# Pathophysiology of purinergic receptors: Investigation of the role of the P2X7 and $P2Y_{12}$ receptors in animal disease models

Ph.D. thesis

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## 1. Introduction

Numerous studies have been published on the importance of purinergic neurotransmission, in which ATP as a neurotransmitter molecule is involved. In pathophysiological conditions, the extracellular level of ATP is increased, which activates further signalization pathways through purinergic receptors. The ATP molecule is the ligand of P2X and P2Y purinergic receptors with different structural and signaling properties. These purinergic receptors are expressed distinct cells of the central nervous system (CNS), such as neurons, astrocyte and microglia cells, so the stimulation of these receptors may have a prominent role in the disorders of the CNS. Based on this approach, P2X and P2Y purinergic receptors may have outstanding importance as potential therapeutic targets in the research of CNS diseases. Our laboratory has focused on the investigation of two purinergic receptor subtypes. One of them is the $P2Y_{12}$ receptor subtype, which is probably involved in the pathophysiology of neuroinflammation and different forms of pain. The other one is the P2X7 receptor, which was examined in a number of animal models of neuropsychiatric disorders, like depression, epilepsy and Parkinson's disease, but until now, have not investigated in the pathophysiology of schizophrenia.

Schizophrenia is a severe, chronic mental disorder with approximately 1% prevalence in the human population. The diagnosis of this neuropsychiatric disorder is complicated because of its complex symptomatology. Guidelines of the diagnostic critera of schizophrenia are published by the World Health Organization (WHO) and the American Psychiatry Association (APA). The phenotype of the disease is not uniform, because several circumstances, like genetic heterogeneity, neurodevelopmental disturbances and environmental factors may all play a role in the development of the pathophysiology of schizophrenia. The molecular background of the disorder was first hypothesised by dysfunction of neurotransmitter systems. Earlier studies recognized the potential involvement of the dopaminergic system, but more recently, the possible participation of glutamatergic, GABAergic and purinergic neurotransmitter systems were also proposed in the pathophysiology of schizophrenia. Nowadays, the treatment of the disease is still not sufficient, because of the extrapyramidal and other side effects of common applied antipsychotics. Therefore, research on new potential therapeutic targets

to the indication of schizophrenia have outstanding importance to improve the quality of life of schizophrenic patients.

Furthermore, neuroinflammation processes may also have possible role in the pathophysiology of several neuropsychiatric disorders. It is suggested that among purinergic receptors, $P2Y_{12}$ receptors may be involved in chronic inflammatory and neuropathic pain, therefore it would be a potential therapeutic target in these indications. So far, the possible role of $P2Y_{12}$ receptors in pathophysiology of neuropsychiatric disorders has not been studied yet. Nonetheless, in the future, it may be worthwhile to investigate the potential involvement of $P2Y_{12}$ receptors in the neuroinflammatory changes underlying schizophrenia.

## 2. Objectives

Research of our laboratory is directed at the understanding of the pathophysiological role of the purinerigic system in the CNS. The objective of my study was to examine the involvement of P2X7 receptors, in the phencyclidine (PCP), as an NMDA (N-methyl-D-aspartate)-type glutamate receptor antagonist, induced animal model of schizophrenia. In addition, inflammatory processes may also have a prominent role in the diseases of the central nervous system and in different forms of pain. Accordingly, the possible role of $P2Y_{12}$ receptors in chronic inflammatory pain was also examined.

**I. Investigation of the potential role of the P2X7 receptor in the phencylclidine (PCP)-induced animal model of schizophrenia. We asked the following questions:**

- In mouse behaviour experiments, we asked are there any effect of the genetic deletion or antagonism of the P2X7 receptor on the schizophrenia-like behavioural pattern of mice?
- In gene expression studies, we asked are there any alterations in the basal P2X7 receptor mRNA expression levels in the prefrontal cortex (PFC) and hippocampus (HPC) during the postnatal development of mice?
- Further gene expression experiments were designed to answer does the PCP treatment impact on the the P2X7 receptor mRNA expression levels in the PFC and HPC of young adult mice?

- In earlier studies, it was demonstrated that the stimulation of P2X7 receptor has effect on the glutamate release from HPC tissue slices of mice. Therefore, we aimed at investigating whether does the PCP treatment influence the P2X7 receptor-mediated glutamate release from PFC slices of mice?
- In addition, we aimed at studying further the relationship of P2X7 receptors and NMDA-type glutamate receptors. Hence, we asked whether genetic deletion or antagonism of the P2X7 receptor affect the NMDA-induced ion currents in PFC of the mouse brain?
- Finally, we aimed at investigating, whether do the deficiency of P2rx7 gene and/or the PCP treatment have an impact on mRNA expression levels of schizophrenia-related genes in PFC and HPC samples of mice?

**II. Investigatiion of the potential role of the $P2Y_{12}$ receptor in the complete Freund's adjuvant (CFA)-induced animal model of chronic inflammatory pain.**

- We aimed at studying what is the effect of the genetic deletion of $P2Y_{12}$ receptors on protein expression levels of distinct inflammatory mediator proteins after CFA-induced chronic inflammation?

## 3. Methods

### 3.1. Animals

The maintenance and application of the experimental animals were carried out according to the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Institute of Experimental Medicine Hungarian Academy of Sciences (IEM HAS) (Budapest, Hungary, ref. No. PEI/001/778–6/2015). In most of our experiments young adult (around 2 months old), male wild-type (P2rx7 +/+) and P2X7 receptor knockout (P2rx7 −/−) mice were used. Homozygous P2X7 receptor P2rx7 +/+ mice were bred on a background of C57Bl/6J. The original breeding pairs of P2rx7 −/− mice were kindly supplied by Christopher Gabel from Pfizer, Inc. (Groton CT, USA). In each experiment of gene expression and electrophysiology younger animals were applied as well. In further experiments, 60-70 days old male wild-type (P2ry12 +/+) and $P2Y_{12}$

receptor knockout (P2ry12 −/−) mice were applied. These mouse strains are bred in the Medical Gene Technology Unit of IEM HAS among specific, pathogen-free conditions.

### 3.2. Behavioural studies

All of the behavioural experiments were carried out by Behavioural and Stress Studies Laboratory of IEM HAS with the management of József Haller PhD. Two distinct experimental setups were performed. At first, mice with different genotypes (P2rx7 +/+ és P2rx7 -/-) were treated intraperitoneally (i.p.) with two distinct doses of PCP (2 or 5 mg/kg, Sigma-Aldrich Kft, Budapest) or with its vehicle (sterile saline, 0.9 % NaCl solution). At the other experimental layout, the wild-type mice were subjected to an i.p. injection of P2X7 receptor selective antagonist, JNJ-47965567 (30 mg/kg i.p., Janssen Research és Development, San Diego, USA) or its vehicle (30% β-cyclodextrin i.p., Cydex Pharmaceuticals, Lawrence, USA) 30 minutes before the PCP (1.5 - 5 mg/kg) or its vehicle administration. 45 mins after the treatments, 10 mins long behavioural tests were performed and the following parameters were measured: distance travelled (locomotor activity), stereotype behavoiur, ataxia and social interaction. Experiments were accomplished in a dark-grey, circular open field. Two unfamiliar mice after the same pharmacological treatment were placed at opposite sides of the apparatus. Each experiment was recorded for 10 mins with a video camera was fixed above the open field. The pharmacological treatment type was unknown for the analyst of behaviour tests. Social interactions were defined as sniffing directed towards the unfamiliar mouse, when the nose of the scored mouse touched (or was very close to) the body of it. Line crossings and social interactions were recorded for the whole duration of experiments by a computer-based event recorder. Animals were randomly selected to different treatment groups (10-12 mice per group).

### 3.3. *In vitro* [$^3$H]glutamate release studies

All experiments were performed by tissue perfusion techniques on PFC slices of young adult wild-type (P2rx7 +/+) and P2X7 receptor genetically deficient (P2rx7 -/-) mice. The animals were received a single i.p. injection of PCP (2 mg/kg) or its vehicle 60 mins before the tissue isolation. The mice were anaesthetized under light $CO_2$ inhalation,

and subsequently decapitated. PFC tissue sample were place into an ice-cold Krebs solution saturated with 95% $O_2$ and 5% $CO_2$. 400 μm tissue slices were made by McIlwain tissue chopper and incubated in 1 ml of modified Krebs solution (113 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, and 11.5 mM glükóz, pH 7.4) with the presence of [$^3$H]glutamate ([$^3$H]GLU) for 45 mins. The medium was bubbled with carbogen gas (95% $O_2$ and 5% $CO_2$) and maintained at 32 °C. After the loading incubation period the slices were continuously perfused with carbogen gas-saturated modified Krebs solution (flow rate: 0.7 ml/min). The washout period following perfusion have taken 90 mins to remove excess radioactivity, perfusate samples were collected over 3 min periods and analyzed for the tissue tritium content. Two distinct stimuli were tested to evoke [$^3$H]GLU efflux. In one experiment layout after 6 mins of the first collection of the perfusate, PFC slices were superfused with P2X7 receptor agonist, BzATP (100 μM, Sigma) for 3 mins without the presence of $Mg^{2+}$. In the other test setup instead of BzATP electrical field stimulation (10 Hz, 1 msec) was applied. After the stimulation period normal Krebs solution was perfused through the PFC slices until the end of the collection period.

### 3.4. *In vitro* electrophysiological studies

All experiments were accomplished by Department of Pharmacology and Pharmacotherapy of Semmelweis University, with the management of László Köles MD, PhD. Prefrontocortical tissues of young (16-20 days old) of wild-type and P2X7 receptor knock-out mice were applied for electrophysiological investigations. Brains of the animals were quickly removed after decapitation and placed into ice-cold artificial cerebrospinal fluid (aCSF) saturated with carbogen gas (95% $O_2$ and 5% $CO_2$) of the following composition (mM): NaCl 126, KCl 2.5, $NaH_2PO_4$ 1.2, $CaCl_2$ 2.4, $MgCl_2$ 1.3, $NaHCO_3$ 25 and glucose 11; pH 7.4. A vibrating blade microtome was used to cut thin coronal slices (200 μm thickness) from a block of tissue containing the prelimbic portion of the medial PFC using. From a single brain of each mouse 6-8 slices were cut out, and transferred to a holding chamber and stored in oxygenated aCSF at 36 °C for 1 h, and then at room temperature (22–24 °C). Directly before use, individual slices were placed into a recording chamber (300–400 μl volume) and continuously perfused (3 ml/min) with oxygenated, $Mg^{2+}$-free aCSF at room temperature. Before all single experiment each slice

was incubated at least 15 minutes. Only one cell in individual brain slice was measured by whole-cell patch-clamp technique. An upright microscope with a ×40 water immersion objective (Axioscope FS; Carl Zeiss) was applied to visualize pyramidal cells in layer V of the PFC. Patch pipettes are made from borosilicate glass capillaries were filled with intracellular solution (potassium gluconate 140 mM, NaCl 10 mM, $MgCl_2$ 1 mM, HEPES 10 mM, EGTA 11 mM, Mg-ATP 1.5 mM, Li-GTP 0.3 mM; pH 7.3 adjusted with KOH solution). Pipette resistances were in the range of 5–7 MΩ. In order to establish the diffusion equilibrium between the patch pipette and inside of the cell the system was left alone for 5–10 min. Currents were registered at a holding potential of − 70 mV, in the voltage-clamp mode of the patch-clamp amplifier (Axopatch 200B; Molecular Devices). To evoke NMDA-type glutamate receptor-mediated currents, NMDA was applied in serial dilution (1-1000 μM). NMDA solutions with distinct concentrations were perfused for 1.5 min, in increasing concentrations. Between two different concentrations of NMDA solutions drug-free aCSF was administered for 10 mins. In those experiments when the influence of the P2X7 antagonist were examined, it was present in the superfusion medium throughout the experiment. All data were filtered at 2 kHz with the inbuilt filter of Axopatch 200B, digitized at 5 kHz, and for the analyisy Digidata 1200 interface and pClamp 10.0 software (Molecular Devices) was applied.

### 3.5. Gene expression studies

Prefrontal cortex and hippocampus tissue samples derived from 4, 18, 35 and 56 days old naive P2rx7 +/+ mice (8 animals/group) were used to investigate the age-related gene expression levels of P2X7 receptors. Examination of the single injection of two distinct doses of PCP (2 or 5 mg/kg, Sigma-Aldrich Kft, Budapest) effects on P2X7 receptor mRNA levels in PFC and HPC, young adult (56 days old) wild-type mice were applied, the animals of the control group were treated with its vehicle (sterile saline, 0.9 % NaCl solution). Young (18 days old) and young adult (56 days old) wild-type (P2rx7 +/+) and P2X7 receptor genetically deficient (P2rx7 -/-) mice were used to study the mRNA expression levels of schizophrenia associated genes in PFC and HPC brain regions, after PCP treatment (2 or 5 mg/kg) of the animals. Approximately 1 hour before the PFC and HPC tissue isolation mice were subjected to a PCP or its vehicle injection. The tissue samples were stored at -70°C until their utilization.

Total RNA isolation from the PFC and HPC samples were performed by RNeasyLipid Tissue Mini Kit (Quiagen, Valencia Ca; kat. szám: 74804) according to manufacturer's protocol. To determine the total RNA concentration and the integrity of the RNA samples, the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) was applied with Agilent RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). To synthesize the first strand cDNA from the RNA samples Tetro cDNA Synthesis Kit (Bioline USA Inc, Taunton, MA) was used with the accordance of the producer's guideline. The relative quantifications of target genes mRNA expression levels were accomplished by quantitative real-time PCR analysis using a ViiA™ 7 Real-Time PCR System (Thermo Fisher Scientific Inc, Waltham, MA, USA). All real-time PCR experiments were completed by the standard protocol of the manufacturer applying TaqMan® Gene Expression Assays (Thermo Fisher Scientific Inc, Waltham, MA, USA) of the target genes. These assays contain primer pairs and Taqman® detection probes (Thermo Fisher Scientific Inc, Waltham, MA, USA) are specific for the requested nucleotide sequence. The mRNA expression level of each gene was normalized to expression level of glyceraldehyde 3-phosphate dehydrogenase gene (Gapdh), as a housekeeping gene.

### 3.6. Multiplex bead array analysis of inflammatory cytokines

To investigate inflammatory mediator protein levels, hind paw samples of young adult, male wild-type and $P2Y_{12}$ receptor knockout (P2ry12 −/−) mice were used. The experimental animals get an intraplantar injection to their right hind paw of freshely prepared complete Freund's adjuvant (CFA, Sigma-Aldrich) solution or its vehicle (0.9% NaCl solution). Tissue samples were collected before (Day 0) and after (Day 3 and 14) the CFA or saline treatment. For further processing the tissue samples were stored in -80°C. The homogenization of the samples were applied in buffer with the following contents: 50 mmol/L Tris-HCl, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 0.02% $NaN_3$, 1% Triton X, Protease Inhibitor Cocktail Set I (Merck KGaA, Darmstadt, Germany), pH 7.4. After tissue homogenization and centrifugation (27000 g, 15 min), supernatants were collected. To measure the total protein concentration of the samples, Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) was applied, and the absorbance was detected at 560 nm with a Perkin-Elmer Victor 3V 1420 Multilabel Counter (Perkin-

Elmer). The levels of inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-10, TNF-α) and one chemokine (CXCL1(KC)) were determined by BD Cytometric Bead Array (CBA) Flex Sets (BD Biosciences, New Jersey, USA). Measurements were performed on a BD FACSVerse flow cytometer, and data were analyzed with FCAP ARRAY v5 software (Soft Flow, Burnsville, MN, USA). The examined cytokine levels were normalized to total protein levels of the samples.

## 4. Results

### 4.1. Influence of the genetic deficiency and pharmacological blockade of P2X7 receptor on PCP-induced behavioural patterns

To investigate the effect of P2rx7 gene deletion and the antagonism of the P2X7 receptor on the PCP treatment-caused behaviour alterations following parameters were measured: distance travelled (locomotor activity), stereotype behavoiur, ataxia and social interaction. The lower dose of PCP (2 mg/kg i.p.) evoked significant changes on all evaluated behavioural patterns in wild-type (P2rx7 +/+) mice. The locomotor activity, stereotype behavoiur and ataxia were enhanced and social interaction activity was decreased by PCP application compared to the vehicle-treated group. Similarly, the higher dosage (5 mg/kg) of PCP had a greater significant effect on stereotype behavoiur and ataxia. However, the 5 mg/kg dose of PCP had an opposite impact in locomotor activity tests and did not altered the social interaction compared to the control group. The absence of P2X7 receptor had no effect on basal locomotor activity and stereotype behaviour in vehicle-treated animals. In contrast, the genetic deficiency of P2X7 receptor changed PCP-induced behavioural patterns. Significant difference was observed between the two genotypes in hyperlocomotion at the lower dose and in stereotype behaviour after the higher dose of PCP addition, and P2rx7 gene disruption alleviated the the effect of PCP. Interestingly, the genetic deletion of the P2X7 receptor enhanced the basal level of social interaction in saline-treated mice. The genetic deficiency of the P2X7 receptor did not alter the PCP-induced ataxia and social withdrawal in comparison with wild-type animals.

In further experiments the pharmacological antagonism of the P2X7 receptor was examined. Wild-type animals were pretreated with the blood-brain barrier permeable, P2X7 receptor selective antagonist (JNJ-47965567, 30 mg/kg i.p.) or its vehicle 30 min

before the PCP or its vehicle administration. Behavioural patterns of vehicle-pretreated mice after PCP addition was similar to previous observations in P2X7 receptor wild-type naïve animals. The lower dose of PCP treatment caused hyperlocomotion, enhanced stereotype behaviour, ataxia, and decreased social interaction time compared to saline-treated mice. In animals, subjected to higher dose of PCP, greater elevation was measured in stereotype behaviour and ataxia. Furthermore, the social interaction time was significantly decreased in response to both doses of PCP when compared with vehicle. Likewise to the effect of genetic deletion of P2X7 receptor, application of P2X7 receptor selective antagonist attenuated hyperlocomotion and stereotype behaviour in lower dose of PCP treated animals. Moreover, JNJ-47965567 eliminated the reduction in social interaction time caused by lower dose of PCP, but this effect was not observed after higher dose of PCP treatment. The PCP-induced alterations in ataxia was not modified by pharmacological blockade of the P2X7 receptor.

To summarize our results of mice behavioural experiments, both genetic deletion and antagonism of the P2X7 receptor could change the schizophrenia-like behavioural patterns, but not in all parameters and doses of PCP.

### 4.2. Developmental differences in P2X7 mRNA expression levels in the PFC and HPC of the mouse brain

P2X7 receptor relative mRNA expression levels were investigated in PFC and HPC regions of naive P2rx7 +/+ mice in four age groups (4, 18, 35 and 56 days old). No significant difference was observed among the gene expression levels of P2rx7 in PFC tissue samples of each age group. Nevertheless, significantly lower (around 30%) mRNA expression levels were detected in HPC regions of 18 days old animals in comparison with 4 and 56 days old mice.

### 4.3. Influence of PCP administration on P2X7 mRNA expression levels in the PFC and HPC of the mouse brain

Effects of two doses of PCP treatment (2 and 5 mg/kg i.p.) on P2rx7 gene expression levels were examined in PFC and HPC samples of wild-type mice. In the prefrontal cortex, the lower, 2 mg/kg dose of PCP slightly elevated P2X7 receptor mRNA level, whilst the higher, 5 mg/kg dose did not change the expression. In contrast, in the

hippocampus, 5 mg/kg dose of PCP lead to statistically significant upregulation in the gene expression level of P2rx7 compared to saline, whereas the 2 mg/kg dose of PCP was without effect . In these gene expression studies, greater basal P2X7 receptor mRNA expression levels were detected in HPC tissue samples than in PFC regions.

### 4.4. Effect of PCP treatment on P2X7 receptor mediated [$^3$H]glutamate release in the PFC of the mouse brain

One hour before the PFC samples isolation derived from mice with two distinct genotypes (P2rx7 +/+ és P2rx7 -/-), animals were subjected to a lower dose of PCP (2 mg/kg i.p.) administration. It is known from previous studies that the stimulation of P2X7 receptors evoked glutamate release from nerve terminals. Accordingly, two types of activation of P2X7 receptors were applied, and BzATP as a P2X7 receptor agonist and electrical field stimulation (EFS) were used on acute PFC tissue slices. There were no difference in basal [$^3$H]GLU release from PFC slices between mice groups with the two genotypes. After a 3 minutes perfusion of BzATP (100 μM) a 1.5-fold transient, reversible increase in [$^3$H]GLU release was observed in comparison with baseline values. This elevation in [$^3$H]GLU efflux was returned to the baseline after washout of the P2X7 receptor agonist. In PFC slices derived from PCP-treated animals, the basal [$^3$H]GLU release was higher than in mice were treated with vehicle. In P2X7 receptor wild-type mice, this baseline value of [$^3$H]GLU release was 1.58 ± 0.06% in the vehicle-treated group, while this value was 4.49 ± 0.25% in animals which subjected to PCP administration. Similar tendency was observed in PFC slices of P2rx7 knock-out mice, after saline treatment the detected baseline of [$^3$H]GLU efflux was 1.26 ± 0.05%, whilst in PCP-treated animals this value was 2.89 ± 0.62%. The BzATP-induced [$^3$H]GLU release elevation in PFC samples of P2rx7 +/+ mice was greater after PCP addition in comparison with animals which received vehicle previously (PCP: Δ[$^3$H]GLU = 2.8%, vehicle: Δ[$^3$H]GLU = 1.1%). In saline-treated animals, the EFS-induced [$^3$H]GLU release from PFC slices was not altered by the genetic deletion of P2X7 receptor compared to wild-type mice. Similarly, in PFC samples of mice subjected to PCP addition, P2rx7 genetic deficiency did not change the EFS evoked [$^3$H]GLU efflux.

### 4.5. Influence of the genetic deficiency and pharmacological blockade of P2X7 receptor on the NMDA-induced currents in the PFC region of the mouse brain

In whole-cell patch clamp studies, the effect of genetic deletion of P2X7 receptor or its antagonism by JNJ-47965567 compound on serial dilution of NMDA (1-1000 μM)-induced inward currents in layer V pyramidal cells of prefrontal cortex was investigated. There was no genotype-related difference in ion current amplitudes measured in cells after application of lower concentration range of NMDA (1-10 μM). In contrast, using higher concentrations of NMDA (>30 μM) significant difference was observed between ion current amplitudes in the two genotypes of mice. In layer V pyramidal cells of PFC of P2rx7 knock-out animals, the detected ion current amplitudes was smaller (1343 ± 238 pA) than in P2X7 receptor wild-type mice (2658 ± 504 pA) after addition of 30 μM of NMDA. To analyze the concentration-ion current amplitude curves of NMDA, the maximal efficacy ($E_{max}$) was greater in P2rx7 +/+ mice (4330 ± 1171 pA) compared to P2rx7 -/- mice (3224 ± 693 pA). Other regularly examined parameters of the concentration-response curves ($EC_{50}$: 24.98 ± 0.27 vs. 41.78 ± 0.26; Hill coefficient: 0.91 ± 0.58 vs. 0.85 ± 0.57; wild type vs. P2rx7− /− mice, respectively) did not show any significant variation. Besides these experiments, in pyramidal cells of PFC of P2X7 receptor wild-type animals the NMDA concentration-response correlation in the presence of P2X7 receptor antagonist, JNJ-47965567 (0.1 μM) was examined as well. In this experiment, the NMDA concentration-ion current amplitude curve was similar to determined curve in PFC of P2rx7 knock-out animals. In P2X7 +/+ mice the calculated $E_{max}$ value was smaller after application of JNJ-47965567 compound (2831 ± 360 pA) compared to absence of the P2X7 receptor antagonist, but the difference of tendencies was not statistically significant, because of the high variability of cells.

### 4.6. Effect of the genetic deficiency of P2X7 receptor on the PCP-induced gene expression alterations

In earlier studies it was described that changes in mRNA expression levels of genes plausible in pathomechanism of several psychiatric disorders could be influenced by the genetic deletion of P2X7 receptor. In our gene expression studies PFC and HPC tissue samples from PCP- or its vehicle-treated, young (18 days old) and young adult (56 days old) naive and P2rx7 -/- mice were collected. According to our previous results from

the examination of the PCP administration on P2X7 receptor mRNA expression levels, in case of PFC samples the lower dose of PCP (2 mg/kg) and in case of HPC region the elevated dosage of PCP (5 mg/kg) was used.

Therefore, to investigate alterations of gene expression levels in prefrontal cortex brain region, animals which subjected to the reduced dose of PCP addition were applied. In PFC samples of young mice, genes coding for each ionotropic, NMDA-type glutamate receptor subunit (Grin1, Grin2a, Grin2b) and one metabotropic glutamate receptor (Grm3) mRNA expression levels were not changed by either the genetic deletion of P2X7 receptor or the PCP treatment. In contrast, in PFC region of young adult animals, both deficiency of P2rx7 gene and PCP application influenced gene expression levels of genes mentioned above. Thus, in PFC samples of P2rx7 +/+ animals the expression level of gene coding for NR1 receptor subunit (Grin1) was approximately 40% lower in PCP-treated animals than after vehicle addition. Similar to the influence of PCP administration, in vehicle-treated P2rx7 knock-out mice Grin1 gene expression level was downregulated significantly compared to their wild-type littermates. Interestingly, in PFC regions of PCP-treated mice, the genetic deficiency of the P2X7 receptor caused an additional 25% significant reduction in the expression level of Grin1. The PCP treatment resulted 50% upregulation of genes coding for NR2A and NR2B receptor subunits (Grin2a and Grin2b) in PFC regions of P2X7 receptor wild-type mice in comparison to the animals are given vehicle. PCP-induced upregulation of Grin2a and Grin2b was decreased to the baseline by P2X7 receptor genetic deletion. However, in the absence of P2rx7 gene the expression levels of Grin2a and Grin2b were upregulated in PFC samples derived from animals subjected to vehicle treatment, but in the case of Grin2a this increase did not reach the treshold of significance. The expression level of gene coding for metabotropic glutamate receptor subtype 3 was significantly downregulated after PCP administration in PFC region of young adult mice with both genotypes compared to the vehicle-treated animals. In animals subjected to PCP the expression level of Grm3 gene was not altered by the deletion of P2rx7 gene.

Gene expression experiments were performed in hippocampal regions of elevated dose of PCP-treated (5 mg/kg) animals, because earlier this dosage of PCP had a significant influence on mRNA expression of P2X7 receptor in the HPC samples of mice. In this tissue region, slight changes in expression levels of Grin1, Grin2a, Grin2b and

Grm3 were observed by both genetic deletion of P2X7 receptor and PCP treatment. Grin1 gene mRNA expression levels were not modified either by P2rx7 gene knock-out or PCP application in young and young adult animals.

### 4.7. Effect of the genetic deficiency of $P2Y_{12}$ receptor on the protein levels of inflammatory mediators

In wild-type animals the complete Freund's adjuvant (CFA) treatment caused substantial increase in the protein levels of three examined proinflammatory cytokines (IL-1β, IL-6, TNF-α,) and in KC in the isolated hind paw samples on the 3rd and 14th day after the injection. Genetic deletion of $P2Y_{12}$ receptors reduced the CFA-induced accumulation in protein levels of the measured mediators the hind paw samples derived from both 3rd and 14th day. On the third day after the CFA treatment, in wild-type animals the injection of CFA effected significant, around 7- and 8-fold growth in the level of KC and TNF-α, respectively, whilst in P2ry12 -/- animals subjected to CFA these protein levels were 37% (KC) and 47% (TNF-α) lower. Similar tendency was observed in the levels of IL-6, but this reduction was not significant. The CFA treatment resulted a significant, 55% decrease in the level of IL-1α in P2ry12 +/+ animals compared to the vehicle treated mice. The level of IL-1α was under the detection limit in $P2Y_{12}$ knockout animals after subjected to CFA. Isolated hind paw samples from wild-type animals on the 14th day, the CFA treatment caused significant increase in protein levels of IL-1β, IL-6, TNF-α, KC compared to the vehicle treated group, and these levels were significantly lower in P2ry12 -/- mice. In P2ry12 +/+ mice, the level of IL-1α was significantly, 70% lower after CFA treatment, and this reduction was not changed by the genetic deletion of the $P2Y_{12}$ receptor. The anti-inflammatory mediator, IL-10 level was downregulated in samples of P2ry12 +/+ mice derived from both 3rd and 14th day after CFA treatment, and the genetic deficiency of $P2Y_{12}$ receptor had no effect on them. However, to compare the vehicle-treated groups in the hind paw samples derived from the 14th day, in the absence of $P2Y_{12}$ receptor the level of IL-10 was significantly, 85% lower in comparison with wild-type animals.

## 5. Conclusion

In our research, the possible role of purinergic P2X7 receptor in the pathophysiology of schizophrenia was investigated. Therefore, an ionotropic, N-methyl D-aspartate (NMDA)-type glutamate receptor antagonist, phencyclidine (PCP)-induced animal model of schizophrenia was used.

In mice behavioural experiments, th majority of PCP-evoked phenotypes (hyperlocomotion, stereotype behavior, social withrawal) were modified by genetic deletion and/or the antagonism of the P2X7 receptor. These observations suggested that the P2X7 receptors might modulate signaling mediated by NMDA-type receptor inhibition.

Identical PCP treatments had an effect on P2X7 receptor mRNA levels in the PFC and HPC in mice. In further experiments, it has been shown that PCP upregulated P2X7 receptors mediated glutamate release in prefrontocortical samples of mice. Moreover, the genetic deficiency and pharmacology blockade of P2X7 receptors attenuated NMDA-induced ion currents in layer V pyramidal cells of prefrontal cortex. These results can support the hypothesis that P2X7 receptor-mediated signaling processes have a tight connection with NMDA-type glutamate receptor-mediatiad signaling.

In our gene expression studies the effect of genetic deletion of P2X7 receptors on the alterations of PCP-evoked mRNA expression levels of schizophrenia-related genes was examined. Overall, the effect of the absence of P2rx7 on gene expression levels was observed mainly in the PFC region of mice. Based on our findings P2X7 receptors would have a prominent role in PCP-induced alterations of mRNA expression levels of each NMDA-type glutamate receptor subunits (NR1, NR2A, NR2B). Furthermore, both PCP treatment and genetic deficiency of P2X7 receptors had effect on gene expression levels of two subtypes of metabotropic glutamate receptors (mGluR2 and mGluR5) in PFC region, so the P2X7 receptors would have impact on signaling processes mediated by these types of glutamate receptors as well. Moreover, P2X7 receptors could modulate indirectly the GABAergic neurotransmission, because the PCP-evoked changes in expression level of $GABA_A$ receptor α1 subunit in the PFC region of wild-type animals, were not observed in the absence of P2X7 receptors. In addition, in the PFC area the deficiency in P2rx7 gene had the prevented PCP-induced increase in the neuregulin 1 coding gene (Nrg1), as a schizophrenia candidate gene mRNA expression level compared

to the P2X7 +/+ mice. It is well-known, that neuregulin 1 protein has regulatory role in neurotransmission and synaptic plasticity in several brain regions. Besides, neuregulin 1 is the ligand of ErbB4 receptor, which was detected in postsynaptic density of glutamatergic synapses and presynaptic terminals of GABAergic neurons, so neuregulin 1 protein could modulate the depolarization-dependent GABA release.

Based on our findings, the following hypothesis can be proposed (Fig.1.): (a) The stimulation of P2X7Rs by ATP/BzATP increased the release of glutamate in the prefrontal cortex. This release of glutamate and NMDA-receptor mediated currents were decreased in the absence or under the pharmacological blockade of P2X7 receptors. These data imply that postsynaptic NMDA receptors are subject to modulation by P2X7 receptors directly or indirectly. (b) The NMDA receptor antagonist, phencyclidine (PCP) evokes schizophrenia-like behavior, through the disinhibition of parvalbumin (PV) containing GABAergic neurons synapsing onto prefrontocortical pyramidal neurons, resulting in increased EFS-induced glutamate efflux detected in release experiments. In parallel with behavioral changes, the mRNA expression of Neuregulin 1 (Nrg1), different NMDA receptor subunits (Grin1, Grin2a, Grin2b), the $GABA_A$ Receptor $\alpha 1$ Subunit (Gabra1) and the metabotropic glutamate receptor 5 (Grm5) are also dysregulated, and all these alterations are subject to regulation by P2X7 receptors. In turn, PCP treatment upregulates and increases the functional responsiveness of P2X7 receptors, resulting in an increased BzATP-induced glutamate efflux.

In addition, we have studied the the role of $P2Y_{12}$ receptors in the regulation of the local cytokine response of the inflamed hindpaw in an animal model of chronic inflammatory pain. Genetic deletion of $P2Y_{12}$ receptors had profound effects on levels of proinflammatory mediators, which explain the antihyperalgesic effect detected under these conditions. Furthermore, it is suggested that it would be interesting to examine the potential role of $P2Y_{12}$ receptors in neuroinflammation processes underlying schizophrenia.

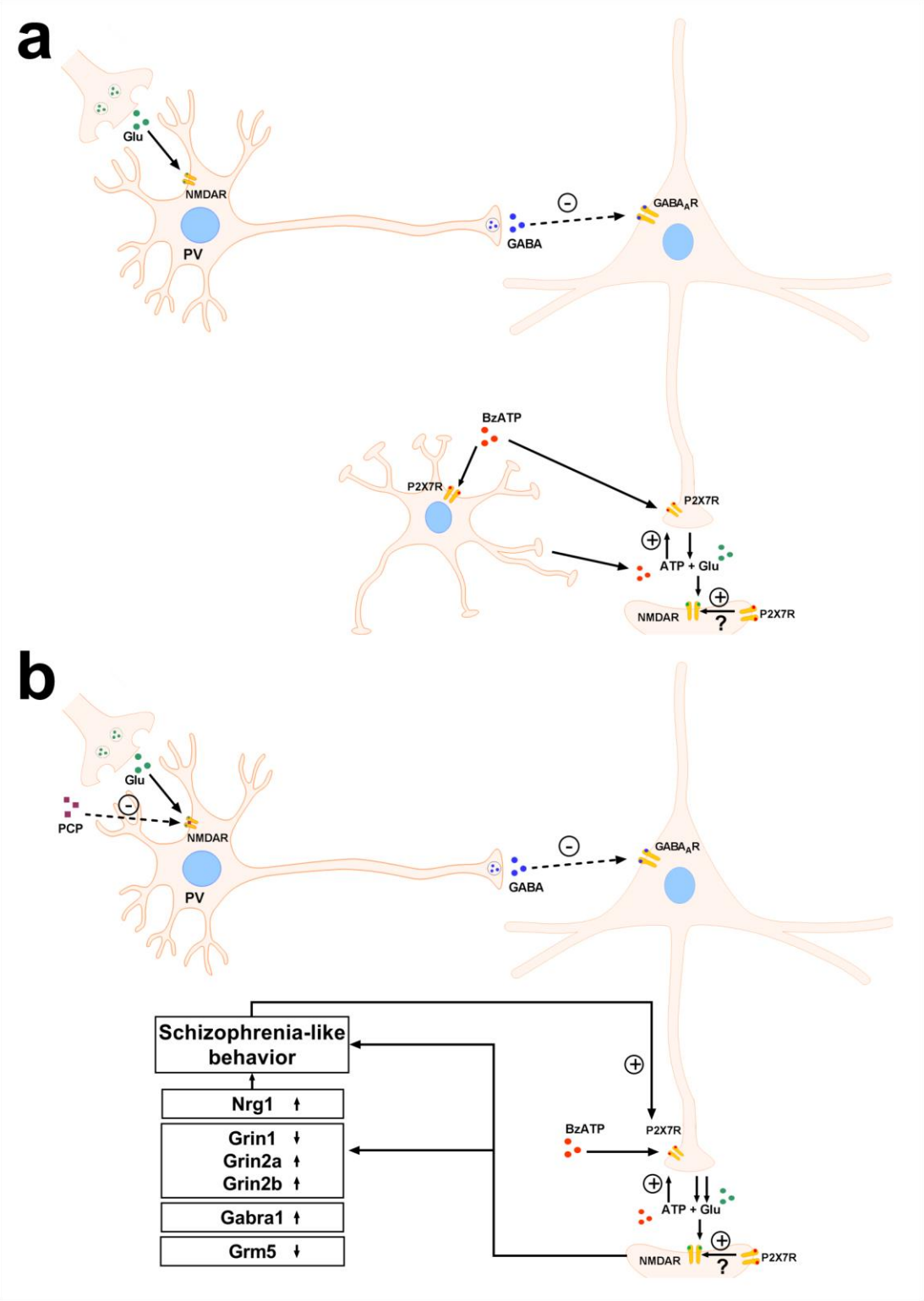


**Fig.1.: The hypothesis for the potential interactions of the P2X7 receptor with NMDA receptor regulation.**

## 6. Publications

### 6.1 Publications in the topic of the thesis

1. **Koványi, B**. Csolle C, Calovi S, Hanuska A, Kato E, Koles L, Bhattacharya A, Haller J, Sperlagh B. The role of P2X7 receptors in a rodent PCP-induced schizophrenia model. Sci. Rep. 6, 36680 (2016).

**IF: 4.259**

2. Bekő, K, **Kovanyi B**, Goloncser F, Horvath G, Denes A, Kornyei Z, Botz B, Helyes Z, Muller CE, Sperlagh B. Contribution of platelet P2Y12 receptors to chronic Complete Freund's adjuvant-induced inflammatory pain. J. Thromb. Haemost. (2017).

**IF: 4.899**

### 6.2 Other publications

1. 1. Horváth, G. Goloncser F, Csolle C, Kiraly K, Ando RD, Baranyi M, **Kovanyi B**, Mate Z, Hoffmann K, Algaier I, Baqi Y, Muller CE, Von Kugelgen I, Sperlagh B. Central P2Y12 receptor blockade alleviates inflammatory and neuropathic pain and cytokine production in rodents. Neurobiol. Dis. 70, 162–178 (2014).

**IF: 5.078**

2. Beamer, E. Goloncser F, Horvath G, Beko K, Otrokocsi L, **Kovanyi B**, Sperlagh B. Purinergic mechanisms in neuroinflammation: An update from molecules to behavior. Neuropharmacology 104, 94–104 (2016). (Review)

**IF: 5.012**

3. Nagy J, Kobolák J, Berzsenyi S, Ábrahám Z, Avci HX, Bock I, Bekes Z, Hodoscsek B, Chandrasekaran A, Téglási A, Dezső P, **Koványi B**, Vörös ET, Fodor L, Szél T, Németh K, Balázs A, Dinnyés A, Lendvai B, Lévay G, Román V. Altered neurite morphology and cholinergic function of induced pluripotent stem cell-derived neurons from a patient with Kleefstra syndrome and autism. Transl Psychiatry. 7(7):e1179 (2017).

**IF: 4.691**