Studies on purinergic signalling in the central nervous system using high resolution techniques

PhD Theses

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1. Introduction

Under normal conditions, pain is associated with electrical activity in small-diameter fibers of dorsal root ganglion (DRG) of the spinal cord. In addition, numerous studies have shown that descending pathways from the brainstem, including the descending noradrenergic pathway play crucial role in the modulation of sensory transmission in the spinal cord and thereby attenuate pain sensation. The ability of ATP to elicit pain was first described more than 40 years ago, and it is now widely recognized that it is an important messenger involved in sensory information processing. ATP is released from spinal cord nerve terminals upon depolarization and probably from damaged or stressed cells upon pathological conditions. The released ATP acts via various subtypes of ionotropic P2X (homomeric P2X1-7, and hetero-oligomeric P2X1/2, P2X1/5, P2X2/3, P2X1/4, P2X2/6, P2X4/6) receptors, and/or metabotropic P2Y ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) receptors. mRNA encoding all subunits of the P2X receptors are expressed along the nociceptive pathways, including the DRG, and among them, P2X1, P2X2 and P2X3 receptor proteins are expressed on different subpopulations of primary afferent neurons. ATP, activating P2X receptors, acts as an excitatory neurotransmitter in the dorsal horn of the spinal cord. Moreover, the activation of P2X receptors not only mediates but also facilitates excitatory transmission, releasing glutamate from primary afferent fibers terminating in lamina II and lamina V of the spinal cord; these actions are mediated by P2X3, P2X1/5 and P2X4/6 receptors. Less is known about the role of metabotropic P2 receptors in the modulation of signal transmission in the spinal cord. All subtypes of the P2Y receptor family are widely expressed in different parts of the nervous system, although the expression of only $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor has been demonstrated so far in sensory neurons. The activation of P2Y receptors causes blockade of the N-type calcium channels in DRG cells. This effect may decrease the release of glutamate from DRG terminals in the spinal cord and thereby partly counterbalance the algogenic effect of ATP. Nevertheless, neurochemical evidence supporting a role for these receptors in the modulation of spinal neurotransmitter release has not been presented so far.

ATP and its extracellular breakdown product, adenosine are important signaling molecules in the CNS under physiological and pathological conditions. ATP, activating ionotropic P2X (P2X1-7) and metabotropic P2Y ($P2Y_{1,2,4,6,11,12,13,14}$) receptors acts as a fast transmitter and as a modulator of synaptic activity in several regions of the brain, including the hippocampus. Moreover, it also participates in long term-synaptic plasticity and regulates the survival of

neurons and the following repair process under pathophysiological conditions, such as ischemia and inflammation. Adenosine, activating metabotropic adenosine receptors (A_1, A_{2A}, A_{2B}, A_3), has also a well-established role in the hippocampus, as a pre- and postsynaptic depressant of excitatory transmission gaining significance under physiological conditions, metabolic stress and seizures.

Accordingly, convincing evidence demonstrate the activity-dependent release of both ATP and adenosine from the hippocampus under stimuli, mimicking physiological neuronal activity such as electrical stimulation or pathological challenge, such as combined oxygen-glucose deprivation. However, due to their ubiquitous nature, the endogenous source of purines for synaptic modulation remained enigmatic under these conditions.

Recent studies highlighted that glial cells, in particular astrocytes, are active players in information processing in the brain and periphery and both adenine nucleotides and nucleosides as well as glutamate play a key role in the glia-neuron cross talk. Thus, ATP and glutamate coordinately activate astrocytes, through the mobilization of their internal Ca^{2+}, which in turn triggers the release of several neuroactive molecules from astrocytes including ATP and glutamate themselves. These 'gliotransmitters' signal either to astrocytes, where they generate Ca^{2+} waves, or to neurons, where they modulate synaptic transmission and neuronal excitability. In the hippocampus, stimulation of Schaffer collaterals leads to the glutamatergic activation of AMPA receptors located on astrocytes, which in turn releases ATP from astrocytes. This ATP, by itself, or by its metabolic degradation to adenosine elicits activity-dependent heterosynaptic depression of neighboring pathways via activation of P2Y and A_1 receptors respectively, which may lead to increased synaptic inhibition within intact hippocampal circuits. Although the astrocytic source of ATP for synaptic modulation has been demonstrated by a transgenic mouse model of the astrocyte-specific, inducible expression of the soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE) instrumental for the release of gliotransmitters, the physiological and pathological trigger for the release of ATP from astrocytes and its mechanism is still unknown.

Being a highly charged molecule, ATP by itself cannot permeate the cell membrane. Nevertheless, in previous studies, several release mechanisms has been identified for both ATP and adenosine. ATP is co- stored in synaptic vesicles, and its classical, Ca^{2+}-dependent vesicular release has been demonstrated in a number of central synapses by both electrophysiological and neurochemical detection. Vesicular exocytosis of ATP has also been demonstrated from astrocytes. In addition, other alternative routes of ATP release have also been identified, such as P2X7 receptor channels gap junction hemichannels, lysosome

exocytosis, and volume sensitive anion channels. However, these latter mechanisms have primarily described in non-neuronal or simplified experimental systems, such as cultured astrocytes, and less information is available, whether these pathways also serve as a conduit of ATP release for purines involved in the modulation of synaptic signaling.

For adenosine, there are also a number of different mechanisms whereby it could accumulate in the extracellular space. Firstly, it can be generated from the released ATP through the actions of extracellular nucleotide catabolizing enzymes, i.e. E-NTPDases, E-NPPases, alkaline phosphatases and ecto5'-nucleotidase. Secondly, it can also be released by its own right, via equilibrative or non-equilibrative transporters. Finally, although direct evidence is lacking, several studies support that adenosine can also be released in a vesicular fashion.

Despite that chemical signaling has a key role in neural function, only a few techniques are available to directly measure the concentrations of neurotransmitters and modulators in the extracellular space. Among them, the recently developed microelectrode biosensor technique allows the detection of the efflux of endogenous neurotransmitters with better resolution than conventional neurochemical methods, and also circumvents the limitations of widely used electrophysiological techniques which indirectly detect the released transmitters. Using these multi-enzymatic microelectrode biosensors, it is possible to measure the concentration of different neurotransmitters and modulators with high temporal and spatial resolution and to detect paracrine and other non-classical release mechanisms as well.

2. Aims of the study

1. We wished to answer to the following questions with the radiolabelled neurotransmitter release experiments:
    a. How the activity of different P2 receptors influence the release of monoamine and amino acid transmitters in the spinal cord?
    b. To what extent the modulation depends on the applied types of agonists and concentration? (concentration-response curves, agonist profile)
    c. Which subtypes of P2 receptor mediate the effect is of agonists? (use of selective antagonists, pharmacological identification of the subtypes of the receptors)

2. We studied with the application of RT-PCR:
    a. Which subtypes of P2Y receptor are expressed in the rat spinal cord, brainstem and dorsal root ganglion?

3. We aimed at answering the following questions with the application of real-time biosensor technique:
    a. What are the sources, mechanisms and exact dynamics of the release of ATP, adenosine and glutamate triggered by depolarization?
    b. What P2 and other receptors (P1, glutamate receptor) are involved in the release of the purines and glutamate evoked by K^+ depolarization?
    c. What is the temporal relationship between of the release of ATP and glutamate?

3. Methods

3.1. RT-PCR amplification of different P2Y receptor mRNAs

Total RNA from the tissue samples was isolated with Trizol Isolation Reagent according to the protocol provided by the supplier. RNA (1 mg, 2 mL) was reverse transcribed using a RevertAid First Strand cDNA Synthesis. Aliquots of the first-strand cDNA template were subjected to PCR using 0.4 mmol/L (1 mL) forward and reverse primers and 2 U (0.3 mL) of Taq DNA Polymerase. The primers used for amplification of P2Y receptor cDNAs were: for $P2Y_{12}$ CAGGTTCTCTTCCCATTGCT (forward primer) and CAGCAATGATGATGAAAACC (reverse primer), for $P2Y_{13}$ GGCATCA ACCGTGAAGAAAT (forward primer) and GGGCAAAGCAGACAAAG AAG (reverse primer), for β-actin ATGGATGACGATATCGCTG (forward primer) and ATGAGGTAGTCTGTCAGGT (reverse primer). The conditions for amplification were as follows: initial denaturation at 95 °C for 5 min, hot start at 80 °C, then 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min, for 40 cycles, with a final extension at 72 °C for 5 min. PCR products were analyzed by agarose gel electrophoresis. The identity of the various amplified PCR products had previously been verified by sequencing. Genomic DNA contamination in RNA samples was ruled out by direct PCR amplification of RNA samples.

3.2. Tritium outflow experiments

[^{3}H]GLUT and [^{3}H]NA release experiments were performed by the application of the method described in our previous studies in the spinal cord. Briefly, male Wistar rats (140-160 g) were anesthetized under light CO_2 inhalation, and then decapitated. The spinal cord was dissected in ice-cold Krebs solution saturated with 95% O_2 and 5% CO_2, and 400 µm thick slices were prepared using a McIlwain Tissue Chopper and incubated in 1 mL of modified Krebs solution (mmol/L: NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11.5; pH 7.4) containing 1 µCi/mL [^{3}H]GLUT or 2.5 µCi/mL [^{3}H]NA, ascorbic acid (300 mmol/L) and Na_2EDTA (30 mmol/L) for 30 min. The medium was bubbled continuously with 95% O_2 and 5% CO_2 and maintained at 37 °C ([^{3}H]NA); in order to minimize the spontaneous firing of excitatory neurons and the metabolic efflux of [^{3}H]GLUT, the bath temperature was kept at 32 °C in [^{3}H]GLUT release experiments. After incubation, the tissues were rinsed three times with 6 mL Krebs solution, transferred to polypropylene tissue chambers and superfused continuously with 95% O_2- and 5% CO_2-saturated modified Krebs solution at a rate of 0.65 mL/min. In order to wash out the excess radioactivity and to

allow tissue equilibration, a 60-min preperfusion time was applied and subsequently, 3-min perfusate samples were collected and assayed for [^{3}H]GLUT or [^{3}H]NA. The slices were electrically stimulated during the collection period using platinum ring electrodes fixed to the top and the bottom of the 100 mL volume tissue chamber, with the following parameters: 40 V, 15 Hz, 3.5 ms, 1 min ([^{3}H]GLUT) and 40 V, 3 Hz, 1 ms, 2 min ([^{3}H]NA). The radioactivity released from the preparations was measured using a Packard 1900 Tricarb liquid scintillation spectrometer (Canberra, Australia).

3.3. P2Y1 receptor immunohistochemistry

Male Wistar rats (140-160 g) were decapitated and the spinal cord was quickly removed and placed into a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at pH 7.4 for 30 min at room temperature. After several changes of the fixative and overnight fixation at 4 °C, fixative was washed out in 0.1 M PB (pH 7.4). Transverse cervical sections (35 µm) were cut by vibratome.

3.3.1. Immunofluorescence staining

Sections were incubated in blocking solution (5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)) for 1 h. An incubation, with the first antibodies vesicular glutamate transporter 1 (VGLUT1) (1:3000, rabbit polyclonal, affinity-purified fusion protein containing amino acid residues 456-560 of rat VGLUT1) or $P2Y_1$ (1:200, rabbit polyclonal corresponding to residues 242-258 of rat or human $P2Y_1$), was performed at 4 °C overnight. After careful washing with PBS, incubation with the second antibodies (1:500 Alexa Fluor 488 goat anti-rabbit IgG or Alexa Fluor 594 goat antirabbit IgG,) were carried out at room temperature for 2h in the dark. After wash in distilled water, mounting in VectaShield, pictures were taken by means of Nikon Eclipse E600 microscope equipped with a SPOT RT color digital camera. Control experiments were performed using fresh blocking serum instead of the first antibody.

3.3.2. Immunohistochemical staining for bright field and electron microscopy

Endogenous peroxide activity were blocked by 3% H_2O_2 (15 min), traces of H_2O_2 were removed with 0.1 MPBS. Triton X100 (0.1%, 15 min) was applied to increase the penetration of the antibodies. Careful washing steps, incubation with blocking serum (5% normal goat serum for 2h) then incubation with the first rabbit polyclonal antibodies, such as 1:3000 VGLUT1 (Synaptic Systems), or 1:200 $P2Y_1$, were performed. After repeated washing,

incubation with biotinylated anti-rabbit IgG for 2h was carried out. An ABC-3,3 diaminobenzidine (DAB, Vector Laboratories) staining kit was used according to the manufacturer's instructions. Sections for light microscopic investigation were washed and dried onto microscopic slides and mounted in Canada balsam. Pictures were taken under a Zeiss Axioplan2 microscope equipped with an Olympus 70D camera using DPC Controller software (Olympus Ltd., Tokyo, Japan). Samples for electron microscopic investigation were washed and post-fixed in 1% OsO_4 for 30 min, dehydrated in graded ethanol (en bloc-stained with 2% uranyl acetate in 70% ethanol for 30 min) and embedded in Taab 812 resin (Taab Equipment Ltd., Aldermaston, Bershire, England). Ultrathin sections were cut (Leica UCT, Leica Microsystems, Milton Keynes, UK) and examined in a Hitachi 2001 transmission electron microscope (Hitachi, Tokyo, Japan). In control experiments the first antibody was omitted from the incubation medium.

3.4.1. Extracellular recording

Young adult male *Wistar* rats (4 weeks of age, 85-110 g) were euthanized by cervical dislocation. After decapitation, the brain was rapidly removed and placed in ice-cold artificial cerebrospinal fluid (aCSF) containing 11 mmol/L Mg^{2+}, wherein 400 µm coronal slices were prepared by a microtome with vibrating blade (Microm HM 650 V).

A single slice was transferred to a recording chamber, fully submerged in aCSF and perfused at 6 mL/min (34-36°C). Field excitatory postsynaptic potentials (fEPSPs) were extracellularly recorded in AC mode using a ISO-80 isolated bio-amplifier, with aCSF-filled glass microelectrode (1B150F-4, < 2 MΩ;), from the *stratum radiatum* of CA1 region in response to stimulation (at 3V- 7V, 0.1ms, 15s intervals; parallel bipolar electrodes) of the Schaffer collateral-commissural fiber pathway. Extracellular DC recordings were performed with the Multiclamp 700A amplifier in DC mode using borosilicate glass pipettes (GC120F-10, 4-7 MΩ, Havard Apparatus, Holliston, MA, USA) filled with aCSF. Electrical signal from the electrode were acquired at 10 kHz (sampling frequency), using the pCLAMP 9 software package and displayed on the laboratory computer. Signal analysis was performed using the Curve Analysis software package. Extracellular recordings were performed simultaneously with ATP and null sensor measurements in a set of experiments.

3.4.2. Biosensor recording using ATP, adenosine and glutamate sensors

The principles and operation of the Pt/Ir microelectrode biosensors for the purines, ATP and adenosine, have been described previously. The biosensors used in this study were obtained

from Sarissa Biomedical. The ATP sensor comprised of two enzymes (glycerol kinase EC 2.7.1.3 and glycerol-3-phosphate oxidase EC 1.1.3.21) and the adenosine sensor comprised of three enzymes (adenosine deaminase EC 3.5.4.4, nucleoside phosphorylase EC 2.4.2, xanthine oxidase EC 1.1.3.22) entrapped within a matrix around a fine platinum wire of 50 µmol/L diameter and 0.5 mm long. Electrochemical sensor can respond not only to the analyte of interest, but also to any electroactive species in the immediate environment. To check whether the sensors were responding to ATP, we used a dual recording configuration. To give a measure of net ATP concentrations, the signal from a NULL sensor (lacking enzymes, but otherwise identical) was used to measure background signals, which were then subtracted from the signals generated by the ATP biosensor. For the adenosine/inosine sensor (ADO/INO), a separate inosine-sensor (INO), lacking adenosine deaminase, is required to yield a signal specific to adenosine. Thus, the signal of adenosine sensor can be described as that from both adenosine and inosine, and the net adenosine signal is achieved by subtracting the signal of inosine sensor from this value. Glutamate release was measured using a glutamate (GLUT) sensor, comprising glutamate oxidase (EC 1.4.3.11) and using NULL sensor, as background. The sensor is selective for GLUT over glutamine, aspartate, dopamine and 5-HT. All microbiosensors were operated at 500-700mV (vs. Ag/AgCl) in a flow system for amperometric detection at 34-36°C temperature. In certain series of experiments ATP, NULL, ADO/INO and INO recordings were performed simultaneously, whereas in other series of experiments simultaneous ATP, GLUT and NULL recordings were done in order to limit the maximal number of recording electrodes in the same measurement location.

The microelectrode biosensors were each inserted through the 400 µmol/L thickness of the CA1 region of the hippocampal slice such that most of the sensing part of the sensors was in intimate contact with the tissue. Previous studies showed that insertion of the sensor is not detrimental to the slice, exhibiting fEPSP activity in its place. Before insertion and after removal, the sensors were calibrated with known concentrations of standards: ATP (10 µmol/L), adenosine (3 µmol/L), inosine (10 µmol/L) and glutamate (10 µmol/L), which allowed quantification of any rundown in sensor sensitivity over the duration of an experiment. Before experiments a standard calibration curve was generated, and a high linear correlation was observed between the peak value of signal and the ATP, adenosine, inosine, and glutamate concentration. To take into account that biosensor sensitivity varies during different experiments, we used the calibration of the sensor to normalize each signal. In addition, we tested all compounds for any interference with the sensitivity of the sensors, but none of them elicited any detectable change in the signal. Besides the sensors, the recording

electrode was placed adjacent to the sensors. Continuous signal from the biosensor was acquired at 10 kHz using the pCLAMP 9 software package and displayed on a PC. For calculation, 'net' signals for each experiment were calculated subtracting the currents of the corresponding baseline, which were expressed in absolute units of concentration (μmol/L). In case of pharmacological treatments, their effects on the peaks of the responses of the biosensors were quantified. Unless otherwise stated, in case of ATP recording, when a biphasic signal was recorded, the effects of treatments on the peak of the second phase of the response were calculated, i.e. the absolute maximum of the signal was taken into account. Calibration curves were constructed using the GraphPad software.

3.4.3. Experimental protocol

After insertion of biosensors, an approx. 20 min equilibration period was allowed to reach a steady-state baseline. Then, K^+ depolarization was applied by subjecting the preparations to modified aCSF containing 26.9 mmol/L K^+ (Na^+: 102 mmol/L) for 270 sec. All drugs were applied to the perfusion solution, from 20 min before the beginning of K^+ depolarization until the end of experiments, except FAc, which was added 10 min before the onset of K^+ stimulation.

3.5. Statistical analysis

Date are expressed as mean ± SEM with *n*= number of identical experiments. Student's *t* test (pairwise comparisons) and one-way analysis of variance (ANOVA) followed by the Dunnett test (multiple comparisons) were used as a statistical analysis, as appropriate. P values of less than 0.05 were considered statistically significant.

4. Results

4.1.1. [^{3}H]GLUT release experiments

The basal neurotransmitter outflow measured in a 3-minn sample in [^{3}H]GLUT release experiments was 3.09 ± 0.16%, of the total tissue content (n = 8), which remained relatively constant during the subsequent sample collections. Electrical field stimulations were applied during the 3rd and 14th sample collections, resulting in a rapid increase in the basal [^{3}H]GLUT efflux, which peaked 3 min after EFS_1 and EFS_2, then gradually declined and returned to the baseline level. The amount of tritium released by the second stimulation period was comparable in amount to the first, resulting in an EFS_2/EFS_1 ratio of 1.01 ± 0.07 (n = 8) in control experiments. When the slices were superfused with Ca^{2+}-free Krebs solution supplemented with 1 mmol/L EGTA, the evoked release of [^{3}H]GLUT was inhibited more than 90%, without affecting the basal efflux. The majority of the release could be therefore regarded as a Ca^{2+}-dependent release. In subsequent experiments, the effect of different P2 receptor agonists on electrically evoked [^{3}H]GLUT release was examined. Among them, ATP, ADP and 2-MeSADP all concentration-dependently attenuated the stimulation-evoked release of [^{3}H]GLUT at the micromolar concentration range with the following rank order of agonist potency: ADP > 2- MeSADP >ATP, whereas they did not affect basal tritium efflux. The maximal inhibition of [^{3}H]GLUT efflux obtained by ATP was 63.15 ± 2.88% (n = 8), and the apparent IC_{50} value was 70.1 mmol/L. Among P2 receptor agonists, 2-MeSATP, the metabolically stable analogue of ATP, at 100 µmol/L concentration significantly inhibited, but in higher concentrations (200-300 µmol/L) increased stimulation evoked [^{3}H]GLUT overflow from the rat spinal cord slices. Next, the effect of ATP and 2-MeSATP on [^{3}H]GLUT efflux was tested in the presence of various antagonists acting on P2 and P1 receptors. The inhibitory effect of ATP (1 mmol/L) on the evoked release of [^{3}H]GLUT was abolished in the presence of the non-selective P2 receptor antagonist suramin (300 µmol/L) and the $P2Y_{12,13}$ receptor selective antagonist 2-MeSAMP (10 µmol/L); moreover, in the presence of 2-MeSAMP, a net potentiation was observed. By contrast, PPADS (30 µmol/L) and MRS2179, the selective $P2Y_1$ antagonist (10 µmol/L) partly reversed the inhibitory effect of ATP (38.96 ± 8.1%, and 32.63 ± 1.36% inhibition in the presence of PPADS 30 µmol/L and MRS2179 10 µmol/L, respectively, n = 4-8, P < 0.01, vs. ATP alone). When the slices were preperfused with the selective P1 (A_1)-adenosine receptor antagonist DPCPX (100 nmol/L), the effect of ATP was not changed. The facilitatory effect of 2-MeSATP (300 µmol/L) was reversed by the P2X1 receptor selective antagonist NF449 (100 nmol/L).

4.1.2. [^{3}H]NA release experiments

Similar to [^{3}H]GLUT release experiments, the basal tritium efflux was constant after preloading the spinal cord slices with [^{3}H]NA (0.437 ± 0.015%, n = 8), and electrical field stimulation (40 V, 2 Hz, 1 ms, 2 min) elicited a rapid and reproducible increase in tritium efflux, with an EFS_2/EFS_1 ratio of 0.93 ± 0.03 (1.363 ± 0.136%, n = 8). Previous studies demonstrated that electrical field-stimulation evoked [^{3}H]NA efflux under identical conditions is $[Ca^{2+}]_o$-dependent. Among P2 purinoceptor agonists, ATP, ADP and 2-MeSADP decreased the stimulation-evoked [^{3}H]NA outflow in a narrow concentration range, with the following rank order of agonist potency: ADP ≥ 2-MeSADP >ATP. In case of ADP the maximal effect was obtained at 30 µmol/L concentration. These agonists did not significantly affect basal release. Just as in the case of [^{3}H]GLUT release experiments, 2-MeSATP did not inhibit, but significantly enhanced the stimulation-evoked efflux of [^{3}H]NA in the concentration range of 100-300 µmol/L. In addition, 2-MeSATP also increased the basal efflux of [^{3}H]NA (at 300 µmol/L: 0.987 ± 0.036%, n = 4, P < 0.01 vs. control). The effect of ATP was prevented by the $P2Y_{12,13}$ receptor antagonist 2-MeSAMP (10 µmol/L) and, similar to [^{3}H]GLUT experiments, a net potentiation was observed. When the slices were preperfused with MRS2179 (10 µmol/L), and DPCPX (100 µmol/L), the effect of ATP was partly reversed. On the other hand, suramin (300 µmol/L) and PPADS (30 µmol/L µmd/L), non-selective antagonists of P2 receptors, did not affect the inhibitory effect of ATP. The facilitatory action of 2-MeSATP (300 µmol/L) on [^{3}H]NA efflux was sensitive to inhibition by the P2X1 receptor selective antagonist NF449 (100 nmol/L) and by PPADS (30 µmol/L). By contrast, the facilitatory effect of 2-MeSATP (100 µmol/L) persisted in the presence of $P2Y_1$ receptor selective antagonist MRS2179 (10 µmol/L); moreover, it was potentiated (21.38 ± 0.33% and 106.63 ± 2.7% facilitation in the absence and presence of MRS2179, respectively, n = 4, P < 0.0001). The antagonists used in these experiments did not significantly change the [^{3}H]NA overflow evoked by stimulation, with the exception of suramin and PPADS, the former enhancing and the latter slightly decreasing the evoked tritium efflux.

4.1.3. RT-PCR study in the rat brainstem, DRG, and spinal cord

Total RNA samples were reverse transcribed and amplified by PCR using primers specific to the two different P2Y receptor subtypes. RT-PCR analysis showed positive signals for both $P2Y_{12}$- and $P2Y_{13}$-receptors in the rat brainstem. These bands were absent when the reverse transcriptase was omitted, and thus were not due to the presence of contaminating genomic DNA. We then explored the mRNA expression of P2Y receptor subunits in the spinal cord.

RT-PCR analysis revealed that the size of the $P2Y_{13}$-specific amplification product was consistent with the expected sequence-based product size, indicating the expression of this P2 receptor subtype in the rat spinal cord. Similarly, we were able to detect mRNA expression of the $P2Y_{13}$ receptor in the rat DRG. Conversely, we were unable to demonstrate mRNA expression of the $P2Y_{12}$ receptor, either in the rat spinal cord or in the DRG.

4.1.4. P2Y1 receptor immunohistochemistry

The pharmacological analysis also indicated that in addition to $P2Y_{12}$ and $P2Y_{13}$ receptors, the inhibitory effect of P2 receptor agonists on [^{3}H]GLUT and [^{3}H]NA release might also be mediated by $P2Y_1$ receptors. However, because previous studies have already clarified that mRNA encoding $P2Y_1$ receptors is expressed in the rat brainstem, DRG and spinal cord respectively, we did not examine the expression of this receptor at the mRNA level. Instead, to explore the cell-type specific distribution of the $P2Y_1$ receptor protein and to reveal whether $P2Y_1$ receptors involved in the modulation of [^{3}H]GLUT release are expressed on primary afferent nerve terminals or interneurons, immunohistochemical experiments were performed using a specific antibody raised against $P2Y_1$ receptors. In order to visualize glutamatergic nerve terminals in the spinal cord, immunostaining selective for VGLUT1 was also performed. Similarly to the recently published results of Persson and coworkers, immunoreactivity of VGLUT1 was present in different densities, but basically in the whole dorsal horn from lamina I-VI at the cervical level. DAB staining showed VGLUT1 immunoreactivity in the membrane of clear vesicles of glutamatergic terminals. Although we cannot exclude a potential co-localization, the distribution pattern of $P2Y_1$ receptor immunolabeling in the transverse cervical section was different from that of VGLUT1 staining. The most intense $P2Y_1$ staining was found in lamina I-II, and the density of immunostaining weakened in the medial part of the dorsal horn. Electron histochemical staining for $P2Y_1$ receptor protein revealed immunoreactive dendrites but not synapses. According to our previous study, DAB precipitates indicating the presence of the $P2Y_1$ receptor were also observable on the luminal membrane of endothelial cells, presumably due to the caveolae docking here.

4.2.1. ATP, adenosine and glutamate released under basal conditions and after K^+ depolarization in the hippocampal CA1 area

The selectivity of the biosensors used versus interferences found in the hippocampus enabled to estimate the basal tone of ATP, adenosine and glutamate in the slice. As the biosensors

have a background current, basal tone was measured with the following method: at first placing the sensors into the tissue chamber, allowing it to equilibrate for at least 20 minutes and then inserting the sensor into the slices, allowing it to equilibrate and measuring the changes and potential differences between these equilibrium states. To convert this difference to absolute concentration of ATP, adenosine and glutamate we used null and inosine sensors as references. Under basal conditions extracellular ATP, adenosine and glutamate basal tones have been estimated to be as low as a few nmol/L, respectively (ATP: 4.1 ± 0.8 pA, n = 10; ADO: 5.4 ± 0.7 pA, n = 10; GLUT: 6.4 ± 0.8 pA, n = 10).

When slices were subjected to K^+ depolarization (25 mmol/L; 270 sec) well detectable signals were recorded on ATP, glutamate and adenosine sensors, respectively. All sensors showed a rapid response; however, the adenosine sensor detected an earlier and longer lasting signal than the ATP and glutamate sensor. The release of adenosine started 0.79 ± 0.01 min after the beginning of the K^+ depolarization and reached its peak at 7.31 ± 0.01 min eliciting a maximal release of 10.28 ± 1.41 µmol/L (n = 8). The efflux of glutamate started 2.68 ± 0.04 min after K^+ depolarization, and reached its peak at 5.62 ± 0.06 min (3.49 ± 0.84 µmol/L, n = 8). Two phases of ATP release were detected, the first occurring at the same time, when glutamate release started, while the onset of the second phase coincided with the maximum peak of glutamate release. ATP efflux started 2.56 ± 0.05 min after the beginning of the K^+ stimulation and reached its absolute peak during the second phase at 8.08 ± 0.01 min (1.23 ± 0.23 µmol/L, n = 8). Null sensors that lacked enzymes in the polymer coating displayed only small fluctuations of the current around the baseline. Evoked signals of field excitatory postsynaptic potentials (fEPSP, in AC mode) recording from stratum radiatum of rat hippocampus simultaneously with the ATP sensor were recovered after the K^+ depolarization (Fig 1E), indicating that slices retained their viability during the experiments. During K^+ depolarization, the gradual loss of biological electrophysiological activity was observed, as expected. fEPSP activity is then completely recovered in the next 15 min during wash out. High K^+-induced spreading depression was detected at 3.81 ± 0.2 min (n = 6) after stimulation from stratum radiatum of area CA1. This large negative deflection on the extracellular DC potential occurred immediately after with the loss of fEPSP, but before adenosine release did not reached the maximum peak. ATP started to release before the beginning of SD, showed a small first peak at the time of SD (0.48 ± 0.16 µmol/L, n = 8) and continued to increase after the peak of SD. Likewise, glutamate release did not reach its plateau before the onset of SD.

4.2.2. Inhibition of extracellular ATP metabolism increases ATP and inhibits adenosine accumulation

The presence of ectonucleotidases that can metabolize ATP to ADP, AMP and adenosine in the hippocampus is well documented. These enzymes could play a role in the production of adenosine during K^+ depolarization (25 mmol/L; 270 sec). We therefore tested the contribution of the hydrolysis of ATP to extracellular adenosine accumulation by the selective ecto-ATPase inhibitor ARL67156. ARL67156 was applied in a concentration (100 µmol/L), which, according to previous studies elicits a substantial, although not complete blockade of extracellular breakdown of ATP in the hippocampus.

Inhibition of extracellular ATP metabolism by ARL67156 (100 µmol/L) enhanced the extracellular level of ATP following K^+ depolarization (8.62 ± 0.76 µmol/L, n = 8, $p<0.01$). Moreover, K^+ depolarization evoked ATP efflux appeared earlier than in the absence of ectoATPase inhibitor (1.69 ± 0.012 min after the beginning of the K^+ stimulation). On the other hand, ARL67156 (100 µmol/L) had no significant effect on the glutamate efflux (2.43 ± 0.63 µmol/L, n = 6, $p>0.05$). The elevation in glutamate efflux in this case started coincidently with the elevation of ATP efflux. In contrast, adenosine release during the stimulation was inhibited in the presence of ARL67156 (0.15 ± 0.04 µmol/L, *n* = 6, $p<0.01$). These results show that ATP efflux detected by the ATP biosensor is subject to extracellular metabolism and contributes to the generation of adenosine that is detected by the adenosine biosensor. When the slices were preperfused with ARL67156 (100 µmol/L) and the adenosine transport inhibitor dipyridamole (50 µmol/L) the adenosine efflux was partly inhibited (ADO: 2.71 ± 0.23 µmol/L, *n* = 5, $p<0.001$ vs. ARL67156 alone), i.e. it was higher, than detected in the presence of the ectoATPase inhibitor alone. These findings indicate a minor additional direct adenosine release in response to K^+ depolarization, which is not derived from the released ATP.

4.2.3. Differential susceptibility of ATP, adenosine and glutamate release to extracellular Ca^{2+}

Because ATP can be released in a $[Ca^{2+}]_o$-dependent manner, we asked whether the efflux of ATP during K^+ depolarization (25mmol/L; 270 sec) was $[Ca^{2+}]_o$-dependent. To examine the Ca^{2+}-dependency of purine release during K^+ depolarization, we omitted Ca^{2+} from the perfusion medium and 1 mmol/L EGTA was added to chelate any residual extracellular Ca^{2+}. Perfusion of Ca^{2+}-free/EGTA and 25 mmol/L K^+ containing aCSF caused a significant decrease in ATP (0.21 ± 0.01 µmol/L, *n* = 6, $p<0.01$) and adenosine (0.22 ± 0.02 µmol/L, *n* =

6, $p<0.01$) efflux during K^+ depolarization. On the other hand, glutamate efflux did not significantly change in response to stimulation (3.64 ± 0.15 µmol/L, $n = 6$, $p>0.05$), when rat hippocampal slices were perfused with Ca^{2+}-free/EGTA aCSF solution.

4.2.4. Involvement of ongoing neuronal activity in ATP, adenosine and glutamate release

To test the involvement of the sodium-channel mediated axon potential propagation, the effect of TTX (3 µmol/L) was examined. Inhibition of the voltage-dependent Na^+ channels by TTX inhibited ATP and adenosine accumulation following K^+ depolarization (ATP: 0.07 ± 0.05 µmol/L; $n = 6$, ADO: 0.08 ± 0.04 µmol/L, $n = 6$, $p<0.01$). Similarly, glutamate release was reduced in the presence of TTX after high K^+ stimulation (0.12 ± 0.03 µmol/L, $n = 6$, $p<0.01$). The Na^+-channel blocker also caused a complete inhibition of the fEPSP activity. These findings indicate that ATP, adenosine and glutamate released in response to K^+ stimulation is associated with ongoing axonal activity. Next, we investigated the involvement of glutamatergic excitatory transmission in K^+ depolarization evoked ATP, glutamate and adenosine efflux by testing the effect of glutamate receptor antagonists. When the slices were perfused with the non-NMDA glutamate receptor antagonist CNQX (10 µmol/L), it almost abolished glutamate efflux, but did not significantly change ATP and adenosine efflux in response to 25 mmol/L K^+ stimulation (GLUT: 0.09 ± 0.06 µmol/L, $n = 5$, $p<0.01$; ATP: 1.17 ± 0.12 µmol/L, $n = 5$, $p>0.05$; ADO: 8.43 ± 0.53 µmol/L, $n = 5$, $p>0.05$). The NMDA receptor antagonist D-AP-5 (10 µmol/L) and the NR2B-selective NMDA receptor antagonist ifenprodil (10 µmol/L) was applied before and during K^+ depolarization (25 mmol/L; 270 sec). Elevation of extracellular ATP level was significantly decreased in the presence of D-AP-5 (0.21 ± 0.03 µmol/L, n=5, $p<0.01$) and ifenprodil (0.13 ± 0.06 µmol/L, n=5, $p<0.01$). Accumulation of adenosine was also inhibited by D-AP-5 (0.11 ± 0.01 µmol/L, n=5, $p<0.01$) and by ifenprodil (0.11 ± 0.02 µmol/L, n=5, $p<0.01$) under these conditions.

4.2.5. ATP, adenosine and glutamate is released from glial cells in response to K+ depolarization in CA1 area

Next, we investigated, whether the source of detected extracellular purines and glutamate is glial or neuronal. Previous reports showed that fluoroacetate (FAc), a mitochondrial gliotoxin selectively impairs the oxidative metabolism of glial cells. The specificity of FAc in the concentration used is due to its selective uptake by acetate transporter present only in glial cells. In addition we used a relatively short time for FAc perfusion period to further ensure glia-selectivity. FAc perfusion (1 mmol/L, n=6) was added 10 min before the beginning of K^+

depolarization. Inhibition of oxidative metabolism in glial cells resulted in almost complete inhibition of the detected increase in the second phase of the efflux of ATP (ATP: 0.21 ± 0.14 µmol/L, *n* = 8, p<0.01), whereas the first phase of the ATP efflux did not significantly change (ATP: 0.48 ± 0.16 µmol/L, *n* = 8, and 0.54 ± 0.20 µmol/L, *n* = 8, in the absence and presence of FAc, respectively, p>0.05). Glutamate efflux was also inhibited by the FAc perfusion (GLUT: 0.11 ± 0.07 µmol/L, *n* = 7, p<0.01). Adenosine accumulation was decreased by FAc treatment, although was still detectable and appeared later than in the absence of FAc (ADO: 3.08 ± 0.89 µmol/L, *n* = 8, p<0.01). The extracellular level of adenosine detected in the presence of FAc (1 mmol/L) was further inhibited in the presence of TTX (3 µmol/L; 0.22 ± 0.1 µmol/L, *n* = 6, p<0.05 vs. FAc alone). The electrical stimulation could evoke fEPSPs in the presence of FAc and the signal remained largely unchanged before and after stimulation, indicating that the treatment mostly affected the glial population. Nevertheless, a slight reduction of the amplitude of the response could be observed when compared to signals recorded from control slices, which might reflect the contribution of glia to the modulation of fEPSP activity or a minor effect of FAc on neuronal survival. These results show that glial and not neuronal cells are the main source of extracellular purines and glutamate under our experimental conditions and this transmitter efflux might affect measured fEPSP amplitudes in the CA1 area.

4.2.6. Mechanism of hippocampal ATP, adenosine and glutamate release

In the next experiments inhibitors of ion channels, known to mediate the release of ATP and/or glutamate were tested in order to identify the underlying mechanism of their transmembrane efflux under our experimental conditions. At first we examined the role of P2X7 receptor ion channels (P2X7R), which are expressed in the hippocampus and which are known to mediate the release of glutamate and ATP from nerve terminals, as well as from cultured astrocytes. To investigate the involvement of P2X7R in ATP, adenosine and glutamate efflux from the hippocampus, the effects of BBG (0.1 µmol/L) and AZ10606120 (0.1 µmol/L), antagonists of P2X7R were tested. Extracellular elevation of ATP was inhibited in the presence of BBG (0.11 ± 0.14 µmol/L, n=5, p<0.01; Fig. 6) and AZ10606120 (0.17 ± 0.02 µmol/L, n=6, p<0.01) after depolarization. On the other hand BBG had partial (4.60 ± 0.74 µmol/L, *n* = 5, p<0.01), whereas AZ10606120 (0.12 ± 0.05 µmol/L, *n* = 6, p<0.01) had complete inhibitory effect on adenosine release in response to K^+ stimulation. The efflux of glutamate was also inhibited by a P2X7R-selective concentration of BBG (100 nmol/L, 0.029 ± 0.06, µmol/L, *n* = 7, p<0.01). Next, the effect of carbenoxolone (CBX) was tested, which is

known as a wide spectrum gap junction inhibitor, but also inhibits P2X7R. High concentration of carbenoxolone (100 μmol/L) completely inhibited the ATP, adenosine and glutamate efflux in response to 25 mmol/L K^+ stimulation (ATP: 0.21 ± 0.02 μmol/L, *n* = 6, p<0.01; ADO: 0.22 ± 0.02 μmol/L, *n* = 6, p<0.01; GLUT: 0.013 ± 0.02 μmol/L, *n* = 6, p<0.01). Because it is known that CBX in low concentration inhibits only the Panx1-evoked current and P2X7R co-immunoprecipitate with Panx1 we also examined a lower, pannexin selective concentration of CBX (20 μmol/L). This treatment completely abolished the adenosine efflux during K^+ depolarization (1.02 ± 0.26 μmol/L, *n* = 5, p<0.01; Fig 7). Administration of probenecid (150 μmol/L), another pannexin inhibitor also caused a remarkable decrease in the extracellular concentration of adenosine (0.2 ± 0.01 μmol/L, *n* = 7, p<0.01). In contrast, the extracellular level of ATP was significantly increased in the presence of low concentration CBX (20 μmol/L; 2.22 ± 0.37 μmol/L, *n* = 5, p<0.01) and in the presence of probenecid (150 μmol/L; 2.70 ± 0.11 μmol/L, *n* = 7, p<0.01). These data demonstrate that the mechanism of the efflux of ATP and adenosine is partly different: although the efflux of adenosine did arise from previously released ATP, through the actions of ectonucleotidase, it might also be released directly via pannexin hemichannels. In contrast, ATP is released through P2X7 receptors into the extracellular space, before its rapid breakdown to adenosine.

As for the underlying mechanism of glutamate efflux, because it was Ca^{2+}-independent the participation of excitatory amino acid transporters (EAAT1-5), present on neuronal and glial membrane was taken into account, and L-trans-2,4-PDC, the wide spectrum EAAT inhibitor was examined. L-trans-2,4-PDC significantly decreased glutamate efflux evoked by K^+ depolarization (1.24 ± 0.66 μmol/L, n = 7, p<0.05).

5. Conclusion

In the first study, the P2 receptor-mediated modulation of [^{3}H]glutamate and [^{3}H]noradrenaline release were examined in rat spinal cord slices. ATP, ADP, and 2-MeSADP decreased the electrical stimulation-evoked [^{3}H]glutamate efflux with the following order of potency: ADP>2-MeSADP>ATP. The effect of ATP was antagonized by suramin (300 μmol/L), the $P2Y_{12/13}$ receptor antagonist 2-MeSAMP (10 μmol/L), and partly by PPADS (30 μmol/L) and the $P2Y_1$ receptor antagonist MRS2179 (10 μmol/L). ATP, ADP, and 2-MeSADP also decreased evoked [^{3}H]noradrenaline outflow; the order of agonist potency was ADP>2-MeSADP>ATP. The effect of ATP was reversed by 2-MeSAMP (10

µmol//L), and partly by MRS2179 (10 µmol/L). By contrast, 2-MeSATP (10–300 µmol/L) increased resting and electrically evoked [^{3}H]glutamate and [^{3}H]noradrenaline efflux, and this effect was prevented by the P2X1 receptor selective antagonist NF449 (100 nmol/L). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that mRNAs encoding $P2Y_{12}$ and $P2Y_{13}$ receptors are expressed in the brainstem, whereas $P2Y_{13}$ but not $P2Y_{12}$ receptor mRNA is present in the dorsal root ganglion and spinal cord. $P2Y_1$ receptor expression in the spinal cord is also demonstrated at the protein level. In conclusion, inhibitory P2Y and facilitatory P2X1-like receptors, involved in the regulation of glutamate ($P2Y_{13}$ and/or $P2Y_1$) and noradrenaline ($P2Y_{13}$ and/or $P2Y_1$, $P2Y_{12}$) release have been identified, which provide novel target sites for analgesics acting at the spinal cord level.

The second study was undertaken to characterize the K^+ depolarization-evoked ATP, adenosine and glutamate outflow in the in vitro rat hippocampal slice. We utilised the microelectrode biosensor technique and extracellular electrophysiological recording for the real-time monitoring of the efflux of ATP, adenosine and glutamate. ATP, adenosine and glutamate sensors exhibited transient and reversible current during 25 mmol/L K^+ depolarization, with distinct kinetics. The ecto-ATPase inhibitor ARL67156 enhanced the extracellular level of ATP and inhibited the prolonged adenosine efflux suggesting that generation of adenosine may arise from the extracellular breakdown of ATP. Stimulation-evoked ATP, adenosine and glutamate efflux was inhibited by tetrodotoxin, while Ca^{2+}-free medium abolished ATP and adenosine efflux. Extracellular elevation of ATP and adenosine were decreased in the presence of NMDA receptor antagonists D-AP-5 and ifenprodil, whereas non-NMDA receptor blockade by CNQX inhibited glutamate but not ATP and adenosine efflux. The gliotoxin fluoroacetate and P2X7 receptor antagonists inhibited the K^+-evoked ATP, adenosine and glutamate efflux, while carbenoxolone in low concentration and probenecid decreased only the adenosine efflux. Our results demonstrate activity-dependent gliotransmitter release in the hippocampus in response to ongoing neuronal activity. ATP and glutamate is released by P2X7 receptor activation into extracellular space. Although the efflux of adenosine did arise from released ATP, it might also be released directly via pannexin hemichannels.

6. References

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