The function of P2X7 receptor in animal models of central nervous system disorders

Doctoral theses

Lilla Otrokocsi

Semmelweis University

Szentágothai János Doctoral School of Neurosciences

Supervisor: Dr. Ágnes Kittel D.Sc. scientific advisor

Official reviewers: Dr. Viktor Román Ph.D. group leader

Dr. Júlia Timár Ph.D. associate professor

Chair of Exam Committee: Dr. László Hársing D.Sc. scientific advisor

Members of Committee: Dr. Ildikó Miklya Ph.D. associate professor

Dr. István Tarnawa Ph.D., group leader

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1. Introduction

Purinergic signalling has a fundamental role in cell-to-cell communication. In pathological conditions or after mechanical injuries extracellular ATP concentration becomes elevated activating purinergic receptors. The ionotropic P2X7 receptor is expressed by immune cells, neurons, astrocytes and microglia cells alike. Only high ATP concentration can induce the P2X7 functions, while extensive activation turns the receptor into a non-selective pore. They have a primary role in inducing inflammatory processes, starting cellular immune responses, and also triggering ROS production and pro-apoptotic genes, therefore P2X7 receptors are involved in cell death and neurodegeneration as well. The receptors modulate neurotransmitter release, since P2X7 activation elicits Ca^{2+} influx followed by increased glutamate and subsequent GABA release. In relation to these functions, P2X7 receptors are implicated in a variety of central nervous system disorders, such as Alzheimer's- and Parkinson's disease, major depression, bipolar disorder, epilepsy, migraine and schizophrenia. In my thesis I investigated the learned helplessness model of depression, the hypothesized role of purinergic signalling in autism spectrum disorder, and tested the efficacy of a newly synthetised MAO-B inhibitor compound in the rotenone induced *in vitro* Parkinson model. Parkinson's disease is a chronic illness caused by progressive neurodegeneration of the dopaminergic system, primarily causing motor symptoms (bradykinesia, resting temor, muscle rigidity), then affecting other transmitter signalling systems, leading to cognitive decline. Currently the therapy involves dopamine precursors or metabolism inhibitors, in order to restore the amount of dopamine in the body. Major depression is a mood disorder influencing a great proportion of the developed population. Its major symptoms are sadness, decreased energy or fatigue, irritability, loss of interest and motivation, feelings of helplessness, disrupted sleep or weight problems that affect the patients' everyday life. Therefore major depression lays severe social and financial burden besides the psychiatric issues. Most antidepressant drugs influence the disrupted monoaminergic neurotransmission, although their effect is often delayed or insufficient in several patients. Autism spectrum disorder is a neurodevelopmental disorder with genetic and environmental factors, and pre- or perinatal events interacting in the pathogenesis. At present, few therapeutic options are available; usually the behavioural

symptoms can be alleviated by psychosocial methods or with antipsychotic drugs in severe cases. All three investigated disorders have inadequate or insufficient treatment; therefore it is especially important to identify new drug targets regarding these anomalies.

2. Objectives

We investigated the influence of genetical and pharmacological inhibition of P2X7 receptor in two complex animal studies, the learned helplesness model of depression and the maternal immune activation model of autism spectrum disorder. Besides, we tested the protective effect of a newly synthetised MAO-B inhibitor in the rotenone induced *in vitro* model of Parkinson's disease.

I. Investigating the function of P2X7 receptors in the learned helplessness model of depression regarding behavioural changes and brain morphology.

Several studies demonstrated the antidepressant phenotype of genetical or pharmacological inhibition of P2X7 receptros. We performed experiments in order to reveal the background mechanisms of this phenotype. We asked:

- How the genetic blockade of P2X7 affects depression-like behaviour in the learned helplessness modell?
- Do the hippocampal spine synapse plasticity differ in the two genotypes?
- How do synaptic markers (structure proteins, excitatory receptor subunit) alter in the learned helplessness model?

II. Participation of P2X7 receptors in the maternal immune activation model of autism spectrum disorder, regarding behaviour, biochemistry and brain morphology.

Immune challange induced neurodevelopmental disturbancy is a possible theory in connection with the emergence of autism. Since P2X7 receptors modulate inflammatory processes, we supposed they may take part in the patogenesis of autistic features. We asked:

- How maternal immune activation influences the behaviour and brain morphology of $P2rx7^{-/-}$ offspring?

- What biochemical changes are triggered by the immune activation?
- Can we prevent the effects of immune activation by maternal pretreatment with P2X7 selective antagonist?
- Can we reverse the autistic phenotype by P2X7 antagonist treatment of the offspring?

III. The protective effect of SZV558 in the *in vitro* rotenone induced Parkinson model

In this part of my thesis our work did not include P2X7 receptors. We tested a newly synthetised heteroarylalkenyl propargylamine compound, named SZV558 in the *in vitro* rotenone induced Parkinson model. We aimed to show:

- Is this novel MAO-B inhibitor compound protective against the dopaminergic cell loss in the substantia nigra? Is it more effective than rasagiline, the compound currently used in clinical practice?

3. Methods

3.1 Animals

For the depression model we used 8-12 weeks old P2rx7$^{-/-}$ mice with C57/Bl6 background, and wild-types as controls, all kept individually one week before the experiments. In the autism model we established mating trios of two primiparous 12-14 weeks old females and one 8-12 weeks old male. Behaviour tests were performed on 8-12 weeks old offspring. All behaviour experiments were carried out between 9 a.m. and 2 p.m. in the Behaviour Analyis Unit of the Institute of Experimental Medicine. For the *in vitro* Parkinson model we used male Wistar rats weighing 200-220 grams. Animals were kept under standard laboratory conditions in 12 hour light-dark cycles with food and water provided *ad libitum*. All efforts were taken to minimise animal suffering and reduce the number of animals used. All experiments followed the ARRIVE guidelines and were conducted in accordance with the principles and procedures outlined in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The local Animal Care Committee of the IEM approved all experimental procedures (Permission No: PEI/001/778-6/2015).

3.2 Depression model

3.2.1. Learned helplessness

The learned helplessness model is based on one of the typical features of depression, becoming resigned to inevitable situations. Experiments are performed in 'shuttle boxes', plexi boxes divided to two compartments with a guillotine door. Animals receive inescapable scrambled footshocks in this box during training, but in the testing phase footshocks can be avoided through the opened door. If the animal is helpless, it will not escape to the other compartment. Control mice were exposed to the box withough receiving inescapable shocks in training. Testing includes 30 trials, when failed escapes and escape latencies are recorded (n=23-27).

3.2.2. Electron microscopy analysis of spine synapses

24 hours after testing learned helplessness, 3 mice/group were subjected to perfusion fixation, then 100 μm coronal sections were embedded for electron microscopy analysis. Small tissue blocks were cut from each section from the molecular layer of the dorsal dentate gyrus. Then 75-nm consecutive serial sections were made at each sampling site, and digitised electron micrographs were taken at 12000x magnification. Appearing spine synapses were counted on image pairs depicting identical regions in adjacent ultrasections. The number of spine synapses was calculated independently by two investigators.

3.2.3. RT-PCR

Total RNA samples were isolated and purified from cell lysates of hippocampus (n=4) using the Qiagen RNeasy Lipid Tissue Mini kit according to the manufacturer's instructions. The integrity and concentration of RNA samples were measured with Agilent 2100 Bioanalyzer. Then 1 μg of total RNA was reverse transcribed using the Tetro cDNA Synthesis Kit with an AB GeneAmp PCR system 2700 instrument. The concentration of cDNA samples was calculated with Qubit ssDNA Assay kit. The expression level of the target gene P2rx7 was determined according to standard protocols using TaqMan Fast Universal PCR Master Mix (2x) and TaqMan Gene Expression Assay Mix (20x). P2rx7 expression was normalized to the level of Gapdh as a reference housekeeping gene.

3.2.4. Western blot

Hippocampi dissected 6 or 24 hours after testing learned helplessness were stored at −70°C until further investigation (n=5). Samples were homogenised in 250 μL lysis buffer (containing 1% protease inhibitor) then centrifuged in 4°C 10 000 rpm for 10 minutes. Resulting supernatants were used for Western blot after measuring their protein concentrations with BCA protein assay. From each sample, 40 μg protein was loaded and separated by SDS-PAGE (10%) and transferred onto a PVDF membrane using a MiniProtean-3 apparatus. The blot was incubated in blocking solution (1% bovine serum albumin, 5% milk, TBST) for 2 hours room temperature, then in primary antibodies (actin, 1:200 goat, synaptopodin 1:200 goat, PSD95 1:500 rabbit) applied overnight at 4°C. After rinsing and washing 3x10 minutes in TBST, horseradish peroxidase-conjugated secondary antibodies were used (anti-goat 1:5000, anti-rabbit 1:4000) for 2 hours at RT, rinsed and washed in TBST for 3x10 minutes, then in TBS for 5 minutes. The specific immunoreactive bands were detected and visualized by chemiluminescence and quantified by densitometric analysis with ImageJ.

3.2.5. Immunohistochemistry of hippocampal granule cells and NR2B/GluN2b subunit

Mice (n = 3) were anesthetized and perfused transcardially with 4% PFA 24 hours after the evaluation of learned helplessness, then postfixed overnight in 4% PFA at 4°C. Then 40 or 60 μm coronal sections from the dorsal hippocampus were used for immunoreaction. Nucleus staining with 1:10000 Hoechst 33342 in TBS for 2 hours at room temperature was applied on 40 μm sections. NR2B/GluN2B immunolabeling was performed on 60 μm sections after permeabilization with pepsin+HCl, and blocking with 10% normal horse serum. Primary NR2B/GluN2B antibody (1:1000) was applied for 24 hours at 4°C, then Alexa568 (anti-mouse, 1:1000) fluorescent secondary antibody was used for 2 hours at room temperature. Sections were washed in TB, mounted on slides and cover slipped with Vectashield antifade reagent. Confocal images were acquired at the same depth of the sections at 20x and 60x magnification, with same acquisition parameters with a Nikon C2 confocal system on a Nikon Ni-E microscope equipped with NIS-Elements C software.

Average intensity of NR2B/GluN2B immunoreaction was quantified with NIH ImageJ software.

3.3. Autism model

3.3.1. Experimental design – maternal immune activation

Neurodevelopmental disturbance was induced with maternal immune activation via a 3 mg/kg (E12.5) and 1.5 mg/kg (E17.5) dose of poly(I:C) intraperitoneal injection to pregnant mouse dams. Control dams received saline injections. Behaviour tests were carried out from 8 weeks of age on male offspring in a pre-defined order (sociability, self-grooming, marble burying, rotarod). After behaviour experiments, mice were sacrificed for morphological investigations. We also collected maternal blood and embrionic brain samples on the E12.5 or E14.5 days. Pharmacological blockade of maternal P2X7 receptors was performed two hours before immune activation with JNJ47965567 (JNJ) injection at 30 mg/kg dose dissolved in 30% captisol solution. Postnatal treatment of adult offspring was done by a single injection of JNJ at 30 mg/kg dose 1 hour before the first behaviour experiment. Mice were randomly assigned to different treatment groups.

3.3.2. Social preference

Social preference was measured by using a 3-chamber Plexiglas arena divided into 3 equal chambers with Plexiglas cages put into both of the side chambers. After habituation, a stranger mouse was placed into one of the cages, while the other remained empty. We measured the time spent interacting with the stranger mouse and the empty cage by the test mouse and defined social preference as the percentage of the interaction with the stranger.

3.3.3. Self-grooming

Test mice were put into clear glass observation cylinders individually for 10 minutes and their spontaneous novelty-evoked grooming behaviour was video recorded. Behaviour was manually scored using Noldus Observer XT software and the cumulative duration of self-grooming in seconds was counted by the software.

3.3.4. Marble burying

Repetitve behaviour can be also measure by marble burying test, when mice were placed into a clean cage filled with 4 cm corn cob bedding and 20 marbles gently placed onto its surface and the number of marbles buried was measured within a 10 minute testing period. Marbles were counted as buried if they were covered by ≥50% bedding.

3.3.5. Rotarod test

Motor cordination and balancing skills were tested with an accelerating rotarod instrument. Mice were trained on the first two days at constant 4 rpm speed for three consecutive trials to achieve the ability to maintain balance on the rod for at least 30 seconds. Acceleration phase testing was performed on two subsequent days four trials/day. The inter-trial interval was 45 minutes. Latency of falling down was measured in seconds.

3.3.6. Open field

Basic locomotor activity was examined in a square open field arena for 10 minutes, when animals were able to freely explore the space, and their total distance covered during the test was given in cm.

3.3.7. Immunohistochemistry of cerebellar Purkinje neurons

After perfusion with 4% paraformaldehyde and further overnight post-fixation of brains at 4°C, 50 μm parasagittal sections of the cerebellar vermis were used for immunoreaction. Slices were permeabilized with blocking solution containing 5% normal horse serum, 1% BSA, and 0.3% Triton X-100 in 0.1 M PB for 2 hours at room temperature, and incubated overnight at 4°C with anti-calbindin antibody (1:12000). Sections were carefully rinsed and washed with PB and stained with fluorescent secondary antibody (Alexa 488 anti-rabbit, 1:3000) for 2 hours at room temperature. Purkinje cells in lobe VII of the cerebellum were imaged with a confocal Nikon C2 microscope and counting was performed manually while the length of the lobe was measured by ImageJ.

3.3.8. Synaptosome preparations and electron microscopy

After decapitation, whole brains were used for synaptosome preparation. Samples were homogenised in sucrose solution, then centrifuged, and the resulting supernatant was re-centrifuged in clean tubes. P2 pellets were resuspended in 45% Percoll-Krebs solution and centrifuged again. Then Percoll-Krebs solution was removed beneath the top layer, and the remainging synaptosome rich fraction was washed twice with Krebs solution. Synaptosome pellets were fixed with 4% paraformaldehyde, and after washing they were post-fixed and embedded for electron microscopy analysis. Electron micrographs were taken at 20000x or 30000x magnifications and intact and malformed synaptosomes were counted manually by an investigator blind to treatments.

3.3.9. Fetal brain immunohistochemistry

Fetal heads were collected 48 hours after the first intraperitoneal injection of dams with poly(I:C) or saline, and samples were immersion fixed in 4% PFA for 24 hours at 4°C. After cryoprotection in 15% sucrose (20 min) and 30% sucrose overnight at 4°C, 20 μm sections were cut using a cryostat. Sections were washed in PB, permeabilized with 100 mM Na-citrate for 30 minutes at 65°C and 0.4% Triton X-100 for 20 minutes at RT, and blocked in 2% normal goat serum and 1% BSA for 1 hour at room temperature. Primary SATB2 and TBR1 antibodies (SATB2 1:100 anti-mouse, TBR1 1:500 anti-rabbit) were applied overnight at 4°C, then slices were rinsed and washed three times in PB, incubated with fluorescent secondary antibodies (1:400 Alexa594 anti-mouse, 1:1000 Alexa488 anti-rabbit) containing 1:10000 Hoechst 33342 for 1 hour at room temperature and rinsed and washed in PB again. Slides were covered with Vectashield mounting medium and the fetal cortex was imaged with a confocal Nikon C2 microscope at 20× magnification. TBR1 intensity was measured in the cortical plate with ImageJ software.

3.3.10. Citokin multiplex bead array analysis

Fetal brain samples and maternal plasma were collected 2 hours after the first intraperitoneal injection of poly(I:C) or saline. After tissue homogenisation and centrifugation, supernatants were collected to measure the levels of the following

inflammatory mediators: IL-1α, IL-1β, IL-6, IL-10, TNF-α and CXCL1 (KC) using BD Cytometric Bead Array Flex Sets. Measurements were performed on a BD FACSVerse flow cytometer and data were analyzed using the FCAP Array v5 software. Cytokine concentrations of brain tissue were normalized to total protein levels measured by photometry using BCA Protein Assay Kit. The cytokine levels of plasma are expressed as pg/ml.

3.3.11. HPLC analysis

Fetal brain samples and maternal plasma were collected 2 hours after the first intraperitoneal injection of poly(I:C) or saline. Pregnant dams were anaesthetised by isofurane and blood was taken from the lower vena cava with a syringe containing K-citrate, then embrionic brains were prepared quickly. Maternal blood was cooled in ice water bath for 15 min, then centrifuged to remove platelets and remaining cells. The resulting plasma samples were treated with 4 M perchloric acid solution containing 100 µM theophylline (internal standard) and centrifuged to remove precipitated proteins. In order to neutralise the pH of the resulting solution, supernatant was treated with 4 M K_2HPO_4. The weighed frozen brain tissue was homogenised in 0.1 M perchloric acid containing 10 µM theophylline. The suspension was centrifuged and the perchloric anion was precipitated by 1 M KOH and removed by centrifugation. The pellet was saved for protein measurement. The levels of adenine nucleotides, adenosine and monoamines were determined by online column switching HPLC using Discovery HS C18 columns with Shimadzu LC-20 AD. The adenine nucleotides, adenosine and theophylline were detected by UV absorption. Concentrations were calculated by two-point calibration curve using internal standard method.

3.4. *In vitro* Parkinson model

3.4.1. Dopaminergic neuron labeling in the substantia nigra

Male Wistar rats were decapitated and brains were quickly removed and incised in the middle, so the hemispheres could be handled separately. Blocks containing the substantia nigra were dissected. There were five treatment groups: control, incubation with rotenone,

rotenone + H_2O_2, rotenone + H_2O_2 + rasagiline (100 nM) and rotenone + H_2O_2 + SZV558 (100 nM). Control tissue was incubated in Krebs solution (60 + 120 min, 37 °C). The rotenone group was treated with 10 μM rotenone (60 min, 37 °C), then perfused with Krebs solution (120 min, 37 °C). The rotenone + H2O2 treated group was treated with rotenone (60 min), then perfused with Krebs solution (70 min) followed by perfusion with Krebs solution containing 250 μM H_2O_2 (50 min). In cases of rotenone + H_2O_2 + rasagiline and rotenone + H_2O_2 + SZV558 groups, after incubation with rotenone, the tissues were perfused with Krebs solution (50 min), then were subjected to 100 nM rasagiline/SZV558 perfusion (20 min), and then to 250 μM H_2O_2 and 100 nM rasagiline/SZV558 perfusion (50 min). Each incubated block was immersion fixed with 4% paraformaldehyde (PFA) overnight, then washed with 0.1 M PB. The whole substantia nigra was sectioned with a vibratome at 40 μm thickness between and all coronal midbrain sections were collected and ten consecutive sections were used for immunostaining. Non-specific endogen peroxidase activity was blocked by 0.3% H_2O_2 in methanol for 20 min. Blocking solution (2.5% normal horse serum) was applied for 2 h at room temperature, the primary antibody, anti-tyrosine hydroxylase (rabbit polyclonal, 1:1000) was applied overnight at 4 °C. After carefully washing three times with 0.1 M PB, the ready-to-use secondary antibody (The ImmPRESS Universal Antibody Kit, anti-mouse/rabbit) and ImmPACT DAB as chromogen was applied according to the manufacturer's instructions. The same areas and number of sections were investigated. Pictures were taken at 40x magnifications and the stained cells were counted manually by two independent investigators, using the marker counter function of the Pannoramic Viewer 1.15.4 software.

4. Results

4.1. P2X7 receptor function in the learned helplessness model of depression

4.1.1. P2rx7$^{-/-}$ mice display antidepressant behaviour

The learned helplessness paradigm was used to study depressive-like behaviour in P2rx7$^{+/+}$ and P2rx7$^{-/-}$ mice. In wild-types, inescapable footshocks provoked increased number of escape failures and escape latency values indicating the development of learned

helplessness. In P2rx7$^{-/-}$ animals, elevated escape failure number and escape latency were found in response to escapable shocks of testing in controls compared with P2rx7$^{+/+}$ littermates. However, there was no change in failed escapes or escape latencies in P2rx7 deficient animals when exposed to inescapable footshocks, thus footshocks did not affect these animals. Regarding the higher baseline of results, P2rx7$^{-/-}$ did not display learned helplessness, because higher shock intensity during testing could evoke active escape behaviour.

4.1.2. Spine synapse density alterations in the model

Using electron microscopic stereology, the analysis revealed quantitative alterations in spine synapse number of the examined dentate gyrus areas 24 hours following the evaluation of learned helplessness. While in the P2rx7$^{+/+}$ mice repeated footshocks evoked a decrease in the spine synapse density in accordance with the observed behavioural alterations, this effect was not observed in the P2rx7$^{-/-}$ groups. This result supports the antidepressant phenotype of P2rx7 deficient mice.

4.1.3. Quantitative analysis of granule cells

Because genotype and treatment related alterations in spine synapse number might be due to changes in the number of granule cells, next we determined whether the granule cells also had gone through any quantitative change. Nevertheless, we could not find any alteration in the number of granule cells.

4.1.4. P2rx7 expression is downregulated following learned helplessness

Learned helplessness was accompanied with a time-dependent downregulation of mRNA encoding P2rx7 in P2rx7$^{+/+}$ mice. At 6 hours after testing helpless behaviour, P2rx7 mRNA decreased significantly in the shock treated group compared with controls. In contrast, 24 hours after testing this effect dissolved.

4.1.5. Western blot analysis of synaptic proteins

PSD95 and synaptopodin protein levels of the hippocampus were investigated 6 and 24 hours after testing learned helplessness. The structural protein actin was used as a positive control. In case of synaptopodin, a significant decrease in the protein level was detected in the shock treated group both 6 and 24 hours after testing in the P2rx7$^{+/+}$, but not in P2rx7$^{-/-}$ animals. We found no change in the amounts of hippocampal PSD95 protein independent of genotype or timing of sample preparation.

4.1.6. Learned helplessnes does not influence hippocampal NR2B/GluN2B subunit levels

The NR2B/GluN2B receptor subunit is not involved in the learned helplessness model, since we did not detect alterations in the intensity of immunolabeling following inescapable footshocks. However, we confirmed our previous results of genotypic difference in the hippocampal NR2B/GluN2B expression, as P2rx7$^{-/-}$ mice showed higher intensity of staining regardless of treatment.

4.2. Participation of P2X7 receptor in the maternal immune activation model of autism spectrum disorder

4.2.1. Comparison of P2rx7$^{+/+}$ and P2rx7$^{-/-}$ mice

Offspring of poly(I:C) treated P2rx7$^{+/+}$ dams displayed decreased social preference, impairment of motor coordination and increased repetitive behaviours reflected in self-grooming and marble burying test when compared to offspring of saline treated P2rx7$^{+/+}$ dams. Collectively, these observations suggested that offspring of poly(I:C) treated P2rx7$^{+/+}$ dams have an autism-like phenotype. These treatment-related behavioural alterations were not observed in offspring of poly(I:C) treated P2rx7$^{-/-}$ mice. Basal locomotor activity was not changed by either maternal poly(I:C) treatment or genotype. Lower number of Purkinje neurons was detected in the cerebellum, and the proportion of malformed synaptosomes also increased after immune activation. Fewer Purkinje cells were found in P2rx7$^{-/-}$ mice, while poly(I:C) elicited a further, but alleviated decrease. P2rx7 deficiency by itself elicited a similar increase in the vulnerability of synaptosomes, yet poly(I:C) lost its effect.

Elevation of ATP levels was observed in both P2rx7$^{+/+}$ maternal blood and embrionic brain samples following poly(I:C) injection. Besides, strong IL-6 induction was detected in wild-type mice in response to maternal immune activation, which was attenuated in P2rx7 deficient mice. A similar, however, lower magnitude of statistically significant increase in IL-6 levels was also observed in fetal brain of P2rx7$^{+/+}$ mice which was not detected in poly(I:C) treated P2rx7$^{-/-}$ samples. There was significant increase of IL-1α and KC in P2rx7$^{+/+}$ maternal plasma after poly(I:C) treatment, which was attenuated in P2rx7$^{-/-}$ mice. KC was also induced by immune activation in fetal brain of P2rx7$^{+/+}$ mice. Weaker intensity of TBR1 immunofluorescence was detected in the developing cortical plate of P2rx7$^{+/+}$ embryos of dams subjected to poly(I:C), indicating disruption of cortical development. In contrast, this change was not observed in P2rx7$^{-/-}$ mice.

4.2.2. Effect of maternal P2X7 inhibition with selective antagonist

A single injection of the potent and selective P2rx7 antagonist JNJ (30 mg/kg i.p.) or its vehicle was administered to pregnant wild-type dams 2 hours before the respective saline/poly(I:C) administration. P2rx7 antagonist treatment alleviated poly(I:C) effect in social preference, rotarod, self-grooming and marble burying tests when compared to vehicle treatment. Interestingly, poly(I:C) induced loss of Purkinje cells and increased synaptosome malformation did not occur after JNJ treatment either. The significant induction of IL-6 in maternal plasma and fetal brain by maternal poly(I:C) treatment was attenuated by pretreatment with P2rx7 antagonist when compared to vehicle. Furthermore maternal JNJ prevented poly(I:C) induced loss of TBR1 intensity in the developing cortical plate.

4.2.3. Postnatal P2X7 antagonist treatment of offspring

All poly(I:C) induced alterations were reversed by a single postnatal injection with JNJ: mice displayed social preference, normal motorcoordination, lower self-grooming and marble burying behaviour. The loss of cerebellar Purkinje cells and abnormal synaptosome structures were counteracted by antagonist treatment as well.

3. Protective effect of SZV558 compound in the rotenone induced *in vitro* Parkinson model

Cell death was induced by *in vitro* rotenone treatment in the substantia nigra of rats, resulting in the loss of 40% of dopaminergic neurons. In order to prevent rotenone induced disruption of the cells, we treated the brains with rasagiline, and a novel MAO-B inhibitor, SZV558. Rasagiline treatment was inefficient in hindering cell death, tyrosine hydroxylase positive cells were still severely damaged. However, the SZV558 compound proved to be completely protective against cell loss, displaying almost control conditions.

4. Conclusion

We have performed experiments modelling three different disorders: the learned helplessness model of depression, the maternal immune activation model of autism, and the *in vitro* rotenone induced Parkinson model. In the first two models we investigated the function of the purinergic P2X7 receptor, while in the Parkinson model we tested the protective effect of a novel MAO-B inhibitor compound. Our results confirmed:

- ✓ P2rx7$^{-/-}$ mice displayed antidepressant phenotype in the complex depression model of learned helplessness, in accordance with previous results. Although these mice showed high baseline values in the behaviour test, they did not develope learned helplessness, since elevated shock intensity during testing evoked active escape behaviour.
- ✓ Learned helplessness downregulated hippocampal P2rx7 mRNS expression, indicating the activation of the receptor in this depression model.
- ✓ Disruption of synaptic connections is typical feature of depression revealed in both clinical and animal studies. P2rx7$^{-/-}$ mice are resistant to learned helplessness induced synapse loss, supporting their antidepressant phenotype. We did not detect alterations of spine synapse specific marker synaptopodin in the P2rx7 deficient brain, confirming the plasticity results.

- ✓ NR2B/GluN2B NMDA subunit is not involved in the learned helplessness model; we could not find changes following footshocks. Although we showed genotype-related differences again, similar to our previous findings.
- ✓ We presented that the activation of P2X7 receptor is necessary to induce autistic-behaviour via maternal immune activation. Either genetic or pharmacological inhibition of the receptor prevented the autism-like behavioural, biochemical and morphological alterations.
- ✓ Poly(I:C) injection increases ATP levels in maternal plasma and embrionic brains, supporting the endogen ligands of purinergic signalling, and P2X7 activation.
- ✓ $P2rx7^{-/-}$ mice displayed attennuated induction of IL-6 and KC by immune activation in maternal blood when compared to saline treatments. There was no change in the level of these immune mediators in $P2rx7^{-/-}$ embrionic brains. We obtained similar results in P2X7 antagonist treated animals. By inhibiting P2X7 receptors, maternal immune activation is insufficient to induce autistic phenotype.
- ✓ Neurodevelopment is unaltered in the offspring of $P2rx7^{-/-}$ or JNJ treated dams, TBR1 labeling in the cortical plate is similar as in wild-type controls.
- ✓ We showed the highly protective effect of the novel heteroarylarkenyl propargylamin compound, SZV558 on tyrosine hydroxylase positive neurons of the substantia nigra treated by rotenone *in vitro*. This compound was able to prevent cell death more efficiently, than the clinically used MAO-B inhibitor rasagiline.

Personal publications

Publications in the topic of thesis:

1. **Otrokocsi** L, Kittel Á, Sperlágh B.: *P2X7 Receptors Drive Spine Synapse Plasticity in the Learned Helplessness Model of Depression* (2017) Int J Neuropsychopharmacol 20(10):813-822. doi: 10.1093/ijnp/pyx046.

2. Baranyi M, Porceddu PF, Gölöncsér F, Kulcsár S, **Otrokocsi** L, Kittel Á, Pinna A, Frau L, Huleatt PB, Khoo ML, Chai CL, Dunkel P, Mátyus P, Morelli M, Sperlágh B.: *Novel (Hetero)arylalkenyl propargylamine compounds are protective in toxin-induced models of Parkinson's disease* (2016) Mol Neurodegener 11:6. doi: 10.1186/s13024-015-0067-y

Manuscript under revision in the topic of thesis:

1. Horváth G*, **Otrokocsi** L*, Bekő K, Baranyi M, Kittel Á, Sperlágh B.: *Maternal and offspring P2X7 receptors drive autism-like behavior in mice* (2018) Biological Psychiatry
* equal contribution, shared first authorship

Other publications:

1. Beamer E, Gölöncsér F, Horváth G, Bekő K, **Otrokocsi** L, Koványi B, Sperlágh B.: *Purinergic mechanisms in neuroinflammation: An update from molecules to behavior* (2016) Neuropharmacology 104:94-104. doi: 10.1016/j.neuropharm.2015.09.019.

2. Lőrincz ÁM, Timár CI, Marosvári KA, Veres DS, **Otrokocsi** L, Kittel Á, Ligeti E.: *Effect of storage on physical and functional properties of extracellular vesicles derived from neutrophilic granulocytes.* (2014) J Extracell Vesicles 3:25465. doi: 10.3402/jev.v3.25465.