VASOPRESSIN-DEFICIENT BRATTLEBORO RAT AS A SCHIZOPHRENIA MODEL: VALIDATION AND POSSIBLE CONTRIBUTING MECHANISMS TO THE DEVELOPMENT OF SCHIZOPHRENIA-LIKE BEHAVIOR

Ph.D. theses

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1 Introduction

Schizophrenia (SCZ) is a chronic mental disorder, which has not only a negative influence on the patient's life, but also on its entire family. Moreover, SCZ has serious economic disadvantages. Take into consideration the high prevalence of SCZ worldwide (0.4 - 0.87 %), and the failures in its treatment further studies are needed on the field.

SCZ symptoms can be grouped into three main categories: *positive* (behaviors not seen in healthy people: hallucinations, delusions, disorganized speech, and behavior), *negative* (disrupted normal behaviors like diminished emotional expression, avolition) and *cognitive* ones (disturbances in working and spatial memory) (Diagnostic and Statistical Manual of Mental Disorders: DSM-V). According to DSM-V, at least two out of the five major symptoms (delusions, hallucinations, disorganized speech, grossly disorganized or catatonic behavior, or negative symptoms) have to last more than a month to diagnose SCZ and one of them has to be delusions, hallucinations or disorganized speech.

The main causes of SCZ are still unclear, and available therapies cannot improve cognitive deficits and have small effect on negative symptoms of SCZ, therefore new drugs should be developed. According to the current state of science, animal models are essential for the preclinical investigation of a new drug to explore the underlying mechanisms as well as to be able to develop new treatment options.

A promising genetic model of SCZ might be the **vasopressin (AVP) deficient Brattleboro rat** (di/di, homozygous for diabetes insipidus). Indeed, AVP was associated to SCZ disorder at many points. Furthermore, Brattleboro rat mimics core behavioral symptoms of SCZ (e.g. sensorimotor gating-, and communication deficit, memory impairment), shows molecular changes (upregulation of striatal dopamine-2 receptors) and has response to antipsychotic treatment like in humans.

All in all, this model may be the most suitable to explore the broad role of this neuropeptide in SCZ-like disturbances. Therefore, we aimed to fully characterize this AVP-deficient rat model.

2 Objective

Our main aim was to confirm the role of centrally released AVP in the development of SCZ-like behavior using AVP-deficient (di/di) Brattleboro rat.

First (**Exp. 1**) to ensure predictive validity of the model we investigated if antipsychotic treatment (acute, subacute, or chronic) normalize SCZ-like behavioral alterations (memory impairment, social avoidance, PPI-deficit) in adult animals.

Rat pups, due to their more permeable blood-brain-barrier (BBB) and low body weight seem to be a good model for novel antipsychotic testing. In relation to SCZ maternal separation-induced ultrasonic vocalization (MS-USV) can be considered as a symptom of reduced communication. We tested if the reduced MS-USV of di/di pups can be improved by antipsychotic treatment (**Exp.2**) (paper is under evaluation in Neuropsychopharmacology). Sedative (by righting reflex and negative geotaxis) and stress hormone elevating (ACTH, corticosterone) side effects were excluded. Next (**Exp.3**) the possible contributing AVP receptor type was investigated in MS-USV.

To study possible brain mechanisms, we investigated (**Exp.4**) the contribution of magnocellularly released AVP in social behavior using AVP rescue in SON by adeno-associated viral vector. As another possible mechanism (**Exp.5**) epigenetic changes (histone H3K9Ac modification) were also examined in the PFC, nucleus accumbens, LS and HC of the Brattleboro rat brain.

3 Methods

3.1 Subjects

Brattleboro rats were maintained at the Institute of Experimental Medicine in a colony started from breeder rats from Harlan, Indianapolis, IN, USA.

We compared 56-70 days old male AVP-deficient di/di with homozygous (+/+) control rats (**Exp. 1, 4 and 5**). Rats were housed 2 animals/cage under standard laboratory conditions (temperature: 23±1°C; relative humidity: 50-70 %) with rat chow and tap water available ad libitum. In Experiment 4, animals were housed isolated for safer healing of surgical wound.

In **Exp. 2 and 3** we compared 7-8-day-old Brattleboro pups from the same litters with homozygous AVP deficient (di/di) fathers and heterozygous (di/+) mother rats; in this way di/+ and di/di rats were born. The litter size was not controlled and in this case the gender was not taken into consideration. The genotype of the pups was assessed after the experiment upon the AVP content of the pituitary by radioimmunoassay. In **Exp. 3** Wistar rats were also used: they were purchased from Charles River (Budapest, Hungary) and were mated in the local animal facility. Pups were housed with the dam in their home cage under standard laboratory conditions (temperature: 23±1°C; relative humidity: 50-70 %). The day/night schedule was 12/12h, with lights on at 07:00h.

All experiments were carried out between 10:00 and 14:00 in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were reviewed and approved by the Animal Welfare Committee of the Institute of Experimental Medicine, Budapest, Hungary.

3.2 Experiment 1.: Do antipsychotic drugs can improve behavioral deficits in Brattleboro rat?

3.2.1 Manipulations

Rats were injected subcutaneously with an antipsychotic drug or vehicle every day for 15 days. On the 1st (D1, acute), 8th (D8, subacute), and 15th (D15, chronic) experimental days rats were injected 30 minutes before the behavioral tests. Different doses were used for *aripiprazole* (5 mg/kg; Gedeon Richter Plc., Budapest, Hungary), *clozapine* (1 mg/kg; Sigma Chemical Co., St. Louis, MO), *haloperidol* (0.1 mg/kg; Sigma Chemical Co., St. Louis, MO), *olanzapine* (0.3 mg/kg; Gedeon Richter Plc., Budapest, Hungary), and *risperidone* (0.25mg/kg; Sigma Chemical Co., St. Louis, MO) (n~6-8 in every group). Aripiprazole was dissolved in 5% acetic acid-saline solution while other drugs in 50 µl of 1N HCl per ml of saline solution and titrated with 3N NaOH to pH 6-7. Control animals got vehicle (HCl or acetic acid solution).

3.2.2 Experimental design

Social discrimination was tested comparing control, haloperidol, clozapine, olanzapine, and risperidone treated animals, while aripiprazole treatment was done separately using appropriate controls. The five different treatment was conducted in five "shifted" series testing approx. 20 animals/day containing rats from each treatment groups while the injections of the series were overlapping. As there was no significant difference

between the controls (HCl or acetic acid containing saline) during subsequent testing HCl containing vehicle was used only. Then - in line with other drugs - we studied the effect of aripiprazole instead of clozapine because this later antipsychotic drug has a similar receptor binding profile to olanzapine and has been extensively tested in previous preclinical studies.

Social avoidance and PPI tests were conducted on the same day right after each other in control, aripiprazole, haloperidol, olanzapine and risperidone treated animals. To avoid influential effect of one test over the other, half of the animals underwent first social avoidance followed by PPI, while the other half was tested in PPI first.

3.2.2.1 Social discrimination test

Social discrimination was performed in a new environment (plexi cage with bedding, 41.3 × 26 × 29.8 cm, GeoMaxi, Ferplast, Italy). Subjects were habituated to the experimental cage for 1 h. 25-30 days old male Wistar rats were used as stimulus animals. The test started with a 4 min sampling phase, when a juvenile (Stim1) was introduced to the test-cage. After 4 min Stim1 was removed and kept individually in a fresh cage with food and water ad libitum. Thirty minutes later - during the 4 min choice phase - test subjects were faced with the already familiar Wistar pup, Stim1, and an unknown, similar one (Stim2). Tests were videotaped and investigatory behavior was analyzed by an event analyzing software (H77) by an experimenter blind to treatment conditions. To allow the observer to distinguish between the two juveniles, one of them was marked with green lines (Edding 30 permanent marker, odorless, green, Edding AG, Germany) at least 30 min before testing. To exclude preference or aversion toward the marked animals we randomized the marking between Stim1 and Stim2. Investigatory behavior defined as direct action towards the juvenile rat including anogenital sniffing, hunting, licking, close pursuing and pawing. A significantly longer investigation duration of Stim2 versus Stim1 during choice phase is interpreted as a manifestation of an intact recognition memory. The discrimination index (DI) was calculated as follows:

DI = (time percentage Stim2 - time precentage Stim1)/(time percentage Stim 1 + time percentage Stim 2).

The result of the index may change between -1 and 1, where 0 = no discrimination. Normally, the animals spend more time with the new stimulus (novelty effect), thus, the index ≤0 is a sign of memory deficit.

3.2.2.2 Social avoidance test

Social avoidance test was introduced by our group as a measure of social anxiety. The test cage contained two subcompartments connected by a sliding door. The cages were open on the upper part; their walls were 40 cm high. The experimental animal was placed into the smaller cage (surface: 15 cm by 50 cm) for 3 min habituation. The larger cage (surface: 40 cm by 40 cm) was divided to two equal compartments by a transparent, perforated plastic wall. The distant compartment contained an unfamiliar, adult +/+ male rat. After the habituation period, the sliding door was removed, and the experimental animal was allowed to explore the small as well as large cages for 5 min. Plastic cages were cleaned with water after each test. The test apparatus did not permit physical contact between the experimental and stimulus animals. Subjects had a clear view of the stimulus rat when the door was removed, but not before. Behavior was videorecorded from above and analyzed by an experimental blind to treatment conditions. Three variables were recorded: the duration of visits made to the compartment containing the opponent, time spent with social interaction, and the latency of social interaction.

3.2.2.3 Prepulse inhibition (PPI) test

We performed the test as described previously. After a weight calibration, subjects were placed in a test cage inside a sound attenuated chamber (Acoustic Startle setup, Coulbourn Instruments, LLC). Following 5 min habituation, subjects were presented a 40 ms long, 120 dB acoustic stimuli ("noise", referred as pulse) for five times in every 20 s to standardize startle. Five trial types were then presented during testing: pulse alone, pulse preceded 80 ms by a 20 ms prepulse of varying intensity (73, 77 or 81 dB "tone"), and a trial with no prepulse but 0 dB pulse. Each trial-type was presented five times in a randomized order. The program automatically recorded the startle responses. Response to the 0 dB pulse was the weight of the subject and was subtracted from subsequent startle response data. Mean of the startle response to the 120 dB pulse without prepulse was calculated for every subjects and served as acoustic startle response. This response was considered 100%, from which PPI was calculated by the following formula: PPI = 100 – (startle after prepulse / startle without prepulse * 100). Mean PPI values were given for different prepulse intensities. In the present study the different prepulse intensity had no significant effect and did not modify the effect of genotype or treatment either, therefore we use the average of the three prepulse intensity for each day and treatment.

3.3 Experiment 2.: Ultrasonic vocalization measurement of Brattleboro rat pups during antipsychotic treatment

3.3.1 Manipulations

Pups were injected subcutaneously with 1µl/g antipsychotic drug or vehicle 30 minutes before the MS-USV test. Different doses were used for *haloperidol* (0.1 mg/kg, 1 mg/kg; Sigma Chemical Co., St. Louis, MO), *clozapine* (1 mg/kg, 10 mg/kg; Sigma Chemical Co., St. Louis, MO), *olanzapine* (0.3 mg/kg, 3 mg/kg; Gedeon Richter Plc., Budapest, Hungary), *risperidone* (0.25mg/kg, 1mg/kg, 0.05 mg/kg, 0.1 mg/kg; Sigma Chemical Co., St. Louis, MO) and *aripiprazole* (0.5 mg/kg, 5 mg/kg, 50 mg/kg; Gedeon Richter Plc., Budapest, Hungary). Haloperidol, clozapine, olanzapine and risperidone were dissolved in 50 µl of 1N HCl per mL of saline solution, while aripiprazole was dissolved in 5% acetic acid-saline solution and titrated with 3N NaOH to pH 6-7. Control animals got vehicle (HCl or acetic acid).

3.3.2 Experimental design

3.3.2.1 Maternal separation-induced ultrasonic vocalization

On the day of the study 7-8-day-old rat pups and their dam were moved from the animal housing room to another room and left undisturbed for at least 1 h before initiating the behavioral test. In order to minimize maternal effects, pups from the same litter were randomly assigned to treatments. Pups from at least three different mothers were used for each experiment. Pups received an injection of the drug or vehicle (1µl/g) one after the other in every 12 min, they were marked and returned to the dam and littermates. Thirty minutes after the administration one pup was brought to a soundproof room and placed in a 2l glass beaker without bedding and heating. USV was measured for 10 min. Individual calls during this period were detected using an ultrasound-sensitive frequency division detector (CDB205, CIEL Electronique, Nice, France) fixed on a holder 25 cm above the bottom of the glass beaker coupled to a computer. The distance between the USV detector and the beaker was 10 cm. Vocalizations were recorded using a free Audacity 2.0.5. software and stored on a personal computer. In previous studies, the large portions of ultrasonic vocalizations emitted by 8-day-old rats were found from 30 kHz to 50 kHz. Thus, signals were filtered and the power spectrum was analyzed ranging from 30 kHz to 50 kHz. Data were automatically counted using a Rat Call Counter software (developed by S. Zsebők). The threshold value was set at a signal amplitude of 0.4 V to

exclude background noise. Total number and total duration of calls per session were measured. In addition, USV frequency was calculated as the total number /10 min.

3.3.2.2 Righting reflex

Immediately after MS-USV, *righting reflex latency (RRL) test* was used for investigating sedative effect of the drugs. Offspring were placed into an unusual supine posture on a smooth, flat surface and the latency to return the normal upright position with all four feet on the table was measured manually. Each animal was allowed a maximum of 30 s to return to prone position.

3.3.2.3 Negative geotaxis

Negative geotaxis test investigated impaired motor coordination. After RRL measurement, offspring were placed on a 45° inclined foam-rubber board with their nose pointing downward and its latency to turn 180° to an upright position was recorded in seconds. Each animal was allowed a maximum of 60 s on the apparatus to complete the task. If the rat fell off the plane, the rat was considered to have failed the task and a maximum score of 60 s was assigned.

Both tests were repeated three times at the termination of the measurement of USV and the mean of the three measurements was taken as a typical parameter.

3.3.3 Hormone measurements

Animals were sacrificed, and from blood serum samples ACTH and corticosterone concentrations were measured by specific radioimmunoassay (RIA). Both antibodies were developed in our Institute as described elsewhere. The intra-assay coefficients of variations were 4.7% and 7.5%, respectively for the two hormones.

The hypophysis of the pups was also collected to determine the AVP content thereby the genotype. The preparation of the samples was as following: they were placed in a boiling water bath for 5 min, and then homogenized by ultrasound, centrifuged, and AVP content was measured from the 100-fold diluted supernatant using specific RIA as described earlier. The rabbit antibodies were donated by Dr. M. Vecsernyés (Szent-Györgyi Medical University, Szeged, Hungary). The limit of detection was 1 pg AVP/assay tube. The intra-assay coefficients of variation was 10.7%.

All the samples from a particular experiment were assayed in the same RIA to avoid interassay differences.

3.4 Experiment 3. Vasopressin receptor antagonists on maternal separation-induced ultrasonic vocalization

3.4.1 Manipulations

Brattleboro rat pups born from heterozygous (di/+) mothers and homozygous diabetes insipidus (di/di) fathers were injected ip. with physiological saline 30 min before MS-USV.

Wistar rat pups were injected with V1aR antagonist (SSR49059), V1bR antagonist (SSR149415) or V2R antagonist (SSR121463B) (a generous gift from the Sanofi-Synthélabo company), which were suspended in 0.4 % Tween 80, then injected ip. 30 min before USV in three different concentrations: 3, 10 or 30 mg/kg. In a further experimental series 10 mg/kg V1aR antagonist was mixed with 10 mg/kg V1bR antagonist. Control treatment was the solvent in 1µl/g volume washed with 15µl saline.

3.4.2 Experimental design

3.4.2.1 Maternal separation-induced ultrasonic vocalization

See 3.3.2.1.

3.4.2.2 Righting reflex

See 3.3.2.2.

3.4.2.3 Negative geotaxis

See 3.3.2.3.

3.4.3 Hormone measurements

See 3.3.3. Additionally, only Brattleboro, but not Wistar rat pup hypophysis was collected for determination of the genotype (di/+ or di/di disposition based upon AVP content).

3.5 Experiment 4.: Magnocellular AVP in social behavior

To rescue magnocellular AVP synthesis, AVP-containing adeno-associated virus (AVP-AAV) vector was injected into the SON of AVP-deficient Brattleboro rats (di/di). We compared +/+, di/di and AVP-AAV treated di/di male rats.

3.5.1 Manipulations

Anaesthesia was performed by an intraperitoneal (i.p.) injection of a mixture of ketamine (50 mg/kg, SelBruHa Allatgyogyaszati Ltd., Budapest, Hungary), xylazine (20 mg/kg, Spofa, Prague, Czech Republic) and promethazinium chloratum (0.2 ml/kg, EGIS, Budapest, Hungary) dissolved in physiological saline.

Anaesthetised rats were fixed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). To rescue local AVP synthesis in the Brattleboro rat, AVP-AAV (Type 2, CMV-GH-SP-AVP-NP-GP-SVPA, $7x10^9$ genome copies/µl, 100 nl/injection site) was bilaterally injected into the SON of di/di rats (stereotaxic coordinates from Bregma: AP: -0.4 mm, ML: +/-1.8 mm, DV: +9.7 mm) (later referred as di/di-AVP). Controls (both +/+ and di/di animals) were injected with saline on the same day and in the same volume as no proper control virus was available.

Only those di/di-AVP animals were included in experiments, which showed a significant drop in water consumption (7 out of 10), as in this strain water consumption clearly defines the correct hits. We considered the drop of water consumption as a proof for successful functional rescue of AVP synthesis in the SON and we therefore started to test the animals two weeks after treatment. The immunohistochemical data confirmed the rescue (in the SON of +/+ rats 23% of the cells showed AVP positivity, while in di/di-AVP animals 17.5%).

3.5.2 Experimental design

3.5.2.1 Social behavioral experiments

Our studies revealed that the MeA, a nucleus known, among others, for its involvement in the control of social behavior in rodents, responded to our rescue of AVP synthesis in the SON. We therefore studied the impact of AVP-AAV SON treatment on social behavior. Eighteen days after AVP-AAV injection the animals were tested for their social investigatory behavior and three days later for aggression.

3.5.2.1.1 Social investigation

More than two weeks after AVP-AAV injection, the rats were transferred to a new cage with fresh bedding 1 h before starting the test. The test consisted of a 4-min exposure to a previously not encountered conspecific juvenile (Wistar rat, ~25 days old). The duration of investigatory behavior of the adult towards the juvenile was measured online by a trained observer blind to the animal's group, using an events recorder (EVENTLOG

1.0 written by Robert Hendersen 1986). Investigatory behavior was defined as the direct action of the adult towards the juvenile rat including anogenital sniffing, hunting, licking, pawing and close pursuit.

3.5.2.1.2 Resident-intruder test (RI)

Subjects were kept in GeoMaxi cages for three days. Rats were then exposed in these (home) cages to smaller, unfamiliar Wistar opponents for 20 min. Their behavior during the exposure was videorecorded and scored later by an experimenter blind to treatment conditions. Behavioral analysis focused on the consummatory phase of aggressive behavior i.e. on biting attacks. The results of the quantitative measures (i.e. attack counts and latency) are presented, as we failed to detect differences in qualitative measures (i.e. attack type and context).

3.5.3 AVP immunohistochemistry

Rats were deeply anaesthetised, then they were transcardially perfused. After overnight post-fixation brains were transferred into a cryoprotective 30% sucrose solution in PBS for 2 nights at 4°C and then stored at –20°C until sectioning. Sequential 30-µm-thick coronal sections of the hypothalamus containing the SON and PVN were cut with a sliding microtome and divided into 6 parallel slice series.

Brain slices containing the SON and PVN were selected and stained for AVP. The AVP antibody was generated by Tamás Görcs. Secondary antibodies were purchased from Jackson ImmunoResearch. Cell nuclei in the slices were visualized by Hoechst 33258 (1:20 000; Sigma-Aldrich). Slides were covered with Mowiol (Sigma-Aldrich).

Images were taken by Nikon C2 confocal laser scanning microscope in the Nikon Microscopy Centre at the Institute of Experimental Medicine in Budapest.

3.6 Experiment 5.: Epigenetic changes in Brattleboro rat brain

3.6.1 Immunohistochemistry

For brain tissue treatment see 3.5.3. Anti-histone H3 (Acetyl-Lys9) antibody (host: rabbit, 1:5000; SAB4500347, Sigma–Aldrich, Inc., Hungary) was used to label this specific epigenetic change. H3K9ac immuno-positive cells were visualized by nickel enhanced 3,3’-diaminobenzidine (DAB).

Microscopic images were digitized by OLYMPUS CCD camera, and stained particles were counted by means of the ScionImage software.

3.7 Statistical analyses

Data were analyzed by two-way ANOVA for factor genotype and treatment using the Statistica 12.0 program of StatSoft, Inc., Tulsa, OK, USA. Post hoc comparison of the data from different experimental groups was performed by the Newman– Keuls test or Fisher Least Significant Difference method and the results were presented on the figures. For DI (discrimination index) a single sample t-test against 0 (no discrimination) was conducted for each treatment group separately. Correlations were calculated by the Pearson method. Multiple regression analysis was conducted to determine the contribution of behavioral and hormonal changes to MS-USV. Data are presented as mean ± S.E.M. The level of statistical significance was taken as $p < 0.05$ in all statistical analyses.

4 Results

4.1 Experiment 1.: Do antipsychotic drugs can improve behavioral deficits in Brattleboro rat?

4.1.1 Social discrimination test

Influence on social interest: During the 4 min sampling period, di/di rats spent the same or even more time with investigating a juvenile (Stim1) as control, +/+ rats (genotype effect). Hence, all rats had something to remember.

We have to admit, however, that antipsychotic treatment diminished the time spent with investigation (treatment effect). This was significant for all (acute, subacute and chronic) aripiprazole and haloperidol treatment, but also after repeated clozapine, olanzapine and risperidone treatment. Thus, after repeated treatment we observed sensitization in contrast to the expected habituation. Nevertheless, this - presumably sedative - effect equally affected +/+ and di/di animals (no genotype x treatment interaction). Therefore, any memory difference between genotypes cannot be attributed to this phenomenon. Moreover, even the lowest sampling time was above 20% (roughly 1 min), which should provide enough time for sampling.

Detection of SCZ-like symptoms: During the whole observation period (i.e. acute, subacute and chronic treatment) vehicle treated di/di rats had poor short term memory as the value of DI was not significantly differed from zero. In contrast the +/+ control group

had intact social memory throughout. Aripiprazole and olanzapine were able to normalize the social memory deficit of di/di rats without influencing the intact memory of the +/+ animals. This effect was independent of the treatment duration. Haloperidol treatment was not effective at all, while the effect of clozapine and risperidone treatment were controversial, significantly increasing the DI in di/di, but reducing it in +/+ rats at most timepoints.

4.1.2 Social avoidance test

Compartment preference: None of the animals preferred the unknown, large compartment containing a sexually mature, male stimulus animal (less than 50% time spent here). During repeated testing (1 week apart) the interest increased (time effect). The treatment (except aripiprazole) significantly reduced the time spent in the "social" compartment, suggesting detrimental side effect. Nevertheless, there was no difference between genotypes and the genotype did not modify the effect of the treatment or time.

Detection of SCZ-like symptoms: On D1 there was a significant difference between vehicle treated di/di and control group in the duration of direct interaction with the stimulus animal. This difference disappeared during subsequent tests. After the 8th vehicle injection the di/di rats started the social interaction significantly later than respective controls. Similar difference was not visible after the 1st or 15th injections.

All treatment reduced the time spent with the stimulus animals. Logically, this interaction started later, except for the aripiprazole treated groups, which was similar to control in this respect. Genotype had no effect and did not modify the effects of treatments either.

4.1.3 Prepulse inhibition test

Detection of SCZ-like symptoms: AVP-deficient vehicle-treated rats showed decreased PPI on each day of examination compared to +/+ C group.

Effects of antipsychotic drugs: In case of all treatments (except the 15th aripiprazole) the difference between the genotypes disappeared at all studied timepoints. However, the treated di/di rats became significantly different from vehicle treated one after repeated treatment only (except for olanzapine, where single treatment was also effective).

4.2 Experiment 2.: Ultrasonic vocalization measurement of Brattleboro rat pups during antipsychotic treatment

The genotype had a significant effect on the number and duration of emitted calls in every case with lower levels in di/di pups. The ACTH levels were always significantly lower in di/di rats than in di/+ animals, while corticosterone levels were always higher.

4.2.1 First-generation antipsychotic effect on MS-USV

Haloperidol treatment did not influence MS-USV, furthermore, it dose-dependently increased the ACTH and corticosterone levels without interaction with the genotype effect.

4.2.2 Second-generation antipsychotic effect on MS-USV

Lower doses of clozapine, olanzapine, risperidone, and aripiprazole normalized the emitted MS-USVs in di/di pups compared to di/+. On the contrary, higher doses reduced the vocalization of di/+ pups to the di/di level. Nevertheless, the higher, but not the lower doses were sedative during the behavioral tests and significantly enhanced the ACTH and corticosterone levels. There were no ACTH elevations in di/di animals.

4.3 Experiment 3. Vasopressin receptor antagonists on maternal separation-induced ultrasonic vocalization

4.3.1 Genetic AVP-deficiency

AVP-deficient Brattleboro rats (di/di) were less anxious based upon their number and duration of emitted calls compared to their heterozygous littermates. In connection their ACTH levels were significantly lower, without significant alterations in corticosterone levels. Righting reflex and negative geotaxis values were comparable in the two genotypes.

4.3.2 Pharmacological AVP-deficiency

4.3.2.1 V1aR antagonist

V1aR antagonist treatment decreased MS-USV in 30 mg/kg concentration only, while the corticosterone levels were significantly higher in the group with 30 mg/kg antagonist treatment. There was no difference between the groups in the latency of righting as well as negative geotaxis.

4.3.2.2 V1bR antagonist

V1bR antagonist treatment decreased the number of emitted calls accompanied by reduced ACTH levels without changes in corticosterone. There was no difference between the groups in the latency of righting and negative geotaxis.

4.3.2.3 V2R antagonist

3 mg/kg V2R antagonist enhanced MS-USV, while the higher doses had no effect on MS-USV. Both stress hormone levels were higher 45 min after a single 30 mg/kg V2R antagonist treatment compared to control injection group. There was no difference between the groups in the latency of righting as well as negative geotaxis.

4.3.2.4 V1aR+V1bR antagonists

The combination of V1a and V1bR antagonist effectively reduced MS-USV, without any effect on stress-hormones. The same dose of V1aR antagonist induced 34.3% and 26.8% non-significant reduction in MS-USV number of calls and duration, respectively, while in case of V1b 51.5% and 54.3% significant reduction was visible. The combination induced 57.1% reduction in MS-USV number of calls and 68.53% reduction in duration. There was no difference between the groups in the latency of righting as well as negative geotaxis.

4.3.3 Correlations

As it could have been expected the number and duration of emitted calls after maternal separation positively correlated with each other in all experimental series. Interestingly, the same was true for ACTH and corticosterone correlation except in case of Brattleboro animals, where there was no correlation at all. In Brattleboro rats the AVP content of their hypophysis showed a significant positive correlation with the MS-USV number of calls and duration. Moreover, in their case the serum ACTH level also showed positive correlation with their MS-USV number of calls. Interestingly, similar ACTH and MS-USV number of calls correlation was detected after V1bR antagonist treatment.

4.4 Experiment 4.: Magnocellular AVP in social behavior

In accordance with previous results AVP-AAV injection into the SON restored the local AVP synthesis both physiologically and functionally.

During social investigation test di/di-AVP rats spent more time investigating the conspecific juvenile than both +/+ and di/di animals.

In the RI test the quantitative measure of aggression (number of bites) was reduced in di/di-AVP rats compared to +/+, also showing a tendency to be lower in comparison with the di/di group.

4.5 Experiment 5.: Examination of epigenetic changes in Brattleboro rat brain by immunohistochemistry

The following brain regions were investigated: nucleus accumbens core (AcbC) and shell (AcbS) regions, prelimbic cortex (PrL), infralimbic cortex (IL) and dorsal peduncular cortex (DP), dorsal (LSD), intermediate (LSI) and ventral (LSV) part of the LS, as well as CA1, CA2 and CA3 fields of the dorsal HC.

In di/di animals AcH3K9 immunostaining showed significantly less labeled cells in DP of the PFC than in +/+ rats. In the other investigated PFC areas (PrL and IL) no differences were detected between the genotypes. AcH3K9 immunohistochemistry did not show significant differences between di/di and +/+ animals neither in the whole Acb nor in the separately investigated core and shell. In LS only in LSV compartment had significantly less AcH3K9 immunopositive cells in di/di than in +/+ rats. In CA1 there was significantly more AcH3K9 labeled cell in di/di rats than +/+, while in CA2 and CA3 there was no genotype effect.

We correlated the values of the investigated brain areas with each other. There was a significant positive correlation between DP area and AcbC, LSI and LSV. The core region of the accumbens significantly correlated with CA3, while the shell region positively correlated with LSD, LSI and LSV.

5 Conclusions

Our studies have extensively examined AVP-deficient Brattleboro rat as a possible model of SCZ from several perspectives to confirm its suitability for preclinical research.

We confirmed that clinically effective antipsychotics alleviated SCZ-like symptoms in adult, male AVP-deficient Brattleboro rat. Thus, therapeutic response of the strain to medication is similar that in human SCZ patients. Therefore, (additional to its *face* and

construct validity) based upon its *predictive validity* this animal might be a good preclinical model of SCZ. Although PPI was assumed to model cognitive deficit, it may resemble also positive and negative symptoms. Additionally, SD models also negative symptoms. As new drugs should target the presently untreatable negative and cognitive symptoms, this might be a special strength of this model as di/di Brattleboro rats showed deficit presumably in these domains.

Besides, 7-8-day-old di/di Brattleboro rat pup can be a good model for the communication deficit of SCZ. This animal model might be an excellent opportunity to test newly developed drugs: all antipsychotics used in our experiment restored low MS-USV in AVP-deficient animals at lower doses. Furthermore, MS-USV of pharmacologically produced AVP-effect deficient Wistar rat pups was also reduced compared to controls and it positively correlated with pituitary AVP level, confirming the role of this neuropeptide in separation-induced vocalization.

AVP originating from the SON might contribute to the fine-tuning of social behavior neuronal network of rodents, possibly via a pathway involving the MeA.

All experienced behavioral deficits in di/di Brattleboro rats are possibly occurred through epigenetic changes, which also might reinforce the validity of di/di rat as a model of SCZ. However, further studies are needed to investigate this possible connection.

6 List of own publications

Publications related to the dissertation:

Török, B., C. L. Fazekas, A. Szabo, and D. Zelena. **2021**. "Epigenetic Modulation of Vasopressin Expression in Health and Disease." Int J Mol Sci 22 (17). doi: 10.3390/ijms22179415 **IF: 5.92**

Török, B., A. Fodor, B. Klausz, J. Varga, and D. Zelena. **2021**. "Ameliorating schizophrenia-like symptoms in vasopressin deficient male Brattleboro rat by chronic antipsychotic treatment." Eur J Pharmacol 909:174383. doi: 10.1016/j.ejphar.2021.174383. **IF: 4.43**

Török, B., A. Fodor, S. Zsebok, E. Sipos, and D. Zelena. **2021**. "The Effect of Vasopressin Antagonists on Maternal-Separation-Induced Ultrasonic Vocalization and Stress-Hormone Level Increase during the Early Postnatal Period." Brain Sci 11 (4). doi: 10.3390/brainsci11040444. **IF: 3.11**

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Other publications:

Fazekas, CL., Bellardie, M., Török, B., Sipos, E., Tóth, B., Baranyi, M., Sperlágh, B., Dobos-Kovács, M., Chaillou, E., and Zelena, D. **2021**. "Pharmacogenetic excitation of the median raphe region affects social and depressive-like behavior and core body temperature in male mice." Life Sci. 2021 Dec 1;286:120037. doi: 10.1016/j.lfs.2021.120037. **IF: 5.037**

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Fazekas, C. L., E. Sipos, T. Klaric, **B. Török**, M. Bellardie, G. N. Erjave, M. N. Perkovic, G. Lauc, N. Pivac, and D. Zelena. **2020**. "Searching for glycomic biomarkers for predicting resilience and vulnerability in a rat model of posttraumatic stress disorder." Stress 23 (6):715-731. doi: 10.1080/10253890.2020.1795121. **IF: 3.10**

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