behavioral changes related to schizophrenia.

In conclusion, further breeding is required to improve our animal model. Molecular biological studies are also required to reveal changes of various neurotransmitter systems and genetic abnormalities. We suppose that capsaicin desensitization together with other treatments (*e.g.* social isolation or ketamine treatment) could further improve the model. However, more work is needed to fully appreciate the role of TRPV1 receptors in the CNS and hence, the potential central consequence of the pharmacological targeting of this channel with either agonists or antagonists with therapeutic activity.

We suggest that the resulting rat line and complex treatment may serve as a potentially powerful model for the examination of the gene-environment interaction in the development of schizophrenia, and it can contribute to identify the symptoms and action mechanisms of the disease.

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Appendix