Involvement of P2X7 receptor in physiological and pathological function of central nervous system: in the pathophysiology of migraine and in the modulation of hippocampal serotonergic transmission

PhD theses

Flóra Gölöncsér

Semmelweis University

János Szentágothai Doctoral School of Neurosciences

Supervisor: Dr. Beáta Sperlágh, D.Sc.

Official Reviewers of the PhD Dissertation: Dr. György Lévay, Ph.D.

Dr. György Bárdos, D.Sc.

Committee of Final Examination:

Chairman: Dr. Éva Szökő, professor, D.Sc.

Members: Dr. Árpád Mike, Ph.D.

Dr. Tamás Tábi, Ph.D.

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1 Introduction

An important communication form in the function of the nervous system is chemical signalling, which is materialized by release of neurotransmitters and neuromodulators from neurons. In addition to classical transmitters (acetylcholine, noradrenaline, dopamine, serotonin, glutamate, GABA), common signalling agents are adenosine 5-triphosphate and adenosine, which implement a non-conventional type of signal transmission in the nervous system and other organs. The biological effects of ATP are mediated through the family of P2 receptors (P2X and P2Y), of which the P2X7 receptor is in the focus of my doctoral thesis.

In my dissertation, I aimed at studying purinergic signalling in the central nervous system. The main purpose of the experimental work presented is to understand the role of neuronal and glial P2X7 receptors in different pathophysiological states. My work is based on my two first authored articles on the investigation of the role of P2X7 receptor, although on two completely different topics:

(1) The participation of purinergic signal transduction in pain management is realized through the activation of P1 and P2 receptors widely expressed in the central nervous system: in neurons and glia cells, smooth muscle and endothelium of the vascular system. In addition, in various pain models successful experiments have been performed with several P2 receptor antagonists, so it is not surprising that their role is also associated with the pathomechanism of migraine. We hypothesized and wanted to prove that the P2X7 receptor plays a pivotal role in the development of headaches and migraine. Thus, in the first part of my work, the role of P2X7 receptor in the pathomechanism of migraine was investigated using P2X7 gene knockout (KO) mice and P2X7 receptor antagonists in the nitroglycerin (NTG) induced migraine mouse model.

(2) In the next part of my work, we also studied the role of P2X7 receptors in the modulation of serotonin release in the brain. Numerous reports have already demonstrated the role of purine receptors in the neurotransmission: in the central nervous system ATP is released at least in micromolar concentrations into the extracellular space during neuronal activity, which can modulate serotonergic transmission with the activation of hippocampal purine receptors. P2X7 receptors modulate the release of several neurotransmitters, however, this has not been clarified

yet regarding serotonin. The serotonergic/glutamatergic raphe-hippocampal pathway from brainstem median raphe can effectively modulate the coding processes in the hippocampus. Furthermore, glutamatergic neurons in median raphe may play a modulating role in the function of ascending serotonergic neurons, but there are few functional data available in this regard. Therefore, to gain better understanding of the characteristics of serotonergic transmission in the median raphe and hippocampus, and to determine the influence of glutamate and ATP, we used specific optogenetic stimulation in our experiments to prove a possible P2X7 receptor-mediated modulation. Wild type (WT) and P2X7 KO mice and P2X7 receptor antagonists were used to investigate changes in serotonergic transmissions by the hippocampal afferents of median raphe stimulated by in vivo and in vivo optogenetic stimulation

2 Objectives

Our investigations focused on the following main topics:

I. In the first part of my dissertation I deal with the unclear aspects of the relationship between purinergic signalling and migraine. Based on literature data it is likely that endogenous purines are potential triggers or mediators of migraine and many research groups have already studied the involvement of P2 receptor subtypes in complicated regulatory processes in migraine. Based on these literature results, it is assumed that P2 receptors play a key role in the development of migraine pain.

One of the aims of this work is to investigate the effects of the genetic deletion and pharmacological inhibition of P2X7 on NTG-induced thermal hypersensitivity and c-fos induction in migraine related areas in central nervous system of mice.

1. Examination of the development of thermal hypersensitivity in NTG-treated mice.

- In our examinations we looked for an answer to the question whether in the NTG-induced migraine mouse model there are differences in the pain threshold of wild type and P2X7 knockout mice?

2. Examination of the role and antagonism of P2X7 receptor in NTG-induced mice migraine model.

- In these examinations our aim was to reveal the P2X7 receptor mediated effects with acute and subacute Brilliant Blue G (BBG) antagonist treatment in WT and P2X7 KO mice.

3. Examination of the change in c-fos expression in migraine-related mouse spinal cord and trigeminal nucleus caudalis (TNC).

- In our subsequent examinations we compared the changes in c-fos expression between the two genotypes and the effects of P2X7 receptor inhibition with the IHC technique.

II. In the second part of my dissertation, I deal with the unclear role of P2 receptors in regulation of serotonergic transmission. Based on literature data it is likely that endogenous ATP modulates serotonin (5-HT) production and activation of P2 receptors inhibits 5-HT release. At the same time, 5-HT modulates ATP levels in the brain, and this modulating effect is also true vice versa. Based on these literary results, it seemed desirable to investigate the role of P2X7 receptor in serotonergic transmission.

Therefore another goal of this work is to examine the nature of serotonergic system of the median raphe region (MRR) and the hippocampus and its relationship with the purinergic system. For this purpose, optogenetic technique was applied to afferents derived from the MRR and its targeting area the hippocampus were selectively activated, and the 5-HT release of the neuron subpopulation in the MRR was investigated in vivo and in vitro. Another aim was to investigate the effect of genetic deletion and pharmacological blocking of P2X7 on the electrically and optically evoked 5-HT release.

1. Investigate the characteristics of 5-HT released from MRR by optical stimulation.

- After releasing serotonin from the MRR of the wild type mice by electric and chemical stimuli, we examined and compared the effect of optical stimulation on serotonin release.

2. Examination of the effectiveness of optical stimulation of MRR on serotonergic system-related behaviour in mice.

- In our investigations we were looking for the answer, how MMR optical stimulation affects the behaviour of the mice expressing ChR2?

3. Examination of the levels of monoamines released by *in vivo* optical stimulation in the MRR.

- In these studies, it was our goal to see how glutamate and GABA levels change beside 5-HT in the MRR due to optical stimulation with different parameters.

4. Mapping the electrically and optically evoked hippocampal 5-HT release.

- After releasing serotonin from the hippocampus of WT mice by electric and chemical stimuli, we examined and compared the effect of optical stimulation on serotonin release.
- We also investigated how 5-HT_1 agonists influence the amount of released 5-HT.

5. Examination of the role and antagonism of the P2X7 receptor in electrically and optically evoked hippocampal 5-HT release.

- In our experiments we sought to answer the question, whether ionotropic P2X7 receptors are involved in the regulation of serotonin release from the hippocampal terminals of serotonergic pathways. To detect this, the P2X7 receptor was blocked pharmacologically before stimulations.

3 Methods

3.1 Animals

In both studies, all experiments used 2-4 months male C57Bl/6J based P2X7 receptor null mutant (knockout, KO) transgenic mice and their wild type (WT) littermates. The original breeding pairs of P2X7 KO mice were supplied by Christopher Gabel from Pfizer Inc. (Groton, CT, USA).

3.2 NTG-induced mouse model of migraine

3.2.1 Increased temperature hot plate test (ITHT)

Responsiveness of mice to nociceptive stimulation was measured by an increasing-temperature hot plate system (IITC Life Science, Woodland Hills, CA, USA). The animals were placed on an electrically heated metal plate that was kept at a constant temperature of 30°C (starting temperature). After the habituation period the plate was

heated from the starting temperature with a constant rate of 6°C/min, until the animals showed nocifensive behaviour (frequent pawlifting and/or pawlicking in both front and back paws, jumping). The temperature at which the animal showed the first sign of nocifensive behaviour was taken as the paw withdrawal threshold (PWT), expressed in °C. Approximately 1 hour later, the measurement was repeated and the average of two values was taken as the baseline thermal nociceptive threshold. After the second measurement, the animals received treatment with drugs as described above, then one and two hours after NTG administration, post-drug nociceptive threshold was measured.

3.2.2 Drugs and treatment

Animals received intraperitoneal (i.p.) injections of 15 mg/kg NTG or vehicle (49 mg glucose monohydrate/ml) after the measurement of the baseline thermal nociceptive threshold. Sumatriptan succinate, dissolved in saline was used to validate our experiments: each animal was given i.p. sumatriptan at the dose of 600 μg/kg or saline 5 minutes after NTG administration. The P2X7 antagonist, Brilliant Blue G or its vehicle (0.9% saline), were applied in an identical way, or using two different prophylactic application protocols:

- Acutely (50 mg/kg), it was applied i.p. 30 minutes prior to NTG treatment and after the measurement of the baseline thermal nociceptive threshold on the day of testing.
- In subacute treatment, mice were treated for 5 consecutive days with the daily doses of BBG (50 mg/kg i.p.) or saline and 30 min after the last injection were subjected to NTG.

All drug solutions were freshly prepared on the day of use.

3.2.3 TNC immunohistochemistry (IHC)

Two hours following i.p. injection of NTG (15 mg/kg) or vehicle, mice were perfused with fixative solution (containing 0.5% borax and 5% paraformaldehyde). Whole brain and spinal cord samples were then freezed and transversal sections were cut at 30 μm. Every fourth section was collected and free-floating c-fos IHC was performed. Mounted and coverslipped sections were microphotographed and fos-immuno-reactive nuclei were counted within both sides of the cervical spinal cord and the TNC using ImageJ software. We calculated the average number of c-fos positive nuclei in each section.

3.3 Optogenetics

3.3.1 Virus injection and optogenetical parameters

40 nl adeno-associated virus vector (AAV) encoding ChR2 (AAV2.5.hSyn.hChR2(H134R)eYFP.WPRE.hGH) was injected from glass capillary (tip diameter 20-30 µm) connected to a MicroSyringe Pump Controller into the MRR (AP: 4.10 mm; L: 0.0 mm; DV: 4.60 mm). The capillary was left in place 5 min after the injections to permit diffusion of the virus and to minimize backflow of the virus after needle retraction. Immediately following injection the incision was closed and the scalp was sutured. In the experiments a 473 nm DPSS laser was used for the optical stimulus.

3.3.2 Optogenetic manipulation of locomotor activity and freezing behaviour of virus-injected mice

Two weeks after injection of the AAV construct mice were implanted with optic fibers above the MRR (in an angle of 10 degrees; AP:-4.80 mm; L: 0.0 mm; DV: 4.062) under ketamine-xylazine anaesthesia. Implants were secured by acrylic resin (Duracryl Plus; SpofaDental, Czech Republic). Behavioural experiments started after a 4-7 days recovery period. Laser beams were generated by low noise diode-pumped solid-state lasers (wavelength: ChR2: 473 nm (blue)), then collimated and guided to the implanted optrode by fiber-optic patch cords. Mice were stimulated continuously at 20 Hz for 5 min in Plexiglas stimulation cages measuring 30x30x30 cm. Controls were injected, not stimulated and light-stimulated mice, which showed no ChR2 expression in the MRR. Behaviour was recorded by a video camera, and the locomotor activity was determined.

Freezing was analysed during the 20 Hz stimulation (Day0), and 1 (Day1) and 7 days (Day7) later, when mice were replaced back to the stimulation cage. After behavioural studies, animals were perfused, and histological analysis was performed. Mice with robust ChR2 expression and with correctly placed optrodes were included into the study.

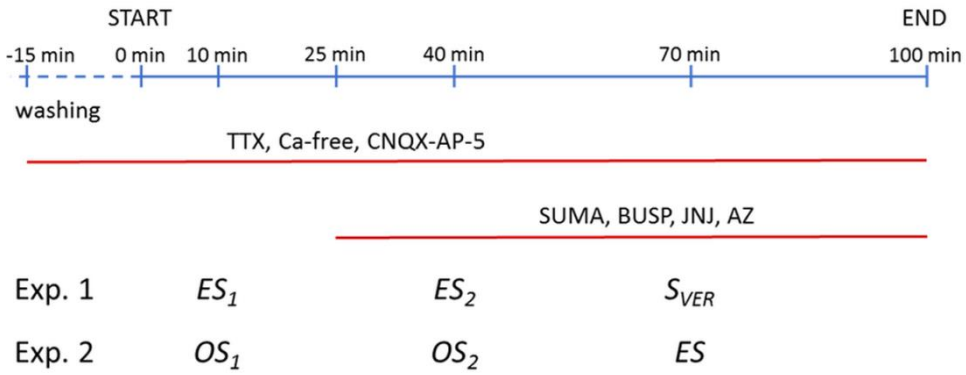
3.3.3 Preparation of survival brain slice

After decapitating the animal, the brain was removed, and Microm HM 650 V vibratome was used to cut 300 µm coronal brain slices in ice-cold Krebs'solution. Brain slices were incubated in 1 ml of Krebs' solution containing triciated isotopes for 60 min

at 37 °C. Then tissues were transferred into low volume superfusion chambers where preperfusion was used for 45 minutes to wash excess radioactivity, subsequent measurements were performed. The incubation and perfusion solutions were also bubbled with a mixture of carbogenic gas at 37 °C to ensure adequate O_2 content and pH of the solution.

3.3.4 Measurements of release of in vitro [^{3}H]serotonine and [^{3}H]glutamate from mouse median raphe and hippocampal slices using tissue perfusion techniques

After incubation and perfusion washing, 1 (Experiment 2) or 3 (Experiment 1) min perfusate samples were collected and assayed for tritium. During the sample collection period stimulation was used 3 times, or in which experiment it was necessary drugs were added to the perfusion solution, for experimental procedures see the figure below.



The radioactivity released from the preparations was measured using a Wallac 1409 liquid scintillation counter (PerkinElmer, USA). Tritium efflux was expressed in Becquerel per gram (Bq/g) and as a percentage of the amount of radioactivity in the slices at the beginning of the respective collection period (fractional release, FR%). Optical or electrical stimulation-induced tritium efflux (FRSx) were expressed by calculating the net release in response to optical or electrical stimulations by the area-under-the-curve method.

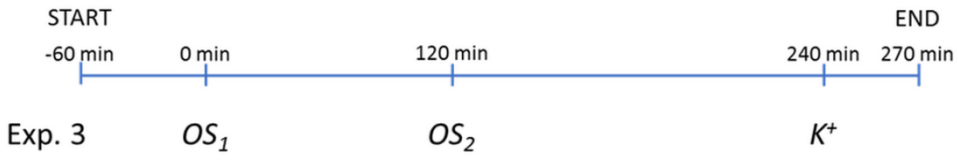
At the end of the experiments the tissues were homogenized in 0.5 ml of 10% trichloroacetic acid and after 15 minutes the radioactivity 0.1 ml aliquots of the tissue samples was determined. The drugs to be tested were added to the perfusion fluid 15 minutes before the second stimulation, while the modified Krebs' solution was perfused from the beginning of the wash period of the tissue to the end of the experiment.

3.3.5 Implantation of microdialysis probe

The microdialysis probe was inserted in mice under 20 % urethane-induced deep anaesthesia. For proper positioning the head of the animals were fixed with stereotaxic device. The microdialysis probes were inserted into the MR (an angle of 10 degrees dorsal, AP:-4.80; L: 0.0 mm; DV: 5.50). An optic fiber was inserted through a guide cannula ending at the top of the membrane. The microdialysis experiments were started after the 2-hour equilibration period.

3.3.6 Microdialysis experiments

During the experiments the head of the animals were fixed in a stereotaxic device and through the microdialysis probe ACSF was perfused. After 2h equilibration period, we started the collection of 30-minute samples. The individual fractions were collected in sample tubes and analysed by HPLC. For each animal, 12 sample fractions were collected, and basal concentrations were determined from the first three samples. Optical stimulation was delivered in the fourth (20 Hz) and eight (50 Hz theta burst) sample starting after 5 min after the beginning of sample collection and lasting 5 min (Experiment 3, in the figure below). In the last sample 100 mM KCl was applied for 5 min.



3.3.7 HPLC analysis of ATP and other neurotransmitters in vivo and in vitro samples (HPLC)

Neurotransmitters serotonin (5-HT), glutamate (Glu) and GABA in dialysates from MRR and endogenously released ATP in tissue over-flow fluid from hippocampus slices were determined by using HPLC method. The extraction solution (PCA) was 0.1 M perchloric acid that contained theophylline (as an internal standard) at 10 µM concentration.

The recovery of the implanted microdialysis probes was evaluated at the end of experiment. The in vitro extraction efficiency for 5-HT, Glu, and GABA were estimated to be 21.1±4.8%, 17.1±2.8%, and 21.9±3.4% respectively. The concentrations of 5-HT,

Glu, and GABA were expressed in absolute amount (nmol/ml or pmol/ml) or as percentage (mean ± SEM) of baseline concentrations to monitor changes from basal levels after optical and chemical stimulation after virus injection of mice.

3.3.8 Histological verification

After behavioural and microdialysis experiments brains were taken out from deeply anaesthetized mice after transcardial perfusion with 0.1M phosphate buffered saline (PBS) for 1 minute, followed by 4% paraformaldehyde in PBS for 10 minutes. The brains were removed and frozen coronal sections (40 µm thick) were cut by sliding microtome. Next on every third section 5-HT immunohistochemistry was performed, and microphotos were taken from the sections. After release experiments the slides were fixed by 4% paraformaldehyde and mounted on slides without further processing for detection of the fluorescent signal of the virus construct.

The sections were evaluated with a Nikon C2 confocal microscope. The position of the optic fiber, microdialysis probe and the virus infected volume were determined on micrographs by using stereotaxic atlas images on the series of images of the MRR.

4 Results

4.1 Effect of genetic deletion and pharmacological antagonism of P2X7 receptor in a migraine animal model

The principal new finding of the present study is that antagonism of P2X7 by treatment with the specific P2X7 antagonist BBG leads to the alleviation of NTG-induced thermal hypersensitivity in mice. Moreover, as BBG treatment was ineffective in mice lacking P2X7, it is reasonable to assume that its effect is mediated by P2X7. The NTG-evoked c-fos expression in the TNC was also attenuated after subacute BBG treatment, which implicates a role for the TNC in mediating the effect of BBG on NTG-induced thermal hypersensitivity. All these findings implicate the therapeutic potential of P2X7 blockade in migraine.

We have used the NTG-induced migraine model described recently by Bates et al. and reproduced findings showing that i.p. injection of NTG elicits thermal hypersensitivity in a time-dependent manner. We have also replicated the finding that NTG-induced thermal hypersensitivity is attenuated by the antimigraine drug, sumatriptan, and is

followed by the expression of c-fos in the trigeminal nucleus of the brainstem and upper spinal cord.

Interestingly, no differences in NTG-induced thermal hypersensitivity in P2X7 KO mice were detected, when compared to their wild-type counterparts. The most likely explanation for this negative finding is the potential developmental upregulation of non-P2X7 P2X receptors in P2X7 KO mice, such as P2X3 or P2X4. In fact, we have previously shown the upregulation of P2X4 mRNA in mice deficient of P2X7.

When applied prophylactically, however, both acute and subacute BBG treatment was effective in the alleviation of NTG-induced thermal hypersensitivity in the P2X7 WT mice. BBG is known to permeate the blood brain barrier and is thought to be specific to P2X7 in the applied dose. Although *in vitro* experiments revealed that BBG can inhibit Na^+ channels in micromolar concentrations, in in vivo studies using a similar dose (45.5 mg/kg), BBG has not reached higher concentrations in the brain than 200 nM, which is selective for P2X7. The finding that its effect was completely lost in P2X7 KO mice also refutes the possibility of other target for BBG than P2X7 in these experiments.

ATP is a well-known danger-signal, which is released in response to cell injury, inflammation, mechanical and metabolic distress, and is per se an algogenic substance. P2X7 is a ligand-gated cation channel with high Ca^{2+} permeability, which participates in pain transmission in various ways. In the dorsal root ganglia P2X7 is expressed on satellite glia and potentiate P2X3 receptor mediated signalling by the release of pro-inflammatory cytokine TNF-α. An analogous mechanism might also play a part in migraine, as satellite glia of the trigeminal ganglion express P2X7 and P2X3 receptors participate in craniofacial pain by interacting with NGF, substance P and CGRP. P2X3 receptors also display an enhanced activity in a genetic animal model of migraine. Alternatively, P2X7 antagonists may act more centrally, at the level of the upper cervical spinal cord or trigeminal nucleus. P2X7 modulates the afferent nociceptive information processing within the dorsal horn of the spinal cord and its activation participates in the central sensitization underlying hindpaw hyperalgesia. Because moderate-high density of P2X7 receptor binding sites were found in the grey matter of the trigeminal nucleus the detected alleviation of NTG induced c-fos expression in response to BBG indicates that the TNC is a potential target area for the action of P2X7

receptor antagonists. The activation of P2X7 releases excitatory amino acids in this area, the blockade of which might underlie the action of BBG.

Furthermore, P2X7 is also expressed in other areas of the brain; therefore, a supraspinal action through diencephalic, brainstem or cortical regions cannot be excluded. Finally, P2X7 is expressed on circulating and locally recruited immune cells and the best characterized action of P2X7 activation is its role in the posttranslational processing of pro-inflammatory cytokines, IL-1β and TNF-α, which are also known algogenic substances.

The data present here indicates that inhibition of P2X7 might be a potential target for the prophylaxis of migraine. Moreover, because BBG is a closed structural analogue of a US Food and Drug Administration (FDA)–approved non-toxic food dye, our data argues for its evaluation in a human NTG-induced migraine model.

4.2 Regulating hippocampal 5-HT release via P2X7 receptors in response to optogenetic stimulation of terminals in the median raphe of mice

In this study we have used optogenetic stimulation to characterize neurotransmitter release in response to selective stimulation of neurons originating in the MRR. Whilst electrical stimulation or chemical depolarization conventionally used to detect transmitter release excites synchronously all cell bodies, axons and nerve terminals in the stimulation field, the optogenetic technique allows the separate activation of a selected neuronal pathway.

20 Hz optical stimulation caused an increase in the locomotor activity of the animals and elicited freezing behaviour 7 days after the stimulation, indicating that this kind of stimulation is behaviourally relevant. Previous studies showed that ascending serotonergic projections play an essential role in such motor acts as walking, moving the head about, or changing posture or in general, in enhanced locomotion. Our recent studies show that median raphe region stimulation alone generates remote, but not recent fear memory traces.

We have used a similar, although not completely identical photostimulation protocol to induce 5-HT release detected radiochemically in perfusate samples of acute MRR and hippocampal slices, derived from virus injected mice. In the MRR optical stimulation

triggered 5-HT release in a reproducible manner, and its amount was comparable, but less than that evoked by electrical and chemical depolarization. This difference is reasonable as electrical stimulation activates the whole incoming nerve bundle, whilst optical stimulation is more specific, and stimulates only those neurons and their nerve terminals that express the ChR2 protein. This observation indicates the presence of serotonergic nerve terminals in the MRR originating outside the area reached by virus injection consistently with the reciprocal innervation MRR and other brainstem serotonergic nuclei, such as the DR.

5-HT release in the MRR evoked by both optical and electrical stimulation was $[Ca^{2+}]_o$-dependent showing that it is mediated by vesicular exocytosis. Both electrically and optically induced 5-HT efflux was also sensitive to antagonists of ionotropic Glu receptors implying that 5-HT is released by Glu from ChR2 expressing MRR neurons. This is compatible with anatomical studies identifying autapses and recurrent axon collaterals of MRR neurons. 5-HT-containing neurons are immunopositive for Glu as well as for phosphate-activated glutaminase, a key enzyme involved in Glu biosynthesis, and 5-HT neurons of raphe nuclei express VGluT3. Further, to support this assumption, here we show the simultaneous [^{3}H]Glu efflux in response to an identical optogenetic stimulation. Because light stimulation-evoked 5-HT efflux was sensitive to TTX, the released 5-HT and Glu are probably originated from distinct nerve terminal populations and 5-HT might also be derived from dendrites and cell bodies. It is well known that 5-HT is secreted from the somata and dendrites of neurons in the raphe nuclei in response to the activation of NMDA receptors in a $[Ca^{2+}]_o$-dependent way, and the majority of 5-HT is released in a non-synaptic manner. The novelty in our study is the identification of MRR activated by the optogenetic technique as the neuronal subpopulation representing the above features.

We have also tested, whether 5-HT is released in a similar way from MRR terminals of one of the target areas of MRR, the HP. All three stimulation evoked 5-HT efflux, comparable to the responses measured in the MRR; however, in absolute amount optical and electrical stimulation released less 5-HT from HP than in the MRR. This is consistent with a less intensive EYFP labelling found in the HP corresponding to a less dense innervation in the remote target area.

Whilst 5-HT efflux by both optical and electrical stimulation was largely dependent on $[Ca^{2+}]_o$ in this area as well, subsequent experiments revealed important differences in the two types of transmitter release in the HP. They displayed a different pattern of frequency dependence, indicating that different transmitter pools are mobilized by subpopulations of nerve terminals activated by the two types of stimulation at different frequencies. In addition, whilst electrical stimulation induced 5-HT release was inhibited by TTX and Glu receptor antagonists, optical stimulation induced 5-HT efflux was not sensitive to the above drugs, which indicates that in the hippocampus ChR2 expressing nerve terminals were directly activated by light stimulation and tritium release represents 5-HT efflux from varicosities originating in the MRR. Because ChR2 is permeable to sodium, it is reasonable to assume that direct ion flux through the channel is responsible for the local depolarization of the nerve terminal membrane and subsequent initiation of transmitter release in this case. An interesting additional observation is that electrical stimulation induced 5-HT release in the HP was sensitive to the blockade of ionotropic Glu receptors. A plausible explanation for that is that electrical stimulation, in contrast to light stimulation, also depolarizes excitatory nerve terminals and releases glutamate, which then acts on ionotropic glutamate receptors and directly or indirectly mobilize 5-HT from nerve terminals originating in the DR.

Midbrain serotonergic neurons are equipped with somatodendritic and nerve terminal inhibitory autoreceptors. These receptors have been widely implicated in the effect of various drugs affecting serotonergic transmission such as serotonin reuptake inhibitor (SSRI) antidepressants and anxiolytic drugs. Whereas activation of somatodendritic autoreceptors decrease the firing rate of the neurons locally, activation of nerve terminal autoreceptors decrease 5-HT release at the target areas, i.e. in the cortex and hippocampus, and both belong to the $5\text{-HT}_{1A/1B/1D}$ subtype. In our experiments sumatriptan, the $5\text{-HT}_{1A/1B/1D}$ agonist decreased 5-HT release by electrical, but not by optical stimulation, whereas buspirone, the selective 5-HT_{1A} agonist was without effect to either response. The most likely explanation for the lack of modulation of OS-induced 5-HT efflux by $5\text{-HT}_{1B/1D}$ receptors is that serotonergic neurons expressing $5\text{-HT}_{1B/1D}$ receptors at their hippocampal nerve terminals are located outside the MRR, and they are most likely in the DR. Our data illustrates the suitability of optogenetic

method to investigate subpopulation-specific modulation of neurochemical transmission, which were largely unknown until know.

The second objective of the study was to reveal the potential modulation of 5-HT from hippocampal terminals by P2X7 receptor. We found that both genetic deletion and pharmacological blockade of P2X7 receptors decreased 5-HT release evoked by optical stimulation as well as focal electrical stimulation of the incoming nerve bundle. These findings indicated that endogenous ATP released by the above stimulation paradigms, promoted the release of 5-HT from serotonergic varicosities through the activation of P2X7 receptors. To support this assumption found that identical stimulation significantly elevated extracellular ATP level in this brain area. Because 5-HT release evoked by both optical and electrical stimulation was decreased by the inhibition of P2X7 receptors, the local control exerted by P2X7 receptor activation in the hippocampus extends on the 5-HT nerve terminals from both median raphe neurons and non-median raphe neurons. Furthermore, since 5-HT release evoked by optical stimulation was insensitive to TTX, a reasonable assumption is that P2X7 receptors driving the modulation of 5-HT efflux from MRR terminals are expressed on the terminals themselves. Nevertheless, the possibility that 5-HT release is modulated by a compound released from nearby astrocytes in response to the activation of astroglial P2X7 receptors cannot be entirely excluded. Our data showing the P2X7 receptor-mediated modulation of [^{3}H]5-HT release are not contradictory with previous findings reporting elevation in endogenous 5-HT level and inhibition of 5-HT transporters in case of genetic deletion and the pharmacological blockade of P2X7 receptors. These changes might be independent and compensatory changes as well, pointing to the complex regulation of serotonergic transmission by P2X7 receptors. The regulation of 5-HT efflux by P2X7 receptors might also gain significance in psychiatric disorders as recent studies pointed out the alleviation of depression- and schizophrenia-like behaviours in rodent animal models by the inhibition of P2X7 receptor.

Finally, we have verified our findings obtained in in vitro slice experiments in vivo, to detect the parallel release of neurotransmitters to a similar optical stimulation. We demonstrate here that 5-HT and Glu are released simultaneously by 20 Hz optical stimulation, whereas GABA was not elevated by a detectable level by this stimulus. On

the other hand, all three neurotransmitters responded equally well to K^+ depolarization, which is not restricted to ChR2 expressing neurons.

In contrast to 20 Hz stimulation, 50 Hz theta bursts lead to significant elevation in the level of GABA, but did not release either 5-HT or Glu. This result illustrates that different patterns of neuronal activity in the MRR results in distinct patterns of transmitter efflux. Since the second optical stimulation was relatively closed to the run-down of the first response, one might assume, that the releasable pool of transmitters have not been replenished yet at this time point. However, this was not the case, because if the order of stimulations was replaced, 50 Hz was still ineffective to release either 5-HT or Glu.

Finally, optogenetic activation is a feasible method to selectively release and directly detect transmitters from an identified pathway in vitro and in vivo by propagating Na^+ channel activity and subsequent Ca^{2+}-dependent exocytosis. Our results show that in the MRR, 5-HT and Glu are both released from ChR2 expressing neurons of MRR and 5-HT release is the result of ionotropic Glu receptor activation. In the HP, 5-HT is released from MRR terminals independently from glutamatergic transmission and is not subject to neuromodulation by presynaptic $5\text{-HT}_{1B/1D}$ autoreceptors. In contrast hippocampal 5-HT release from both MRR and non-MRR terminals is subject to neuromodulation by endogenous activation of P2X7 receptor.

5 Conclusions

In our research, we sought evidence for the involvement of the purinergic P2X7 receptor in the migraine pathophysiology in central nervous system. While in the study of hippocampal serotonin release, we were looking for the modulating role of the P2X7 receptor.

In the following few points, I would like to highlight our main findings:

Migraine model:

1. In the migraine model we demonstrated there is no difference in the pain phenotype of WT and P2X7 KO mice. The two genotypes did not show

significant difference in the NTG-induced thermal hypersensitivity. P2X7 KO mice are not more resistant to pain than their WT counterparts.

2. Acute and subacute BBG treatment influenced the nocifensive behaviour of WT mice, but not P2X7 KO mice. This suggests that the active function of P2X7 receptor is needed in the processing of pain.

3. NTG activates the brain regions which involved in cephalic pain, where affects c-fos expression. We have shown that NTG treatment increased c-fos expression in spinal cord (C1-2) and TNC in wild type and P2X7 KO mice. This suggest that the model and the c-fos expression are related to each other.

4. Furthermore, it was found that subacute BBG treatment reduced c-fos expression in TNC in wild type mice. With this we further strengthened the role of P2X7 receptor activation in the migraine pain and in the induction of characteristic symptoms.

Modulation of serotonin release:

1. In our experiment, we demonstrated that both electrical and optical stimulation increased the release of 5-HT in MRR and HP. Therefore, optogenetic activation is a suitable model for the in vitro and in vivo selective neurotransmitter release.

2. We also investigated glutamate involvement in serotonin release. The glutamate receptor antagonists have no effect on optical stimulation induced 5-HT release from the hippocampal afferents of the median raphe.

3. We also investigated the involvement of 5-$HT_{1A/1B/1D}$ receptors in the hippocampal 5-HT release, but the 5-$HT_{1A/1B/1D}$ and 5-HT_{1A} agonists has no effect on optical stimulation induced 5-HT release.

4. In our experiments, we demonstrated that the active function of P2X7 receptor is necessary for the release of serotonin from hippocampal serotonergic terminals which originate from the median raphe and non-median raphe region.

5., Endogenous ATP released by both optical and electrical stimulation paradigms promotes the release of 5-HT from serotonergic varicosities further enhances the role of P2X7 receptor activation.

Summarizing our results, the effect mediating role of P2X7 receptors has been demonstrated in the migraine pathophysiology in NTG-induced migraine mouse model and the regulation of hippocampal 5-HT release induced by electrically and optically.

6 My publications

Thesis related publications:

Gölöncsér F, Baranyi M, Balázsfi D, Demeter K, Haller J, Freund TFF, Zelena D, Sperlágh B. (2017) Regulation of hippocampal 5-HT release by P2X7 receptors in response to optogenetic stimulation of median raphe terminals of mice. Front Mol Neurosci. 2017 Oct 12;10:325. doi: 10.3389/fnmol.2017.00325. eCollection 2017.

Gölöncsér F, Sperlágh B. (2014) Effect of genetic deletion and pharmacological antagonism of P2X7 receptors in a mouse animal model of migraine. J Headache Pain. 2014 May 1;15:24. doi: 10.1186/1129-2377-15-24.

Other publications:

Balázsfi DG, Zelena D, Farkas L, Demeter K, Barna I, Cserép C, Takács VT, Nyíri G, Gölöncsér F, Sperlagh B, Freund TF, Haller J. (2017) Median raphe region stimulation alone generates remote, but not recent fear memory traces. PLoS One. 2017 Jul 14;12(7):e0181264. doi: 10.1371/journal.pone.0181264.

Bekő K, Koványi B, Gölöncsér F, Horváth G, Dénes Á, Környei Z, Botz B, Helyes Z, Müller CE, Sperlágh B. (2017) Contribution of platelet P2Y12 receptors to chronic Complete Freund's adjuvant-induced inflammatory pain. J Thromb Haemost. 2017 Mar 27. doi: 10.1111/jth.13684.

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Horváth G, Gölöncsér F, Csölle C, Király K, Andó RD, Baranyi M, Koványi B, Máté Z, Hoffmann K, Algaier I, Baqi Y, Müller CE, Von Kügelgen I, Sperlágh B. (2014) Central P2Y12 receptor blockade alleviates inflammatory and neuropathic pain and cytokine production in rodents. Neurobiol Dis. 2014 Oct;70:162-78. doi: 10.1016/j.nbd.2014.06.011. Epub 2014 Jun 25.

Csölle C, Baranyi M, Zsilla G, Kittel A, Gölöncsér F, Illes P, Papp E, Vizi ES, Sperlágh B. (2013) Neurochemical Changes in the Mouse Hippocampus Underlying the

Antidepressant Effect of Genetic Deletion of P2X7 Receptors. PLoS One. 2013 Jun 21;8(6):e66547. doi: 10.1371/journal.pone.0066547. Print 2013.

Sperlagh B, Csolle C, Ando RD, Goloncser F, Kittel A, Baranyi M. (2012) The role of purinergic signaling in depressive disorders. Neuropsychopharmacol Hung. 2012 Dec;14(4):231-8. Review.

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Kováts N, Ács A, Gölöncsér F, Barabás A. (2011) Quantifying of bactericide properties of medicinal plants. Plant Signal Behav. 2011 Jun;6(6):777-9. Epub 2011 Jun 1.

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Additional publications which are not included in the Library Data Sheet:

Stephan G, Huang L, Tang Y, Vilotti S, Fabbretti E, Yu Y, Nörenberg W, Franke H, Gölöncsér F, Sperlágh B, Dopychai A, Hausmann R, Schmalzing G, Rubini P, Illes P. (2018) The ASIC3/P2X3 cognate receptor is a pain-relevant and ligand-gated cationic channel. Nat Commun. 2018 Apr 10;9(1):1354. doi: 10.1038/s41467-018-03728-5.

Beamer E, Gölöncsér F, Horváth G, Bekő K, Otrokocsi L, Koványi B, Sperlágh B. (2016) Purinergic mechanisms in neuroinflammation: An update from molecules to behavior. Neuropharmacology. 2016 May;104:94-104. doi: 10.1016/j.neuropharm.2015.09.019. Epub 2015 Sep 16. Review.

Kováts N, Gölöncsér F, Ács A, Rafaey M. (2010) Quantification of the antibacterial properties of Artemisia absinthium, A. vulgaris, Chrysanthemum leucanthemum and Achillea millefolium using the Vibrio fischeri bacterial bioassay. Acta Bot Hung. 2009 Sep;52(1-2). doi: 10.1556/ABot.52.2010.1-2.e2