

**Involvement of P2 receptors in physiological and
pathological function of central nervous system: from
neurotransmitter release to whole genom microarray
analysis**

PhD theses

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Introduction

ATP as a synaptic transmitter and presynaptic modulator has important role in neuronal information processing in the central nervous system under physiological and pathological conditions. The action of ATP are conveyed by ionotropic P2X (P2X1-7) and metabotropic P2Y (P2Y_{1,2,4,6,11,12,13,14}) receptors in the extracellular space. It is known that ATP is co-released with catecholamines in not only the peripheral but also in the central nervous system. However, the number of monoaminergic cells are rather low in the brain, yet this neuron subpopulation has crucial role in the regulation of functioning of central nervous system. The main population of catecholaminergic cells are located in A1-A3 nuclei (medulla), and in A4-A7 nuclei (pons). Among these, the most important group is the A6 nucleus in the locus coeruleus, which provides a diffuse noradrenergic innervation of the hippocampus. Previously, our group showed that noradrenergic nerve terminals express facilitatory P2X₁ and P2X₃ receptors in the periphery and in central nervous system. The hippocampus is well known to be especially sensitive to hypoxia and ischemia and under pathological conditions noradrenaline (NA) is released in excess to the extracellular space contributing to imbalance of neurotransmitter release and to neuronal cell death. Excess NA release could be an important pathogenetic factor contributing to free-radical induced damage by autooxidation and monoaminoxidase-driven oxidation during reperfusion. By the identification of presynaptic release modulatory receptors on hippocampal noradrenergic nerve terminals, new therapeutic targets could be proposed for ischemic neurodegeneration and other therapeutic fields related to the noradrenergic system (depression, hypertension, regulation of sleep-wake).

However, modulation of NA release is not the only potential purinergic target which could be utilized for neuroprotection. Among P2X receptors, the slowly desensitizing P2X₇ receptor has distinct structural, functional and pharmacological features: its carboxil terminal domain is longer than those of other P2X receptor subunits containing a lipopolysaccharide (LPS) binding site; ATP activates it in higher concentration (1mM) and its sustained or repeated

activation causes the formation of a reversible membrane pore permeable up to 900 Da. P2X7 receptors are expressed by microglia, astrocyte cells and nerve terminals and may be involved in neurodegeneration and in the following repair process. P2X7 receptors may affect neuronal cell death, through their ability to regulate: (1) the processing and release of inflammatory cytokines (IL-1 β , TNF- α , INF- γ) and chemokines (MIP) (2) the neurotransmitter release (glutamate) (3) and to activate signal transmission cascades (MAPKs, PLA2/PLD, caspases, NF- κ B, AP-1, CREB), known as key mediators of neurodegenerative and inflammatory diseases of nervous system (ischemia, Alzheimer's dementia, multiple sclerosis). In addition, P2X7 receptors may participate in the phenomena of learning and memory (LTP, LTD) as a molecular sensor of the increased neural activity. It is well known that proinflammatory cytokines, such as IL-1 β probably expressed by microglia and astrocyte cells contribute to neuronal cell death under ischemic-like conditions and reperfusion. IL-1 β has also a direct inhibitory effect on the development of LTP impairing synaptic plasticity underlying memory formation in the hippocampus. Maturation of IL-1 β requires two activation stimuli: while bacterial endotoxin (LPS) *per se* leads to the expression of pro-interleukin-1 β (pro-IL- β), cellular release of IL-1 β requires a secondary signal, which triggers the cleavage and subsequent rapid release of fully processed IL-1 β . A major immunomodulatory function of P2X7 receptor is that it acts as this necessary secondary, external co-stimulus for posttranslational processing and release of IL-1 β in periphery. Several previous in vitro studies have demonstrated the role of P2X7 receptors in the production of detectable and mature IL-1 β in response to LPS.

Gene polymorphism studies and ongoing animal studies in our and other research groups shed light on another potential indication field, where drug development program targeting P2x7 receptors may be promising: these are mood disorders, including major depression (MDD) and bipolar disorder (BPD). Hence, both genetic deletion and pharmacological inhibition of the P2X7 receptors leads to antidepressant-like effect in rodents, and P2X7 receptors expressed

on non-hematopoietic cells appear to be responsible for this behavioral phenotype

Objectives

I. To explore the inhibitory purinergic regulation of hippocampal noradrenaline release under physiological conditions and to identify the underlying receptor subtypes. We have studied how the activation of the ionotropic P2 receptors affect the release of noradrenaline (NA) from the catecholaminergic terminals of the the hippocampus and, which purinoceptor subtypes mediate this effect.

II. To examine the role of P2 receptors in the inhibitory regulation of NA release in the rat hippocampus under pathological, ischemia-like conditions.

III. To study the involvement of P2X7 receptors in the regulation of IL-1 β production in the rodent hippocampus using an in vivo inflammatory model. We tried to find evidence for the participation of P2X7 receptors in the regulation of central IL-1 β production and asked whether the P2X7 receptors responsible for this regulation are primarily located peripherally or centrally.

IV. To explore the effect of P2X7 receptor deficiency on gene expression related to depression using whole mouse genom microarray analysis. We have identified new biological pathways which are associated with P2X7 receptor activation and plausible for the pathomechanism of depression.

Methods

All experiments used male Wistar rats and 2–3 months old, homozygous (P2rx7^{-/-}) male mice and their wild-type (WT) littermates (P2rx7^{+/+}) were used as controls. The original breeding pairs of P2rx7^{-/-} mice (C57BL/6J based) were supplied by Christopher Gabel from Pfizer Inc. (Groton CT, USA).

In vitro [³H]NA release experiments from rat hippocampal slices

Experimental animals were decapitated and the hippocampus was dissected in ice-cold Krebs' solution and 400 μm thick slices were prepared by a McIlwain tissue chopper and incubated in 1 mL of modified Krebs' solution (mmol/L: NaCl 113, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, and glucose 11.5, pH 7.4) containing 2.5 μCi/mL [³H]NA (specific activity 30 Ci/mmol), ascorbic acid (300 μmol/L), and Na₂EDTA (30 μmol/L) for 45 min and maintained at 37°C.

IL-1β assay

After 6 hours LPS treatment the collected mouse and rat hippocampal tissue supernatant and mouse serum IL-1β production were evaluated using an enzyme-linked immunosorbent assay (ELISA) kit, DuoSet IL-1β (R&D System, Minneapolis, MN, USA), specific for rat and mouse IL-1β protein, respectively, according to the manufacturer's instructions.

Microarray based gene expression experiment

Whole Mouse Genome custom-commercial microarray (Agilent Technologies; 4 x 44K slide format) printed with Agilent SurePrint® technology was used. Each 42,152-feature microarray slide contains 41,041 probes. All animals were given an intraperitoneal (i.p.) injection of saline or LPS and animals were killed by decapitation 7h after LPS injection and amygdala was dissected. Gene expression measurement, including the RNA quality control, linear amplification and hybridization to oligonucleotide expression

microarrays, washing followed by scanning and data prenormalization were performed the in Agilent Microarray Core Facility (Semmelweis University, Budapest Institute of Genetics, Cell and Immunobiology, <http://www.dgci.sote.hu/microarray>) according to the protocols specified by the manufacturers (Agilent Technology). Further bioinformatical data transformation and statistical evaluation were performed according to the protocols and parameters specified by Mono-Color gene expression platform by the manufacturers. Gene expression data were analyzed with GeneSpring GX 9.5 software. The threshold of fold change (FC) analyzed by statistical evaluation was at least 2.0. (up-or downregulation).

GO analysis

The microarray data were further analyzed using the bioinformatics analysis tool GeneCodis. Gene Ontology TM (GO) is a consortium database application for monitored system of functional analysis of genes and their product lists that integrates different biological function and their involvement in various biological processes. Each GO term shows the association of the given genes using the defined algorithm to determine biological annotations or combinations of annotations that were over-represented with respect to a reference list deposited in the NCBI GenBank database.

TaqMan based real-time PCR

TaqMan low-density array (TLDA) was designed and performed on 72 selected genes to confirm the gene expression data obtained with the microarray analysis. The TLDA microfluidic card was preloaded with selected gene expression assays for the genes of interest according to the Agilent Probe ID. Thermal cycling conditions were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 30 sec at 97°C and 1 min at 59.7°C

Results

I. Pharmacological mapping of the presynaptic inhibitory purinergic regulation of hippocampal noradrenaline release.

1. In the first part of the thesis, we examined the presynaptic inhibitory purinergic regulation of hippocampal noradrenaline release by P2 receptors. At first, we tested our experimental model, whether it is suitable to investigate the neuronal release of [³H]NA. Because low frequency electrical field stimulation elicited a reproducible amount of tritium release, resulting in an FRS2/FRS1 ratio of 1.086 ± 0.033 , and the evoked release was largely TTX-dependent ($1-3 \mu\text{M}$), the majority of [³H]NA release has been proved to be action potential dependent and of neuronal origin. According to Dale's principles, neurotransmitters are stored in synaptic vesicles and released upon physiological neuronal activity, and the classical way of transmitter release is exocytosis preceded by calcium influx. In our experiments, when the slices were superfused with Ca^{2+} -free Krebs' solution supplemented with 1 mmol/L EGTA, the electrical stimulus evoked release of [³H]NA was almost totally inhibited ($\text{FRS2/FRS1} = 0.47 \pm 0.01\%$). The majority of the evoked release could be therefore regarded as conventional neurotransmitter release under neuronal activity.

2. We examined the effects of the following agonists on electrical stimulus-evoked release of [³H]NA: the non-selective P2 receptor agonist ATP ($3 \mu\text{M} - 1 \text{ mM}$) and ADP ($0.1- 3 \mu\text{M}$), the selective P2Y receptor agonist 2MeSADP ($3-30 \mu\text{M}$) and the selective P2Y₁ receptor agonist MRS2365 ($0.1-100 \text{ nM}$), which were administered in increasing concentrations into the perfusion solution from 18 min before the second field stimulation. All agonists decreased concentration dependently the release of [³H]NA evoked by electrical field stimulation. In case of ATP, the maximal response was obtained at $300 \mu\text{M}$ which resulted in 43% inhibition. The effect of ATP was concentration-dependent with an IC_{50} value of $30 \mu\text{M}$. Further analysis of concentration-response relationship of the inhibitory effect of agonists resulted in the following rank order of agonist potency: $\text{MRS2365} \gg \text{ADP} > 2\text{MeSADP} \geq \text{ATP}$.

3. In the following experiments, pharmacological characterization of the effect of ATP was performed in order to identify the underlying receptor subtypes. The inhibitory effect of ATP (300 μ M, FRS2/FRS1=0.652 \pm 0.025) on [³H]NA outflow was examined in the presence of various P2 receptor antagonists. All antagonists, including the non-selective P2 receptor antagonist PPADS (30 μ M), the P2Y₁₂ and P2Y₁₃ receptor antagonist 2-MeSAMP (10 μ M), and the selective P2Y₁ antagonist MRS2179 (10 μ M) reversed the inhibitory effect of ATP. Among antagonists, MRS2179 is a selective P2Y₁ receptor antagonist in the concentration used and the activation of P2Y₁ receptors inhibits N-type Ca²⁺ channels in neurons, which may underlie the inhibitory effect of nucleotides on vesicular transmitter release. P2Y₁₂ and P2Y₁₃ receptors are both blocked by 2-MeSAMP, with similar potency, while the antagonism by PPADS favors the involvement of P2Y₁, but not P2Y₁₂ receptors. Taken together, our present findings suggest that P2Y₁ and P2Y₁₃ receptors mediate the inhibitory effect of ATP on the electrical stimulation-evoked NA release. The involvement of P2Y₁₂ receptor in the regulation of the inhibitory effect of ATP is less likely, although cannot be completely excluded. Interestingly, in the absence of agonists, none of the P2 antagonists affected significantly the electrical stimulation-evoked release of [³H]NA. These findings indicate that endogenous ATP released stimulation-dependently by EFS from the hippocampus does not activate tonically inhibitory P2Y receptors under our experimental conditions. Because the release of ATP in the hippocampus is highly frequency-dependent, and the ectoATPase activity is high it is conceivable to suggest that at low frequency stimulation the biophase concentration of ATP might not reach levels high enough to activate inhibitory P2Y receptors. Therefore, an attempt was made to explore conditions, which result in higher ATP concentrations in the extracellular fluid, and thereby leads to the endogenous activation of P2 receptors involved in the modulation of NA release. Previous studies have demonstrated that one such condition might be ischemic-like conditions, which is well known to elevate the extracellular level of ATP *in vitro* and *in vivo*.

II. Effect of combined oxygen and glucose deprivation on the [³H]NA release in rat hippocampal slices

1. In rat hippocampal slices which are especially sensitive to ischemic insult, in vitro ischemic-like conditions, simulated by 30 min combined oxygen and glucose deprivation (OGD) elicited a long-lasting and remarkable increase in the outflow of [³H]NA (8.32±1.97%), which returned to the baseline after 30 min. To investigate the underlying mechanism of [³H]NA release, we applied Ca²⁺ free modified Krebs' solution at the beginning of perfusion and the effect of energy deprivation was more pronounced: the evoked release of [³H]NA was dramatically increased (20.03±0.09). In contrast, TTX (1 μM) totally inhibited (2.11±2.45%) the combined OGD-induced excessive release of [³H]NA. These results show that effect of 30 min OGD on [³H]NA release in rat hippocampal slices is enhanced in the absence of extracellular calcium and was sensitive to TTX, consistently with the concept that neurotransmitter release in response to ischemic-like conditions has non-vesicular, but cytoplasmic origin and is associated with voltage dependent Na⁺ channel activation, axonal depolarization and with subsequent intracellular Na⁺ load. Our observations are in compliance with previous data which showed the TTX-dependence and cytoplasmic origin of NA release in hippocampal and spinal cord slices under in vitro ischemic-like conditions. Combined glucose and oxygen deprivation results in energy deprivation and subsequent inhibition of Na⁺/K⁺-ATPase, intracellular Na⁺ accumulation, and sodium-dependent reversal of the monoamine uptake carrier.

2. Then, the involvement of P2 purinoceptors was examined in the modulation of excess NA release under ischemic-like conditions. MRS2179 (10 μM), the selective P2Y₁ antagonist significantly enhanced the effect of ischemia on the efflux of [³H]NA (18.19±1.05%, vs. ischemic control 8.32±1.97%) indicating the pathological activation of P2Y₁ receptor. PPADS (30 μM), the nonselective P2 receptor antagonist did not affect significantly the release of tritium under ischemia-like conditions. The reasonable

explanation to the lack of the effect of PPADS is that other PPADS sensitive and facilitatory P2X receptors are also co-activated by endogenous ATP and the two opposite modulation counter-balanced each other. Since it is known that the activation of P2X1 or P2X3 receptors by endogenous ATP facilitates NA release in the hippocampus, we hypothesized that ischemia evoked ATP efflux via P2X1 and P2X3 activation contribute to ischemia induced [³H]NA release. Supporting this assumption we examined the potential P2X receptor mediated facilitatory regulation under ischemic like condition. PPNDS (100 μM) the P2X1 receptor antagonist significantly decreased the ischemia induced [³H]NA release, indicating the involvement of P2X1 receptor subtypes (PPNDS 0.74±0.711%, n=8 vs. ischemic control 8.32±1.97%, 90% inhibitory effect). On the contrary, 2-MeSAMP((10 μM), the P2Y_{12/13} receptor antagonist did not affect the [³H]NA outflow (8.20±0.711%), excluding the involvement of these P2Y receptor subtypes in the inhibitory modulation of NA release evoked by ischemic-like conditions.

III. The role of P2X7 receptor in the regulation of IL-1β production in the rodent hippocampus in an in vivo inflammatory model.

1. Systemic LPS treatment caused a remarkable dose-dependent (300μg/kg and 500μg/kg) and time-dependent (2-6h) elevation in central IL-1β level (6 h basal IL-1β: 70.32. ±5.92 pg/ml; LPS 300μg/kg: 227.6±14.4 pg/ml, 500μg/kg: 332.9±35.2 pg/ml, n=4). Similar to our data other reports also showed that IL-1β is expressed at detectable levels in the hippocampus and is subject to i.p. LPS priming, which elicits a substantial increase in its level.

2. It is known that the defensive system of blood-brain barrier may be damaged by bacterial infections, which increases its permeability, and peripheral cytokines may participate in this mechanism. Therefore the action of peripheral LPS on hippocampal cytokine

production could be both direct (in the central nervous system) and indirect (induced in periphery). However, the contribution of peripherally and centrally processed IL-1 β production to the detected hippocampal cytokine level has been so far unknown. To address this question we examined whether the IL- β production is also subject to P2X7 receptor regulation in the serum. Moreover we performed transcatheterial perfusion, which prevents the contamination of brain by blood cells and circulating IL-1 β and compared the IL-1 β levels in the serum and in the hippocampus with and without transcatheterial perfusion in wild type mice. In our blood cytokine analysis, we found that i.p. LPS caused a remarkable increase in level of IL-1 β after 6h, and the detected pg/ml values were similar to that observed in the hippocampus experiments (307.6 \pm 36.1 pg/ml vs. saline treatment: 19.2 \pm 9.6 pg/ml). Although in case of transcatheterial perfusion, 6h intraperitoneal LPS injection was still able to significantly elevate the hippocampal level of IL-1 β , the level of both basal and LPS induced IL-1 β were substantially lower (LPS: 24.3 \pm 0.3 pg/ml, saline 11.9 \pm 0.9 pg/ml). This remarkable difference was also detected in the case basal IL-1 β production, i.e. after saline treatment. Based on these results it is very likely that the origin of hippocampal IL-1 β production upon peripheral LPS challenge is mixed with a predominant peripheral contribution; however the local IL-1 β response was also correlated with the inflammatory stimulus.

3. The primary aim of this series of experiments was to explore the role of P2X7 receptors in the regulation of hippocampal IL-1 β level in an in vivo model. In order to explore any potential species difference we pharmacologically characterized the modulation of IL-1 β production both in rats and mice and examined various selective and non selective P2X7 receptor antagonists as well: the non-selective antagonist PPADS (25 mg/kg) and the P2X7 receptor selective antagonists BBG (100 mg/kg) and oATP(0.9 mg/kg). All three antagonists diminished the LPS induced IL-1 β level in rat hippocampus at 6h (PPADS+LPS: 169.61 \pm 5.92 pg/ml; oATP+LPS: 124.9 \pm 93.37%; BBG+LPS: 58.84 \pm 12.85% vs. LPS: 327.34 \pm 10.91%, of the corresponding saline values). Similar results were obtained in P2X7 receptor wild-type mice in response to systemic endotoxin

(α ATP+LPS: $75.14 \pm 10.91\%$; BBG+LPS: $28.14 \pm 11.11\%$ vs. LPS $395.52 \pm 13.10\%$)

4. In rat experiments, to identify the regulatory role of P2X7 receptors, at first the actions of agonists were tested in putative P2X7 receptor selective doses (ATP, 9 mg/kg, BzATP 6.4 mg/kg). ATP had a time-dependent effect on IL-1 β level. When it was added 4h after endotoxin treatment, IL-1 β level was significantly higher which corroborates with other observations in macrophages and microglia cells (ATP+LPS: $185.71 \pm 14.70\%$ vs. LPS treatment $151.11 \pm 1.61\%$ of the corresponding saline values). In contrast, when we applied longer treatment time, i.e. the agonist was injected prior to LPS, both ATP and BzATP significantly diminished IL-1 β level. We assume that the detected inhibitory agonist action is due to the action of their breakdown product, adenosine and Bzadenosine, respectively, because the half life of both ATP and BzATP is very short in the circulation. Previous studies pointed to the inhibitory role of A₁ receptors in the periphery, although the role for A₁ receptors in the regulation of central IL-1 β level has not been demonstrated yet. In our rat experiments, DPCPX (10mg/kg) the selective A₁ adenosine receptor antagonist, reversed the inhibitory effect of ATP which confirmed our hypothesis (ATP+DPCPX+LPS: $140.59 \pm 11.11\%$ vs. ATP+LPS: $76.33 \pm 7.29\%$ of the corresponding saline values).

5. A more definitive proof for involvement of P2X7 receptor was obtained by the utilization of genetical knockout of P2X7 receptor: the systemic bacterial endotoxin treatment evoked cytokine response detected in P2rx7^{-/-} mice significantly diminished when compared to wild type mice (LPS: 320.4 ± 51.3 pg/ml in wild type mice vs. 215.5 ± 16.1 pg/ml in P2rx7^{-/-} mice). Moreover, the inhibitory effect of antagonists was also significantly attenuated in these mice, indicating that their effect is at least partly related to the specific inhibition of P2X7 receptor. These data are consistent with the findings of an other study showing that i.p. LPS evoked IL-1 β mRNA expression was less elevated in the hypothalamus of P2rx7^{-/-} mice as compared to WT mice. IL-1 β levels are increased to similar extent in the serum upon systemic LPS challenge to that observed in

the hippocampus, and genetic deletion of P2X7 receptor attenuated the serum IL-1 β response as well (307.6+36.1 pg/ml vs. saline treatment 19.2+9.6 pg/ml in wild type mice, 136.99+39.54 pg/ml vs. saline treatment 27.47+3.86 pg/ml in P2rx7^{-/-} mice). Furthermore, after transcatheter perfusion there was no significant difference between hippocampal IL-1 β protein levels of wild type mice and P2rx7^{-/-} mice. Involvement of P2X7 receptor activation in LPS induced peripheral IL-1 β production have been previously demonstrated and our data confirm this peripheral regulation. Our data suggest that participation of P2X7 receptors expressed by peripheral macrophage, leukocyte and other blood cells are predominant in the regulation of central cytokine production followed by LPS injection.

IV. Effect of P2X7 receptor deficiency on the alteration of depression related gene expression: a whole mouse genom microarray analysis

In our functional genomic study, we explored the alteration of gene expression elicited by P2X7 receptor (P2rx7) deficiency in the amygdala, an intrinsic part of the limbic system. Therefore, a whole mouse genome gene expression microarray analysis was performed on the amygdala after 6h peripheral LPS treatment (250 μ g/kg).

1. Our data show that the expression of a surprisingly high number of genes was changed significantly by genetic deletion of P2rx7, compared to wild type mice using classical microarray data analysis and statistical analysis. Deficiency of P2rx7 changed the expression of 8165 transcripts, which showed at least two-fold changes as well. These microarray data are *per se* of interest, however, to understand the functional impact of P2rx7 deficiency on biological processes, microarray data were further subjected to gene ontology analysis.

2. GO analysis showed remarkable upregulation in the following annotation gene groups: synaptic transmission, ion-transport, G-protein coupled receptor protein signaling pathway, ATP synthesis coupled proton transport, and regulation of transcription. Using the data generated by the gene ontology analyses and searching biological plausibility for depression, we generated a further gene list for TaqMan based real-time PCR validation.

3. Microarray experiments were validated by assessing the expression of more than 60 genes of interest, applying both sample and treatment validation. Our real-time PCR results correlated with previous microarray data, and 29 genes were confirmed, which has biological plausibility in synaptic signaling and neuroplasticity and could be linked to the pathogenesis of depression. Among these genes, 25 genes were downregulated in P2rx7^{-/-} mice, including genes encoding ionotropic glutamate receptors (AMPA2, AMPA4), the metabotropic glutamate receptor 7 (Grm7) and the glial cell-derived neurotrophic factor (GDNF), which are consistent with the presumed dysfunction of glutamatergic transmission and consequent neuroplasticity changes in depression. In contrast, an opposite direction, upregulation of the NR2B subunit of NMDA receptor (Grin2b) was found in P2rx7^{-/-} mice. Genes related to GABAergic transmission were also affected by genetic deletion, causing a subtype-specific up- and downregulation of various GABA_A receptor subunits (while Gabrb2, Gabrb3, Gabrg2, Gabrg3 genes were downregulated, similar to the the GABA synthesizing enzyme, the glutamic acid decarboxylase (GAD), whereas the GABAC subunit encoding gene, Gabrr1 was upregulated). These results indicate that P2X7 receptor has subtype-specific regulatory role in the alteration of glutamate and GABA receptor mRNA expression.

4. Relatively modest gene expression changes were observed after peripheral bacterial endotoxin stimulus compared to effect of P2X7 receptor deficiency. In amygdala samples, among transcripts that were affected by endotoxin treatment, 287 transcripts had a significant LPS treatment effect regardless of the effect of genotype and showed at least two-fold, significant upregulation. One potential explanation is that 6h after the relatively low dose of LPS treatment

only inflammatory and immune response related genes, including cytokines and chemokines and other signaling proteins were upregulated. Our results correlate with previous observations, in which genes encoding proteins responsible for chemokine and cytokine profile of inflammatory immune response, increased humoral immune response and phagocytosis and other signal molecules were upregulated in response to similar systemic endotoxin treatment.

Conclusions

I/1. We demonstrated the involvement of inhibitory P2Y receptors in the presynaptic purinergic regulation of hippocampal noradrenaline release.

I/2. According to classical transmitter release, electrical field stimulation released NA from vesicular source in a $[Ca^{2+}]$ -dependent way. NA release in response to field stimulation was associated with ongoing neuronal activity and agonists of P2Y receptors inhibited the evoked outflow. Pharmacological characterisation of P2Y receptors, responsible for the effects of agonists indicated the involvement of P2Y receptors with pharmacological profile similar to that of the cloned P2Y₁ and P2Y₁₃ receptor subtypes. In conclusion, we show here that noradrenaline release is under the control of a dual and opposite modulation by pre-synaptic P2X and P2Y receptors under physiological conditions.

II/1. Combined oxygen and glucose deprivation (OGD) simulated by in vitro ischemic-like conditions caused a remarkable increase in the release of hippocampal NA. We found that the effect of 30 min ischemic-like stimulation on NA release is enhanced in the absence of extracellular calcium. Both facilitatory P2X₁ and inhibitory P2Y₁ receptors are involved in the dual and opposite purinergic modulation of the excessive cytoplasmic release of NA under ischemic-like conditions. Therefore, hippocampal NA release is

under the control of a dual P2 receptor mediated modulation under both physiological conditions (low frequency field stimulation) and under pathological conditions (combined oxygen and glucose deprivation).

III/1. We showed that pathological activation of P2X7 receptors are involved in not only peripheral but also central IL-1 β production. Level of hippocampal IL-1 β increased in a P2X7 receptor dependent way in response to systemic endotoxin stimulation. Activation of A₁ adenosine receptors has an inhibitory role on hippocampal IL-1 β level, which may participate in the neuroprotective action of adenosine.

III/2. After transcordial perfusion, although systemic LPS treatment was still able to significantly elevate the hippocampal IL-1 β production, the level of both basal and LPS induced IL-1 β was substantially lower. Our results indicate that the origin of hippocampal IL-1 β level is mixed with a predominant peripheral contribution. In addition, after transcordial perfusion there was no significant difference between hippocampal IL-1 β protein levels of wild type mice and P2rx7^{-/-} mice. Taken together, we revealed the crucial role of the P2X7 receptors in the modulation of systemic LPS-induced hippocampal IL-1 β protein level, however, P2X7 receptors responsible for this regulation appears to be located peripherally, presumably on immun-competent cells or other blood cells.

IV/1. The expression of some 8165 transcripts was affected significantly by genetic deletion of P2rx7 using the selection filters of more than 2.0-fold changes in the amygdala

IV/2. Among downregulated genes in P2rx7^{-/-} mice, genes encoding ionotropic glutamate receptors (AMPA2, AMPA4), and the metabotropic glutamate receptor (Grm7) were validated, which are consistent with the presumed overactivation of glutamatergic transmission and consequent neuroplasticity changes in depression. These changes are also consistent with previous results showing that the activation of P2X7 receptors in the brain leads to an increased

glutamate and subsequent GABA release from nerve terminals and astrocytes. Therefore, it is tempting to speculate that the increased activation of P2X7 receptor may result in glutamatergic overactivation which would lead to depressive symptoms. Conversely, the lack of these effects in the absence of the P2rx7 results in compensatory changes in the mRNA expression of glutamate and GABA receptor subunits. This assumption is strengthened by observations from animal and human studies indicating that excessive glutamate transmission might be involved in the pathophysiology of affective disorders. These include abnormalities in the level of glutamate, genetic polymorphisms and the differential expression of glutamate receptor subunits in bipolar and major depressive disorders. Previously demonstrated gain-of-function mutations of the *P2RX7* gene and subsequent receptor activation may be associated with overactivation of glutamatergic transmission. On the basis of previous and our recent results we speculate that P2X7 receptor overactivation may contribute to underlying pathomechanisms of depressive symptoms.

My publications

Thesis related publications

1. Csölle C, Heinrich A, Kittel A, Sperlagh B.: P2Y receptor mediated inhibitory modulation of noradrenaline release in response to electrical field stimulation and ischemic conditions in superfused rat hippocampus slices. (2008) *J Neurochemistry.*: 106:(1) 347-360, IF: 4.500

2. Csölle C, Sperlagh B.: Peripheral origin of IL-1beta production in the rodent hippocampus under in vivo systemic bacterial lipopolysaccharide (LPS) challenge and its regulation by P2X(7) receptors. (2010) *J Neuroimmunology*: 219(1-2):38-46., IF:3.159

3. Csölle C., Andó RD., Kittel Á., Gölöncsér F., Baranyi M., Soproni K., Zelena D., Haller J., Németh T., Mócsai A., Sperlágh B. (2013): The absence of P2X7 receptors (P2rx7) on non-haematopoietic cells leads to selective alteration in mood-related behaviour with dysregulated gene expression and stress reactivity in mice. *Int J Neuropsychopharmacol.*: 16(1):213-33, IF:4.578

Other publications

1. Sperlágh B, Heinrich A, Csölle C.: P2 receptor-mediated modulation of neurotransmitter release--an update. (2007) *Purinergic Signaling*: 3: 269-284

2. Heinrich A, Kittel A, Csölle C., Vizi ES, Sperlagh B.: Modulation of neurotransmitter release by P2X and P2Y receptors in the rat spinal cord (2008) *Neuropharmacology*: 54:(2) 375-386 , IF: 3.383

3. Csölle C., Sperlágh B.: Endocannabinergic modulation of IL-1 β level/production in mouse hippocampus under basal conditions and after in vivo systemic LPS stimulation. (2011) *Neuroimmunomodulation*: (4), 226-231, IF: 2.383

4. Hracskó Zs, Baranyi M, Csölle C., Gölöncsér F, Madarász E, Kittel Á and Sperlágh B.: Lack of neuroprotection in the absence of P2X7 receptors in toxin-induced animal models of Parkinson's disease' (2011) *Mol. Neurodegener.*:6: 28: IF: 5.09

5. Andó RD., Bíró J, Csölle C., Ledent C., Sperlágh B.: The inhibitory action of exo- and endocannabinoids on [3H]GABA release are mediated by both CB1 and CB2 receptors in the mouse hippocampus. (2011) *Neurochem Int.*: 60(2):145-52 Volume , IF: 2.857

6. Sperlagh B, Csölle C., Ando RD, Goloncser F, Kittel A, Baranyi M. The role of purinergic signaling in depressive disorders. (2012) *Neuropsychopharmacol Hung.*: (4):231-8.