Development and characterization of chronic animal models of schizophrenia

Summary of Ph.D. Thesis

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1. Introduction

Schizophrenia is a devastating psychiatric disorder that impairs mental and social functions and affects approximately 1% of the population worldwide. 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. Several other, non-specific sings can also be observed in schizophrenia; such as sensory gating disturbance, motor behavioral changes and decreased pain sensitivity.

Genetics play a major role in the etiology of schizophrenia, several "structural" chromosomal abnormalities have been described in this disorder, however, none of the identified risk genes are specific to schizophrenia. Dopaminergic, serotoninergic, glutamatergic and GABAergic deficits have been proposed as pathophysiological factors in schizophrenia. The 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. The NMDA receptor system has an effect on several transmitter systems in the cortico-limbic-striatal network, and also plays a crucial role in brain plasticity during early development, therefore, the developing brain is more susceptible to a chronic, low-dose blockade of NMDA receptors: it causes synaptic weakening and elimination by over-pruning in several brain regions, including the prefrontal cortex and the hippocampus. These genetic, biochemical and neurodevelopmental disorders make people more vulnerable, which may further impair by psychological (eg. stress, trauma) and environmental (social status, family, maternal behavior) effects. Gene-environment interactions have an important role in the development of psychiatric disorders, but the exact pathomechanism is still unknown.

In order to understand the biological mechanisms underlying a complex disorder and in search of novel drug targets, valid animal models are necessary. There are four main groups of chronic animal models for schizophrenia: pharmacological-, lesion-, environmental- and genetic models, and a few studies applied certain combinations of these. 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. There are many developmental models at prenatal or postnatal life,

which cause disorders of different brain structures. Large body of evidence has accumulated to suggest that postweaning social isolation, as environmental model, has profound, long-term effects on rodent brain and behavior. 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. As a pharmacological method, NMDA receptor antagonists (e.g. ketamine, phencyclidine, MK-801) worsen symptoms in schizophrenia or can induce schizophrenia-like symptoms in normal individuals, furthermore the capsaicin desensitization or the lack of transient receptor potential vanilloid1 (TRPV1) receptors can also lead to different behavioral changes, some of which are related to schizophrenia, but investigations into these effects have been scarce. 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 subjects with schizophrenia.

Thus, 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.

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. Methods

Animals

Wistar rats were used for the experiments. To generate and validate a new substrain of rats with signs related to schizophrenia, we used selective breeding after postweaning (at 3 weeks of age) social isolation (between 4-7 weeks of age) and chronic ketamine treatment (30 mg/kg intraperitoneally, from 5 to 7 weeks of age) through several generations. At the end of these interventions, animals were re-housed in a group setting and 1 week of recovery followed. Behavioral assessment started at the age of 9 weeks. 5-7 animals of both sexes with impaired pain sensitivity, sensory gating and memory were selected for the further breeding lines. Male rats of the 15^{th} generation were involved in the present experiment and four experimental groups were compared: naive socialized rats without any treatment (NaNo), or with isolation and ketamine treatment (NaTr) and 15^{th} generation selectively bred animals without any treatment (SelNo), or with isolation and ketamine treatment (SelTr).

In the capsaicin model, male Wistar rats after weaning, were treated on four consecutive days with increasing doses of capsaicin (10, 20, 50 and 100 mg/kg subcutaneously; s.c.) or its vehicle. From these animals four experimental series were formed.

Tests

We performed the wiping test and measured the urinary bladder to verify the effect of capsaicin desensitization. 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.

Ultrasound examination of the urinary bladder was used by sonography, and the bladder volume was estimated by substituting the diameters into the ellipsoid equation formula.

Acute nociceptive threshold was assessed by the TF test. The reaction time was determined by immersing the distal 5 cm portion of the tail in hot water (46, 48 and 52 °C) until a tail-withdrawal response was observed.

Mechanical and thermal inflammatory pain sensitivities were recorded with von Frey (VF) and the paw-withdrawal (PWD) tests in the capsaicin model. The baseline pain thresholds were detected and then unilateral inflammation was induced by intraarticular injection of carrageenan (300 μg / 30 μl) into the right ankle joint. 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.

Sensory gating, as measured by prepulse inhibition of the acoustic startle reflex (PPI), were applied in both models; three different trial types: a PULSE ALONE (PA; 95 dB white noise burst); PREPULSE ALONE (PPA; 20 ms 76 dB); and the PREPULSE-PULSE PAIR (PP) was presented 10 times. %PPI values were calculated as percentages using the following formula: %PPI = [1 − (*startle response for PP trial*) / (*startle response for PA trial*)] × 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)).

Novel object recognition test (NOR) is used to evaluate cognition and motor behavior in both animal models. The following parameters were scored in each phase (habituation, sample and test phases): frequency of occurrence of stereotypic behaviors (such as rearing and selfgrooming), and the time of exploratory activity of sample (S) and new objects (N), walking and inactivity. 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*]).

The telemetry method is appropriate to monitor gross locomotor acitivity in freely moving animals for long period. In the capsaicin model the animals were peritoneally implanted with Minimitter transmitters under ketamine-xylazine anesthesia. After a one-week recovery period the animals were placed on receiver platforms and motor activity was monitored continuously for 5 days.

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

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. As for the effect of capsaicin desensitization on bladder capacity, ANOVA with repeated measures revealed a significant effect of treatment and time ($p < 0.05$), thus capsaicin treated animals had larger bladder volumes compared to the control group.

Pain sensitivity

Acute heat pain sensitivities

Regarding the selectively bred animals, ANOVA revealed a significant effect of strain ($p< 0.05$), time ($p<0.001$) and significant interaction ($p<0.05$) on the TF latencies measured at 3 and 9 weeks of age; thus, the TF latency significantly increased in all groups with time. 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.

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

Inflammatory pain sensitivity and efficiency of morphine

Regarding the threshold for mechanical allodynia, significant effects of treatment ($p < 0.005$), side ($p < 0.001$), time ($p < 0.001$) 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.

In the case of thermal hyperalgesia, significant effect of treatment ($p < 0.001$), side ($p < 0.001$), time ($p < 0.001$) 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. Furthermore, morphine caused a significant increase in the nociceptive threshold on the non-inflamed paw in the capsaicin-pretreated animals.

Sensory gating

ANOVA revealed a significant effect of prepulse stimulation ($p < 0.0001$), and strain ($p < 0.05$) 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. Regarding the sensory gating, the effect of strain was significant ($p<0.005$); thus, both groups of the substrain (SelNo and SelTr) had lower %PPI compared to the naive groups (NaNo and NaTr). 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. Therefore, the %PPI did not show significant differences between the two groups.

Motor behavior

Regarding the motor behavior during NOR test in the complex model ANOVA revealed a significant effect of strain ($p<0.05$), phase ($p<=0.001$) and interaction between phase and strain ($p<0.05$) on the rearing activity. Rearing activity decreased with time (phase) in all groups, and the 15^{th} 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 ($p<0.05$). Analysis of walking duration revealed a significant effect of strain ($p<0.01$), phase ($p<0.001$) and a phase-treatment interaction ($p<0.05$); thus, walking activity decreased with time (phase), and it was lower in the new substrain, while the NaTr group showed enhanced activity in the sample and test phases. No significant differences were observed in the any types of motor behavior (e.g. rearing, grooming) and inactivity in any phases between the two groups in the capsaicin model. However, in the telemetry the separate analysis of dark and light phases showed a significant effect of treatment ($p < 0.05$) and a close to significant effect of phase ($p = 0.06$;), which is to say that the desensitized animals exhibited enhanced motor activity during the active phase compared to control rats.

Memory functions in novel object recognition test

Both NaTr and SelTr groups showed an increased exploring time of the objects. As for the DI, ANOVA revealed a significant effect of phase and 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.

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. 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 the presence of the new object in both groups of capsaicin series.

Categorization

ANOVA revealed significant differences between the four groups ($p<0.001$) in the summarized score of the different groups, *i.e.* the NaNo group had the lowest score, while the SelTr group scored the highest. 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.

5. Discussion

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; *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.

Sensory functions

Clinical reports pointed out that many patients with schizophrenia are less sensitive to pain. Our study demonstrated that selective breeding led to a significant increase in pain threshold, but the complex treatment 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. The presence of persistent changes in this 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. 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. 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. However, the antinociceptive effect of morphine was enhanced and prolonged during joint inflammation in both mechanical and thermal tests; it might be due to the decreased nociceptive input to dorsal horn neurons because of the absence of TRPV1-expressing

afferent fibers. Furthermore, sensory dysfunction was indicated by impairment of urinary bladder function as well. Capsaicin desensitization enhanced the bladder capacity, suggesting that sensory transmission from the bladder in the micturition reflex depends on TRPV1 receptors at all stages of development.

Sensory gating

Previous studies revealed that repeated NMDA-antagonist treatment of neonatal or adult rats, or long-term postweaning social isolation or selective breeding may lead to the disruption of PPI in some but not all the animals; however, the data are somewhat inconsistent. It is assumed that changes in the prefrontal cortex and/or imbalances between neural connections within the cortico-striato-limbic circuitry lead to the observed PPI disturbances. 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; which suggests that genetic factors played the major role in the development of PPI disturbance.

The only piece of literature we have found on the effect of neonatal capsaicin desensitization on PPI supports our finding that desensitization has no effect on PPI. The ineffectivity of capsaicin desensitization 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 in different conditions.

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. The extensive reduction of afferent information together with the damage to the areas involved in memory processes should consequently bring about 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. Furthermore, rats treated with high dose capsaicin as neonates had reduced hippocampal volume and cortical thickness and they exhibited signs of learning impairment. 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. 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.

Motor activity

Altered motor activity has been also reported in schizophrenia. Both the dopaminergic and the glutamatergic systems in the prefrontal and subcortical areas are involved in these abnormalities. 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.

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. 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 some types of schizophrenia.

6. Conclusion

1. 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.
2. Reduced pain sensitivity, disturbed sensory gating, altered motor activity and decreased memory function were observed in the 15^{th} generation of the substrain.
3. 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.
4. 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.
5. 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. 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 TRPPV1 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 this disease.

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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

Poster related to the Thesis

1. Horváth G, Petrovszki Z, Kékesi G, Benedek G, Kéri S. Behavioral changes in a new substrain developed by selective breeding. (MITT 2013.)

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

3. 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)

4. 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.)