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High resolution detection of ATP release from single cultured mouse dorsal horn spinal cord glial cells and its modulation by noradrenaline

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Abstract

HEK293 cells stably transfected with the rat P2X2 receptor subunit were preincubated with 200 nM progesterone (HEK293-P2X2-PROG), a potent positive allosteric modulator of homomeric P2X2 receptors, and used to detect low nanomolar concentrations of extracellular ATP. Fura-2-loaded HEK293-P2X2-PROG cells were acutely plated on top of cultured DH glial cells to quantify ATP release from single DH glial cells. Application the $\alpha 1$ adrenoceptor agonist phenylephrine (PHE, 20 μ M) or of a low K^+ (0.2 mM) solution evoked reversible increases in the intracellular calcium concentration ($[Ca^{2+}]_i$) in the biosensor cells. A reversible increase in $[Ca^{2+}]_i$ was also detected in half of the biosensor cells following the interruption of general extracellular perfusion. All increases in $[Ca^{2+}]_i$ were blocked in the presence of the P2X2 antagonist PPADS or after preloading the glial cells with the calcium chelator BAPTA, indicating that they were due to calcium-dependent ATP release from the glial cells. ATP release induced by PHE was blocked by -L-phenylalanine 2-naphtylamide (GPN) that permeabilizes secretory lysosomes and bafilomycin A1 (Baf A1), an inhibitor of the H^+ -pump of acidic secretory vesicles. By contrast ATP release induced by application of a low- K^+ solution was abolished by Baf A1 but not by GPN. Finally, spontaneous ATP release observed after interrupting general perfusion was insensitive to both GPN and BafA1 pretreatment. Our results indicate that ATP is released in a calcium-dependent manner from two distinct vesicular pools and one non-vesicular pool coexisting in DH glial cells and that noradrenaline and PHE selectively target the secretory lysosome pool.

Introduction

The dorsal horn of the spinal cord (DH) is an important structure for the integration and the transmission of peripheral nociceptive information {Basbaum, 2009 #29;Cordero-Erausquin, 2016 #101;Millan, 1999 #1}. It is also a strategic site where potentially painful messages can be modulated, in particular by descending controls originating from supraspinal centers {Fields, 2004 #34;Millan, 1999 #1;Pertovaara, 2006 #3}. Noradrenergic projections from the brainstem have been shown to modulate nociceptive information at the spinal level {Budai, 1998 #6;Pertovaara, 2006 #3}. These descending noradrenergic projections to the DH originate mainly from the *Locus Coeruleus* (LC) {Lyons, 1989 #5} and are recruited during sustained activity of A δ primary nociceptors {Hitoto, 1998 #7} or during peripheral inflammation {Tsuruoka, 1999 #35;Tsuruoka, 2012 #36}.

Noradrenaline (NA) can inhibit glutamate release from primary afferents {Feng, 2002 #39;Funai, 2014 #8;Hickey, 2014 #37;Kawasaki, 2003 #11;Sonohata, 2004 #12}, decrease the excitability of DH neurons/interneurons {North, 1984 #19;Sonohata, 2004 #12} and facilitate inhibitory (GABAergic and glycinergic) transmission in the DH {Baba, 2000 #9;Baba, 2000 #10;Seibt, 2015 #41}. From a behavioral point of view, NA and α adrenoceptor agonists induce antinociceptive effects when administered intrathecally {Pertovaara, 2006 #3;Yaksh, 1985 #13}.

Adrenoceptors are expressed by DH interneurons {Baba, 2000 #9;Kawasaki, 2003 #11;North, 1984 #19;Sonohata, 2004 #12} as well as by DH glial cells, at least in cultures of whole spinal cords {Hosli, 1982 #14}.]. In the DH, NA fibres form synaptic contacts with fine dendrites of intrinsic DH neurons but not with primary afferent terminals (REFXX Satoh CellTissueres 1982, Hagashida Brain Res 1990, evt Holstege ProgBrainRes 1991). However, a large proportion of the contacts of noradrenergic fibres do not have the features of classical synapses suggesting the involvement of volume transmission (REFXX Rajaofetra JNeurocytol 1992).

Such “non-classical” contacts seem also common for serotonergic and dopaminergic fibres in the DH (REFXX Ridet Brain Res 1992, Ridet Neuroscience 1993). Most importantly, more than 60% of contacts formed by noradrenergic and serotonergic fibres in the DH appear to be of the “non-synaptic” type and to be established with astrocytic profiles {Ridet, 1993 #16}. These morphological results suggest that DH glial cells might represent important targets of the descending noradrenergic projections and therefore might participate in the modulation of nociceptive messages by NA at the spinal level.

Recently, we have shown that NA selectively increased inhibitory synaptic transmission in the DH {Seibt, 2015 #41}. This phenomenon involved α_1 and α_2 adrenoceptors and functional communication between deep and superficial laminae of the DH {Seibt, 2015 #41}. Moreover, blocking the metabolism of DH glial cells with fluorocitrate suppressed the modulatory effect of NA on inhibitory synaptic transmission {Seibt, 2015 #41}, indicating a fundamental role of DH glia-to-neuron communication during noradrenergic modulation of inhibitory synaptic transmission. In addition, our data indicated that ATP seemed to play a major role as a signaling molecule in this interaction {Seibt, 2015 #41}.

Therefore, the aim of the present work was to determine whether NA was able to trigger/modulate ATP release from DH glial cells and to characterize the mechanisms involved in this phenomenon. To this end, we developed and improved a sniffer cell/biosensor system that allowed us to detect low nanomolar concentrations of extracellular ATP and to quantify ATP release at the single cell level with excellent spatial and temporal resolutions.

Methods

Animal procedures

Mice were bred and housed in the animal facility Chronobiotron, UMS3415, accredited according to EU Directive 2010/63 and French regulations 2013-118. All procedures were conducted in conformity with the rules of the European Communities Council Directive 2010/63/EU, and the French regulations 2013-118. Pups were kept with their mothers until euthanasia by decapitation which is considered as an ethical method for very young animals.

Primary cultures of DH glial cell

Cultures of primary DH spinal cord glial cells were prepared from neonatal 3 to 6 day old postnatal C57BL/6 mice pups of either sex. Animals were decapitated and a laminectomy was performed in order to expose the dorsal part of the lumbar spinal cord. After having removed the meninges with thin forceps, the dorsal half of the spinal cord (i.e. the region of the dorsal horn) was harvested and placed in cold (4°C) Dulbecco's Modified Eagle Medium / F-12 containing 15 mM HEPES (Gibco, France).

The collected spinal tissue was then dissociated mechanically using fire-polished Pasteur glass pipettes of decreasing tip diameters until obtaining a turbid solution containing the dissociated cells and tissue debris. This solution was centrifuged at 500 RPM for 5 min. The supernatant containing large debris was removed and discarded and the pellet containing the glial cells was re-suspended in 2 mL of culture medium composed of: Dulbecco's Modified Eagle Medium supplemented with 10 % (v/v) heat inactivated horse serum (Gibco, France) and 0.5 % (v/v) streptomycin/penicillin (at 5.000 µg/ml and 5,000 U/mL respectively, Gibco, France). The cell suspension was seeded in the center of sterile 35 mm standard plastic culture dishes (Corning, USA) in which a central hole (18 mm) had been drilled and to the bottom of which a glass coverslip had been glued, thus delimiting a central well in the culture dish. The internal part of the glass bottom of the dishes was coated with poly-D-lysine (0.02 mg/mL, Sigma, France) in order to favor the attachment of the cells. After seeding, the cultures were placed and

maintained in a 37 ° C water-saturated incubator in an atmosphere containing air and 5 % CO₂. Experiments were performed on confluent cells 2-3 weeks after seeding.

Maintenance of Human Embryonic Kidney 293 (HEK293) cells

In order to detect ATP release from cultured DH glial cells we used a human embryonic kidney (HEK293) cell line that was stably transfected with the rat P2X₂ receptor subunit {Stoop, 1999 #24}. This cell line, which we will refer to as HEK293-P2X₂ later in the text, was kindly provided by Dr. François Rassendren (Institut de Génomique Fonctionnelle, Montpellier France). These cells express homomeric P2X₂ receptors {Stoop, 1999 #24} and were previously used by us to characterize P2X₂ receptor-mediated currents and their modulation by progesterone {De Roo, 2010 #102}. HEK293-P2X₂ cells were cultured in sterile 75 mm² flasks (Thermo Scientific, Denmark) in a culture medium consisting of: Dubelcco's modified Eagle medium with 25 mM HEPES (Gibco) to which were added foetal calf serum (10% v/v, Gibco), penicillin-streptomycin (50 IU/mL each, Gibco) and glutamax (1% v/v, Gibco). The transfected sequence contained the rat P2X₂ subunit sequence as well as a neomycin resistance cassette {Stoop, 1999 #24}. This allowed for the selection of P2X₂-expressing HEK293 cells by culturing them in the presence of geneticin (0.5 mg/mL, G418, Gibco). The cultures were kept at 37°C in a water saturated incubator in an atmosphere composed of air and 5% CO₂. Before calcium imaging experiments HEK293P2X₂ cells were mechanically detached from the bottom of the culture flask by flushing the cells with a stream of culture medium by means of a 10 ml sterile plastic pipette. The medium containing the detached cells was then transferred to a 15 mL sterile plastic tube (Greiner Bio-One, Germany) and the volume was adjusted to 10 mL with standard culture medium. This solution was centrifuged at 500 RPM for 5 min and the pellet was re-suspended in culture medium or extracellular medium used for calcium imaging experiments (see below). In order to prepare cultures of HEK293-P2X₂ cells alone, cells were

re-suspended in 2 mL standard culture medium, seeded on poly-D-lysine coated glass-bottom culture dishes and maintained as described for DH glial cultures in the preceding section. These cultures were used to determine the intrinsic properties of HEK293-P2X2 cells in calcium imaging experiments. Alternatively, HEK293-P2X2 cells were used as biosensor cells to detect ATP release from cultured DH glial cells. To this end, the dissociated HEK293-P2X2 cells were re-suspended in 2 mL of the serum-free HEPES buffer-based extracellular solution used for calcium experiments, loaded with fura-2 and plated on top of cultured DH glial cells just before the beginning of the experiments (see below).

Calcium imaging

I. Loading of cells with fura-2 Cultured DH glial cells or HEK293-P2X2 cells were washed several times with the extracellular HEPES-based extracellular solution used in all the calcium imaging experiments. This extracellular solution was composed of (in mM): NaCl, 135; KCl, 5; CaCl₂, 2.5; MgCl₂, 1; glucose, 10 and HEPES, 5. Loading of cells was achieved by incubating them for 1 h at room temperature (22-25°C) in extracellular medium containing 4 µM fura-2 acetoxymethyl ester (fura-2 AM) (Molecular Probes, USA), 0.001% (w/v) pluronic acid (Molecular Probes, USA) in order to facilitate fura-2 AM uptake by the cells. After loading, the cells were washed with extracellular medium and used for calcium experiments on the experimental set up.

In experiments in which HEK293-P2X2 cells were used as biosensors for ATP release from DH glial cells, the loading with fura-2 was performed on suspensions of HEK293-P2X2 cell (see above). This protocol was performed at room temperature (22-25°C) following a protocol similar to that described above for glial cultures, except that the “loading-medium” also contained 200 nM progesterone (PROG, Sigma Alkadrich, USA) in order to potentiate P2X2

receptor function {De Roo, 2010 #102}. After 1 h incubation, this suspension of HEK293-P2X2 cells was diluted with 9 mL of extracellular solution and centrifuged at 500 RPM for 5 min. The pellet was rinsed two times with 10 mL of extracellular solution and re-suspended in 2 mL of extracellular solution. This suspension of fura-2- and progesterone-loaded HEK293-P2X2 cells was then seeded on top of cultured DH glial cells (200 μ L per dish).

II. Measurements of changes in intracellular free calcium concentration ($[Ca^{2+}]_i$) To monitor changes in $[Ca^{2+}]_i$, culture dishes were transferred to the stage of an inverted fluorescence microscope (Axiovert 35; Zeiss, Gottingen, Germany) and the cells were visualized with a x40 oil-immersion objective (Fluor 40; NA, 1.30; Nikon, Tokyo, Japan). The cultures were continuously superfused with extracellular medium, the temperature of which was maintained at 34°C. Cells were allowed to habituate to this temperature for at least 15 min before starting the experiments. In experiments using HEK293-P2X2 cells as ATP biosensors, the fura-2 and progesterone loaded cells (HEK293-P2X2-PROG cells) were seeded on top of the glial cells once the culture dishes of glial cells had been placed on the microscope stage. This procedure was performed at room temperature and in the absence of extracellular superfusion. Fifteen min after seeding, HEK293-P2X2-PROG cells had attached sufficiently to the glial cell layer and superfusion with heated extracellular solution was started. It should be noted that in biosensor experiments, only HEK293-P2X2-PROG cells were loaded with fura-2. Thus, the measured changes in $[Ca^{2+}]_i$ were solely due to changes in calcium levels in HEK293-P2X2-PROG cells since glial cells did not contain fura-2.

Calcium signals were acquired with a quantitative real- time imaging system comprising a cooled CCD camera (CoolSNAP HQ; Roper Scientific, Tucson, AZ, USA) and an image analysis software package (Imaging workbench 4.0; Axon Instruments, Molecular Devices).

Cells were alternately excited at wavelengths of 350 and 380 nm with a lambda-10 filter wheel (Sutter instruments, USA), and emitted light was collected above 520 nm. Pairs of images were acquired every 1.1 seconds. Throughout the manuscript, intracellular calcium levels and their variations are expressed as the ratio of fluorescence signals (ratio F_{350}/F_{380}) measured at 520 nm after alternate excitation at 350 and 380 nm. This ratio was calculated after background signal subtraction. All experiments were performed at 34°C.

III. Calcium imaging analysis For analysis, regions of interest (ROIs) corresponding to individual cells were selected manually offline using the Imaging workbench 4.0 software (Axon Instruments, Molecular Devices). ROIs were chosen based on cell morphology and cell responsiveness to a positive control in order to avoid any selection bias by the experimenter. For cultured glial cells, this positive control consisted in the existence of a large transient increase in $[Ca^{2+}]_i$ following the local application of 30 μ M ATP at the end of the recording session. For HEK293-P2X2-PROG cells, only those that responded with an increase in $[Ca^{2+}]_i$ to a low (200 nM) concentration of exogenously applied ATP were considered in the analysis. This response corresponded to the change in $[Ca^{2+}]_i$ triggered by the selective activation of P2X2 receptors and attested cells ability to sense low concentrations of extracellular ATP (for details see Results section and Fig. 2). In a given field, all cells that displaying an increase in $[Ca^{2+}]_i$ following the application of ATP were selected for analysis regardless of the fact that they responded or not to other substances applied during the experiment.

The acquired fluorescence signals were analyzed in more detail with the Clampfit 10.7.0 software (Molecular devices) which allowed us to quantify baseline F_{350}/F_{380} ratio values as well as their changes during application of pharmacological substances. Measurements of area under the curve (AUC) of the calcium signals in fura-2 loaded HEK293-P2X2-PROG cells in

contact with glial cells were used to quantify ATP release from glia under control and different experimental conditions (e.g. after treatment with vesicular release inhibitors). To this end, the experimental fluorescence values were divided by the mean F_{350}/F_{380} ratio value measured before application of the tested substance. This value was determined over a period of 500 s preceding substance application and this procedure therefore normalized the basal fluorescence ratio value to 1 and the area under the curve (AUC) value corresponding to this control period to 500 (ratio of 1 during 500 s). Changes in ATP release were quantified by the changes in the AUC value in HEK293-P2X2-PROG cells that were induced by local application of noradrenaline, different adrenoceptor agonists or a low K^+ containing extracellular solution (see below). These applications lasted 150 s and the AUC was measured over a 500 s period, which included the 150 s lasting application of the substance and a subsequent 350 s period outlasting the application time in order to include delayed/prolonged effects on ATP release from glial cells. The AUC determined during the 500 s period before application was compared to that measured during the 500 s following the onset of application of the substance to be tested. The increase in AUC triggered by the substance reflected the increase in ATP release from glia, since it was blocked by a P2X receptor antagonist (see Results section and Fig 3) and was never observed in HEK293-P2X2-PROG cells cultured alone (i.e. in the absence of glia, data not shown). (XX add suppl fig effect of Phe on HEK cells alone + positive control with ATP)

Pharmacological substances

Pharmacological substances to be tested were prepared as 1000 times concentrated stock solutions and diluted to their final concentration in extracellular solution just before the beginning of the experiments.

D,L - Noradrenaline hydrochloride, phenylephrine hydrochloride, clonidine hydrochloride, isoproterenol hydrochloride, adenosine triphosphate disodium salt (ATP), propranolol

hydrochloride, prazosin hydrochloride, yohimbine hydrochloride (all Sigma-Aldrich, USA) and pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid (PPADS) (Tocris, UK) were prepared in distilled water and stored at -20°C.

Fura-2-AM (Molecular Probes, USA), 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (BAPTA-AM) (Sigma-Aldrich, USA), bafilomycin A1 (Abcam, Cambridge, UK), glycyl-L-phenylalanine 2-naphthylamide (GPN) (Abcam, Cambridge, UK) were prepared in dimethyl sulfoxide (DMSO) and stored at 4°C. Progesterone (PROG, Sigma Aldrich, USA) was prepared in ethanol 70% (v/v) and stored at 4°C.

Application of substances

During all calcium imaging experiments the cells were continuously superfused with extracellular solution preheated at 34°C. The total bath volume was 2 mL and perfusion rate was 3 mL.min⁻¹. Substances to be tested were applied locally by means of a gravity driven multichannel perfusion device consisting of independent 5 mL syringes connected via separated polyethylene tubings to a common final outlet, i.e. a 0.9 mm metal needle placed just above the cells. The substance to be applied was selected by opening a tap placed between the syringe and the polyethylene tube connected to this syringe. Outside application periods, standard extracellular solution was continuously flowing through the application system to the cells via one of the application channels that remained open. During application of a substance, the tap of the control extracellular solution channel was closed simultaneously with the opening of the tap of the substance to be tested. The reverse procedure was followed at the end of the application period of the substance.

In all experiments we checked that switching between two channels containing extracellular solution did not produce mechanical artifacts that could trigger an increase in $[Ca^{2+}]_i$. We also

verified that application of vehicle/solvents such as ethanol, H₂O, or DMSO in which stock solutions of substances were prepared, did not induce changes [Ca²⁺]_i. In some experiments increases in [Ca²⁺]_i were triggered by applying a low K⁺ solution {Dallwig, 2000 #98;Hartel, 2007 #63}. To this end the concentration of KCl in the extracellular solution was reduced from 5 mM to 0.2 mM and the NaCl concentration was increased from 135.0 mM to 139.8 mM. Application of this low K⁺ solution never increased [Ca²⁺]_i in HEK293-P2X2-PROG cells cultured alone (in the absence of glia, data not shown). (XXadd suppl fig absence of effect of low K on Hek cells + positive control ATP)

Incubation of cells with substances

In some of the experiments DH glial cells were pre-incubated with pharmacological substances in order to block increases in [Ca²⁺]_i (BAPTA-AM), release of secretory lysosomes (GPN) or release of acidic vesicles (bafilomycin A1). All incubations were performed at room temperature and the substances were added at their final concentration to the extracellular HEPES-based medium used for calcium imaging experiments. The concentrations and times of incubation were as follows: BAPTA-AM (10 μM, 1h); GPN (100 μM; 20 min) and bafilomycin A1 (0.2 μM; 90 min). Concentration and incubation times for the different substances were chosen according to those used in similar studies and published in the scientific literature (XX give refs for GPN, Baf A1 and BAPTA: BAPTA-AM Innocenti JNeurosci 2000,GPN: Li J Neurosci 2008 et Zhang Nat Cell Biol 2007, BafiloYoshimori JBC 1991)

Fitting of dose-response curves

Dose-response curves for ATP were described with the following equation: $y=100/(1+(EC_{50}/[ATP])^{A_H})$ where y is the proportion of responding cells and A_H a Hill coefficient.

Statistical analysis

Statistical data analyses were performed using the statistical software Kyplot 5.0. (KyensLab, Tokyo, Japan). Proportions in contingency tables were compared with Fisher's exact test (<http://quantpsy.org/fisher/fisher.htm>). Comparison between means was performed with paired or unpaired (as appropriate) Student's t-test. The significance level for statistical tests was set as $\alpha = 0.05$. Symbols to indicate P-values that are used in the figures are as follows: ****P < 0.00001 < ***P < 0.0001 < **P < 0.001 < *P < 0.05 < n.s (not significant).

Most results are expressed as proportion of responsive cells given as X/n where X is the number of responsive cells and "n" the total number of cells tested.

The other numerical results are given as mean \pm s.e.m. (standard error to the mean).

Results

DH glial cell cultures

Our aim was to use a mixed DH glial cell culture rather than isolated “purified” glial cell populations in order to maintain the coexistence and the interaction between the major DH glial cell types in culture. Different considerations indicate that our cultures contained no neurons and mainly astrocytes. The absence of neurons was suggested by calcium imaging experiments showing that the application of a depolarizing extracellular solution containing a high (50 mM) K^+ concentration did not induce an increase in $[Ca^{2+}]_i$ that is typical for neurons in culture, including for DH neurons in culture {Dallwig, 2000 #98;Petitjean, 2014 #18}. . (XX include suppl Fig showing absence of effect of High K on glila and effect of ATP) The absence of neurons was most probably due to the purely mechanical dissociation protocol that we used. Indeed, we have a long standing experience in the preparation of primary postnatal DH mixed neuron-glia cultures {Hugel, 2000 #21;Jo, 1999 #22} and we have clearly established that, in order to obtain viable neurons, it is necessary to use an enzymatic dissociation followed by a very mild mechanical dissociation. When mechanical dissociation is used alone or is too intense, neurons do not survive.

In addition, it has been clearly established that, among glial cells, α_1 adrenoceptors are expressed by astrocytes but not by microglia even in cell culture {Hertz, 2010 #72}. In our cultures, 90% of the cells responded to the α_1 adrenoceptor specific agonist phenylephrine (PHE, see below) suggesting that astrocytes were the main glial cell type present in these cultures. Moreover, during calcium imaging experiments, we observed that the cells displaying an increase in $[Ca^{2+}]_i$ upon stimulation with PHE had a flattened star-shaped morphology that is characteristic of astrocytes in culture.

Taken together, these arguments indicate that the majority of cells present in our cultures appeared to be astrocytes.

Effect of noradrenaline (NA) and adrenoceptor agonists on intracellular free calcium concentration ($[Ca^{2+}]_i$) in DH glial cells

Local application of NA (20 μ M, 50 s) induced an increase in $[Ca^{2+}]_i$ in 86.0% of the cells tested (172/200). Three types of responses were observed (Fig. 1a). In the majority of cases, cells responded with a type 1 response that consisted of an initial fast transient peak followed by lower amplitude plateau phase that slowly declined but persisted during the whole duration of NA application. Type 1 responses were observed in 85.5% (147/172) of the NA-responsive DH glial cells. A second type of response (type 2) consisted of an initial peak followed by oscillations in $[Ca^{2+}]_i$ that occurred during the plateau phase. Such oscillatory responses were recorded in 11.0% (19/172) of the cells. Finally, a small proportion of cells, i.e. 3.5% (6/172), displayed a type 3 response that consisted of a single initial peak with no detectable plateau phase or oscillations in $[Ca^{2+}]_i$.

We next tested the classical α_1 , β and α_2 adrenoceptor agonists phenylephrine (PHE, 20 μ M), isoproterenol (ISO, 10 μ M) and clonidine (CLO, 20 μ M), respectively. As illustrated in Fig. 1b, PHE, ISO and CLO induced increases in $[Ca^{2+}]_i$ in 95.0% (132/139), 23.7% (47/198) and 6.1% (7/114) of the cells, respectively. The proportion of responses to PHE was significantly reduced to 5.1% (4/79) in the presence of the α_1 adrenoceptor antagonist prazosin (2 μ M) (Fisher's exact test, $P = 9.4 \cdot 10^{-45}$). Similarly, the proportion of responses to ISO was significantly reduced to 7.0% (4/57) by the β adrenoceptor antagonist propranolol (20 μ M) (Fisher's exact test, $P = 0.03$). By contrast, the proportion of responses to CLO was very low (6.1% see above) and not significantly modified by the α_2 adrenoceptor antagonist yohimbine

(1 μ M) (1.7%, 1/58, Fisher's exact test; $P = 0.265$). In agreement with these observations, we found that a cocktail of prazosin, propranolol and yohimbine virtually abolished the responses to NA (5.1%, 4/79, Fisher's exact test; $P = 4.0 \cdot 10^{-38}$). Fig. 1c summarizes the proportions of type 1, 2 and 3 responses observed following the application of NA, PHE, ISO and CLO. Interestingly, in the majority of cases (90.2%, 101/112), the responses triggered by PHE could be reproduced at least 2 times in the same cells when applications were separated by a delay of 1000 s (data not shown). (XX add suppl fig showing reproducibility and histo of quantification of PHE response in glia)

Taken together, our results suggest that NA increased $[Ca^{2+}]_i$ in about 90% cultured DH glial cells and that this effect was largely mimicked both qualitatively and quantitatively by the $\alpha 1$ -agonist PHE. In addition, about 25% of the cells displayed a response to ISO but virtually none to CLO. We therefore decided to investigate in more detail the effects of PHE and NA on the release of ATP from DH glial cells.

Detection of extracellular ATP by an improved biosensor system

In order to detect the release of ATP from DH glial cells and its modulation by NA and PHE, we used a biosensor/sniffer cell approach. We chose this option because, once released in the extracellular space, ATP is rapidly metabolized by ectonucleotidases {Dunwiddie, 1997 #42; Zimmermann, 1999 #43; Zimmermann, 2000 #45} making it difficult to detect accurately this short-lived molecule. We decided to use HEK293 cells stably transfected with rat P2X2 receptors (HEK293-P2X2) {Stoop, 1999 #24} as a detection system for extracellular ATP, since these receptors are highly permeable to Ca^{2+} ions {Egan, 2004 #28} and Ca^{2+} influx through these receptors can be detected by calcium indicators such as fura-2. The general idea

of our approach was to plate fura-2-loaded HEK293-P2X2 cells on top of cultured DH glial cells. Under these conditions changes in $[Ca^{2+}]_i$ measured in HEK293-P2X2 cells will reflect and allow to monitor the changes in ATP release from glial cells following the application of NA and PHE (see below) with good spatial and temporal resolutions.

When ATP was applied to HEK293-P2X2 cells cultured alone and loaded with fura-2, we observed an increase in $[Ca^{2+}]_i$. The dose-response relationship illustrating the proportion cells displaying an increase in $[Ca^{2+}]_i$ for increasing concentrations of ATP applied is illustrated in Fig. 2a. The apparent EC_{50} of ATP was $0.75 \pm 0.02 \mu M$. Under these conditions, it was difficult to detect low nanomolar concentrations of ATP. Indeed, application of 200 nM ATP triggered small calcium responses in only 5.3% (4/76) of the cells (Fig. 2b), corresponding approximately to the EC_5 of our dose response curve. However, we have previously shown that rat P2X2 receptors can be strongly and selectively potentiated by the steroid/neurosteroid progesterone (PROG) {De Roo, 2010 #102}. Fig. 2b shows that acute application of PROG (5 μM) to HEK293-P2X2 cells for 100 s did not evoke any significant increase in $[Ca^{2+}]_i$. However, when ATP (200 nM), that initially triggered small and rare responses before PROG application, was applied to HEK293-P2X2 cells after they had been exposed to PROG (5 μM), it evoked large increases in $[Ca^{2+}]_i$ in the large majority of the cells (75.9%, 41/54, Fig 2b). PROG, PROG-derived metabolites as well as other steroids are lipophilic molecules that solubilize easily in the hydrophobic part of the plasma membrane where they remain for considerable periods of time {Akk, 2007 #64;Akk, 2005 #65}. In agreement with this, we noticed that the potentiating effect of PROG (5 μM) persisted after the end of the application of PROG. This phenomenon was quantified in a sample of 54 cells (Fig 2c) and the results show that the potentiating effect of PROG on responses triggered by 200 nM ATP persisted without significant attenuation for at least 90 min after removing PROG from the extracellular medium. Similar observations were

made when HEK293-P2X2 cells were incubated with a much lower concentration of PROG (200 nM) for 1 hour. Under these conditions 75.7% (53/70) of the cells responded with large increases in $[Ca^{2+}]_i$ following the application of 200 nM PROG and the potentiating effect of PROG lasted for at least 3 hours without significant attenuation (data not shown) (XX add suppl Fig illustration non fading response for 3h). The effect of PROG was due the positive modulation of P2X2 receptors because ATP receptors mediating the increase in $[Ca^{2+}]_i$ were blocked by the P2X2 receptor antagonist PPADS (50 μ M) (Fig. 2d). After PROG exposure, none of the cells tested (0/41) displayed an increase $[Ca^{2+}]_i$ to ATP (200 nM) in the presence of PPADS (50 μ M) (Fisher's exact test, $P = 1.6 \cdot 10^{-17}$). Moreover, ATP (200 nM) rarely induced an increase in $[Ca^{2+}]_i$ in non-transfected HEK293 cells (5.3%; 2/38) and these responses were not inhibited by PPADS (50 μ M). The complete dose-response relationship for ATP after PROG (200 nM, 1 h) is illustrated in Fig. 2a. After PROG pretreatment, the EC_{50} value decreased to $0.10 \pm 0.01 \mu$ M yielding a 7.5 fold increase in the sensitivity of ATP detection in our system.

Taken together these results suggest that PROG-potentiated P2X2 receptors stably expressed by HEK293 cells represent an excellent tool to detect low nanomolar concentrations of extracellular ATP.

Quantification of ATP release from cultured DH glial cells

In order to detect and quantify ATP release from cultured glial cells, HEK293-P2X2 cells were loaded with fura-2-AM, and incubated with 200 nM PROG for one hour at room temperature and then plated on top of confluent DH glial cell cultures (see Methods section). Since only HEK293-P2X2 cells were loaded with fura-2, changes in $[Ca^{2+}]_i$ reflected either spontaneously

occurring calcium fluctuations in these cells or/and activation of P2X2 receptors by ATP released from DH glial cells in close contact with the HEK293-P2X2 cells.

In an initial set of experiments, we quantified the spontaneously occurring changes in $[Ca^{2+}]_i$ in HEK293-P2X2 cells pre-incubated with PROG but cultured alone (i.e. in the absence of DH glial cells). Under these conditions, the $[Ca^{2+}]_i$ levels were usually very stable and rarely displayed spontaneously occurring peaks in $[Ca^{2+}]_i$ (6.8%; 10/146). When observed, the frequency of peaks was very low ($2.8 \cdot 10^{-3} \pm 4.4 \cdot 10^{-4}$ Hz, $n=10$). Moreover, these cells did rarely displayed increases in $[Ca^{2+}]_i$ following the application of NA (20 μ M) nor PHE (20 μ M). Indeed, we observed an increase in F_{350}/F_{380} ratio in only 2% (2/99) of the cells, and this change consisted of a single peak (type 3 response in Fig. 1a). Calcium peaks occurring spontaneously in HEK293-P2X2-PROG cells plated on top of DH glial cells were also relatively rare and represented 11.9% of the cases (19/160). This proportion of cells was not significantly different from that of HEK293-P2X2-PROG displaying calcium peak in the absence of DH glial cells (Fischer's exact test; $P = 0.17$). Moreover, the mean frequency of peaks was not different from that in the absence of glia ($3.0 \cdot 10^{-3} \pm 2.1 \cdot 10^{-4}$ Hz, $n=42$; Fisher's exact test; $P = 0.75$).

In contrast, when PROG loaded HEK293-P2X2 cells (HEK293-P2X2-PROG) were seeded on top of DH glial cell cultures, application of NA (20 μ M) or PHE (20 μ M) induced the appearance of peak-like increases of $[Ca^{2+}]_i$ in the HEK293-P2X2-PROG cells (Figs 3a1, b1). In 23 cases, HEK293-P2X2-PROG cells displayed spontaneous calcium peaks before the application of NA (20 μ M). The mean frequency of peaks was of $3.0 \cdot 10^{-3} \pm 2.8 \cdot 10^{-4}$ Hz ($n=23$). After application of NA (20 μ M), the frequency of peaks significantly increased to $4.3 \cdot 10^{-3} \pm 3.3 \cdot 10^{-4}$ Hz ($n=23$, Fisher's exact test; $P = 0.007$). Similarly, in 19 cases, HEK293-P2X2-PROG cells displayed spontaneous calcium peaks before the application of PHE (20 μ M). The mean frequency of peaks was of $3.0 \cdot 10^{-3} \pm 3.2 \cdot 10^{-4}$ Hz ($n=19$). After application of PHE (20 μ M), the

frequency of peaks significantly increased to $4.4 \cdot 10^{-3} \pm 5.6 \cdot 10^{-4}$ Hz ($n= 19$, Fisher's exact test; $P = 4.0 \cdot 10^{-3}$).

In order to facilitate the representation of the effects of NA and PHE on calcium peaks, data from all responsive cells were pooled. These data are presented in Fig. 3a2 and Fig. 3b2 under the form of histograms of the number of calcium peaks as a function of time before, during and after the application of NA and PHE. Application of NA (20 μ M) or PHE (20 μ M) induced an increase in the number of $[Ca^{2+}]_i$ peaks that outlasted the duration of NA or PHE application. Interestingly, an increase in the number/frequency of calcium peaks by NA or PHE could be induced only by the first application of these agonists. A second application, even if performed 1000 s after the first application did not induce the appearance or the increase in frequency of $[Ca^{2+}]_i$ peaks, whereas effects on calcium responses in DH glial cells alone could be reproduced at least two times (see above). The calcium signals induced by PHE were never observed when DH glial cells were pre-loaded with BAPTA-AM (0/87, Fig. 3c) or when PPADS (50 μ M) was added to the extracellular medium (0/71, Fig. 3d).

In order to quantify more precisely the changes in $[Ca^{2+}]_i$ that occurred in HEK293-P2X2-PROG cells, we determined the area under the curve (AUC) of the fura-2 signal and normalized each signal by dividing it by the value of the baseline, thus allowing to compare the results between different cells (see Methods section for more details). This was done for equivalent periods of time (i.e. 500 s) before and after application of PHE. As shown in Fig. 3e, PHE significantly increased the AUC from 504.8 ± 1.1 a.u. (arbitrary units) to 518.2 ± 5.0 a.u. ($n=68$, paired Student's t-test; $P = 6.2 \cdot 10^{-4}$). The effect of PHE was abolished by PPADS (50 μ M) or after loading the DH glial cells with the calcium chelator BAPTA-AM (Fig. 3e). In the presence PPADS (50 μ M), the AUC was of 503.8 ± 1.6 a.u. before and of 502.0 ± 2.3 a.u. after application of PHE (20 μ M) ($n= 70$). These values did not differ significantly (paired Student's t-test; $P = 0.37$). When DH glial cells were preincubated with BAPTA-AM, the AUC was of

505.0 ± 1.1 a.u. before and of 506.0 ± 0.9 a.u. after application of PHE (20 μM) (n= 87). These values did not differ significantly (paired Student's t-test; P = 0.27).

These results show that the increase in $[Ca^{2+}]_i$ involved the activation of PPADS-sensitive P2X2 receptors expressed by HEK293-P2X2 cells. Moreover, BAPTA specifically loaded into DH glial cells blocked the changes in $[Ca^{2+}]_i$ observed in HEK293-P2X2 cells indicating that these changes were due to a calcium-dependent release of ATP from the cultured DH glial cells.

Effect of reducing extracellular K⁺ concentration on the release of ATP

Since NA and PHE triggered calcium-dependent release of ATP from cultured DH glial cells (see preceding section), we wondered whether other stimuli that increase $[Ca^{2+}]_i$ were able to induce ATP release from these cells.

It has been reported that application of an extracellular solution containing a low concentration of K⁺ ions (low K⁺) can induce the a rise in $[Ca^{2+}]_i$ in astrocytes by activating Ba²⁺-sensitive Kir4.1 inwardly rectifying K⁺ channels {Dallwig, 2000 #98;Hartel, 2007 #63}. We therefore first tested the effect of applying a low K⁺ solution (in which the concentration of K⁺ was reduced from 5 mM to 0.2 mM) to cultured DH glial cells loaded with fura-2. Application of this low K⁺ solution rapidly induced a rise in $[Ca^{2+}]_i$ that lasted for the whole duration of the application (Fig. 4a). Increases in $[Ca^{2+}]_i$ were observed in a large majority cultured DH glial cells tested (86.9%, 54/62). Moreover, this increase in $[Ca^{2+}]_i$ could be reproduced several times in the same cells when applied at an interval of 1000 s (Fig. 4a). This low K⁺-induced response was totally blocked (0/31) when 2 mM Ba²⁺ was added to the extracellular solution. Interestingly, when tested under the same experimental conditions, the low K⁺ solution never induced a rise in $[Ca^{2+}]_i$ in PROG treated HEK293-P2X2 cells cultured alone (0/150).

When we applied a low K^+ medium to fura-2-loaded DH glial cells, we observed an increase in $[Ca^{2+}]_i$ in 87.1% (54/62) of them (Fig 4b). This proportion was unaffected in the presence of PPADS (50 μ M) in the extracellular medium (88.6%, 39/4, $P = 1.0$) but significantly reduced after preloading of the glial cells with BAPTA-AM (3.2%, 1/31, $P = 6.0 \cdot 10^{-16}$)

Application of the low K^+ solution to co-cultures of DH glial cells and PROG treated fura-2-loaded HEK293-P2X2, resulted in an increase in $[Ca^{2+}]_i$ the HEK293-P2X2 cells (Fig. 4c). Such an effect was observed in 64.1% (84/131) of the recorded cells. None of the of HEK293-P2X2-PROG on top DH glial cells displayed an increase in $[Ca^{2+}]_i$ in the presence of PPADS (50 μ M) in the extracellular medium (0.0%, 0/42, Fisher's exact test, $= 1.5 \cdot 10^{-15}$) and the proportion of HEK293-P2X2-PROG with a calcium response was significantly reduced when the DH glial cells were preloaded with BAPTA-AM (0.0%, 0/37, Fisher's exact test; $P = 8.6 \cdot 10^{-14}$) (data not illustrated).

We further quantified this response by determining the relative AUC for each response (Fig. 4d). In the absence of any treatment, the AUC of the calcium response induced by low K^+ was of 523.0 ± 4.0 a.u. ($n = 131$). The AUC was of 503.7 ± 0.6 a.u. ($= 42$) in the presence of PPADS and of 503.2 ± 1.1 a.u. ($= 37$) after preloading the DH glial cells with BAPTA-AM (Fig. 4d). These values were significantly different from the AUC of the low K^+ calcium response measured in the absence of any treatment: PPADS, unpaired student's t-test; $P = 4.9 \cdot 10^{-4}$; and BAPTA-AM, unpaired student's t-test; $P = 1.9 \cdot 10^{-4}$).

Taken together, these results suggested that application of a low K^+ solution activated Ba^{2+} -sensitive (most probably Kir4.1) channels that triggered an increase in $[Ca^{2+}]_i$ which in turn led to a calcium-dependent release of ATP that was sensed by the transfected P2X2 receptors.

Distinct vesicular pools of ATP are mobilized by NA and low K^+ stimulation of cultured DH glial cells

In order to check whether calcium-dependent ATP release involved intracellular vesicles, we tested the effects of bafilomycin A1 and glycyl-L-phenylalanine 2-naphtylamide (GPN). Bafilomycin A1 blocks the activity of the proton pump present on acidic vesicles that generates the pH gradient across the vesicle membrane that is necessary for the loading of the vesicles with the transmitter. Bafilomycin A1 acts on all types of acidic vesicles including light clear vesicles and secretory lysosomes {Bowman, 1988 #66;Yoshimori, 1991 #67}. GPN is a substrate of cathepsin C, a lysosomal peptidase present in lysosomes {Jadot, 1984 #70}. GPN is taken up by secretory lysosomes and following hydrolysis by cathepsin C it generates fragments that accumulate inside the secretory lysosomes and rapidly induces an intra-lysosomal hypertonic condition that leads to the permeabilization of these secretory lysosomes to low molecular weight substances {Li, 2008 #69;Zhang, 2007 #68}. Incubation with GPN therefore induces a selective functional perturbation of secretory lysosomes {Haller, 1996 #100;Li, 2008 #69;Zhang, 2007 #68}. We first verified that pretreatment with GPN and/or bafilomycin A1 did not directly affect the change in $[Ca^{2+}]_i$ triggered by PHE or low K^+ solution in DH glial cells cultured alone.

Fig. 5a summarizes the effects of GPN (Fig. 5a2) and bafilomycin A1 (Fig. 5a3) on changes in $[Ca^{2+}]_i$ measured in fura-2 loaded, PROG treated HEK293-P2X2 cells plated on top of cultured DH glial cells. When glial cells were pretreated with GPN, the proportion of responses to PHE was strongly decreased (6.3%, 5/80, Fisher's exact test; $P = 2.8 \cdot 10^{-18}$) and that responding to low K^+ was slightly reduced (41.3%, 33/80, Fisher's exact test; $P = 2.0 \cdot 10^{-3}$). After preincubation of the glial cells with bafilomycin A1, there was a significant reduction of the proportion of the responses to PHE (11.8%, 8/68, Fisher's exact test; $P = 3.7 \cdot 10^{-13}$) as well as to low K^+ (5.9%, 3/51, Fisher's exact test; $P = 7.7 \cdot 10^{-12}$).

When glial cells were pretreated with GPN, the baseline value (BL) of the AUC was of 503.4 ± 1.1 a.u. (n= 80) and that after PHE (20 μ M) application was of 503.8 ± 1.7 a.u. (n=80). These values were not significantly different (paired Student's t-test; $P = 0.84$). In the experiments with low K^+ applied to GPN-treated glial cells, the AUC measured in HEK203-P2X2-PROG cells on top of the glia under resting conditions was of 502.7 ± 1.7 a.u. (n= 80). This value significantly increased to 514.2 ± 3.1 a.u. (n= 80, Paired Student's t-test; $P = 3.1 \cdot 10^{-6}$) after application of low K^+ .

When glial cells were pretreated with bafilomycin A1, the baseline value (BL) of the AUC was of 504.0 ± 1.0 a.u. (n= 68) and that after PHE (20 μ M) application was of 505.0 ± 1.3 a.u. (n=68). These values were not significantly different (paired Student's t-test; $P = 0.16$). In the experiments with low K^+ applied to bafilomycin A1-treated glial cells, the AUC measured in HEK203-P2X2-PROG cells on top of the glia under resting conditions was of 501.9 ± 1.1 a.u. (n= 51) and of 501.2 ± 1.2 a.u. (n= 51, Paired Student's t-test; $P = 3.1 \cdot 10^{-6}$) after application of low K^+ . These values were not significantly different (paired Student's t-test, $P = 0.39$).

To summarize, pretreatment of cultured glial cells with GPN suppressed the increase in $[Ca^{2+}]_i$ induced by PHE (20 μ M) application but not that induced by the low K^+ solution, whereas pretreatment with bafilomycin A1 suppressed both responses to PHE and low K^+ . These results indicated that ATP can be released from a secretory lysosomal (GPN-sensitive) pool as well as from and a non-lysosomal (bafilomycin A1-sensitive) pool. Our data also show that PHE targets selectively the secretory lysosomal pool.

Spontaneous release of ATP from cultured DH glial cells

As mentioned above, HEK293-P2X2-PROG cells plated on DH glial cells rarely displayed spontaneously occurring peaks/changes in $[Ca^{2+}]_i$, i.e. in the absence of an application of NA or PHE, indicating that spontaneous vesicular release was a rare phenomenon under our basal experimental conditions. One reason for apparently not detecting spontaneous release might be that ATP released from DH glia under basal conditions did not reach a local concentration sufficient to significantly activate P2X2 receptors in the detector cells. We therefore decided to favor accumulation of ATP released from DH glial cells by temporarily interrupting (stopping) the constant superfusion of the cells with extracellular solution. Such stop/flow experiments have been used previously to demonstrate ATP release from cultured astrocytes following mechanical or electrical stimulation of the glial cells {Hassinger, 1996 #71}. Fig. 6a, b shows that stopping the general and local superfusion induced a significant increase in the F_{350}/F_{380} fluorescence ratio in 55.4% (46/83) of the detector HEK293-P2X2-PROG cells. When extracellular superfusion was switched on again, the fluorescence ratio returned to control levels, i.e. to a value close to that recorded before stopping the flow of extracellular solution, thereby indicating that the increase in $[Ca^{2+}]_i$ in the detector cells was reversible. This increase in $[Ca^{2+}]_i$ was due to the activation of P2X2 receptors by ATP because the fraction of cells displaying an increase in fluorescence ratio was significantly reduced (7.6%; 4/60, Fisher's exact test $P = 3.7 \cdot 10^{-10}$) in the steady state presence of PPADS (50 μ M) in the extracellular solution (Fig. 6a, b). There was also a significant reduction of the fraction of cells displaying an increase in F_{350}/F_{380} ratio when glial cells were preloaded with the calcium chelator BAPTA-AM (4.5%, 2/44, Fisher's exact test $P = 2.3 \cdot 10^{-9}$; Fig. 6). In contrast, pretreating the DH glial cells with bafilomycin A1 in order to block vesicular release of ATP (see above), did not significantly change the fraction of detector cells displaying an increase in $[Ca^{2+}]_i$ following the interruption of extracellular superfusion (Fig. 6a) (67.7%; 25/37, Fisher's exact test $P = 0.23$; Fig. 6).

Taken together our results suggest the existence of a spontaneous ATP release from DH glial cells. This release is calcium-dependent but does not involve acidic vesicles.

Discussion

Our results demonstrate that NA induced/stimulated the release of ATP from cultured DH glial cells. This effect relied on the activation of α_1 adrenoceptors and the calcium-dependent fusion of secretory lysosomes with the plasma membrane. The detection of the release of low nanomolar concentrations of ATP at the single cell level was possible owing to the development of an improved sniffer cell system consisting of HEK293 cells expressing rat P2X2 receptors that are strongly and selectively potentiated by the steroid progesterone (PROG). This system provided very good sensitivity to ATP and excellent spatial and time-resolutions. It also allowed us to demonstrate at the single cell level the coexistence of distinct vesicular and non-vesicular calcium-dependent release mechanisms of ATP {Blum, 2008 #103;Dubyak, 2012 #96}.

Improved sniffer cell detection system for the detection of ATP release from DH glial cells

ATP is an important extracellular signaling molecule in the nervous system that can be released from various cell types including neurons and glial cells {Burnstock, 2006 #46;Fields, 2006 #48;Guthrie, 1999 #74;Hassinger, 1996 #71;Verkhratsky, 2018 #73}. ATP is also recognized as a major gliotransmitter that underlies calcium wave propagation in glial networks and modulation of electrical activity in neuronal networks {Boue-Grabot, 2017 #49;Guthrie, 1999 #74;Hassinger, 1996 #71;Haydon, 2001 #20;Lalo, 2014 #51;Verkhratsky, 2018 #73} including in the DH of the spinal cord {Hugel, 2000 #21;Jo, 1999 #22;Scemes, 2000 #60}. A major limit

for the precise detection and quantification of ATP release is its rapid degradation by extracellular ectonucleotidases {Dunwiddie, 1997 #42; Zimmermann, 1999 #43; Zimmermann, 2000 #45} which strongly reduce the lifetime of ATP in the extracellular space once it has been released. This is a serious challenge when trying to quantify low concentrations of extracellular ATP, in particular at the single cell level. Several approaches have been developed to try to resolve this issue. One of these consists of using calcium-permeable ionotropic ATP receptors (P2X receptors) as detection/detector elements. HEK293 cells transfected with P2X2 or mutant P2X3 receptors have been used successfully for detecting ATP release from glial cells {Lalo, 2014 #51; Pangrsic, 2007 #76}. We found, when using a calcium imaging approach, that simply expressing P2X2 receptors in HEK293 cells did not improve the ATP detecting capacity of these cells that naturally express metabotropic P2Y receptors {Atwood, 2011 #77; Fischer, 2005 #78}. Indeed, our results show that non-transfected and HEK293 cells transfected with rat P2X2 receptors displayed a similar EC_{50} for ATP that was in the micromolar range (0.75 μ M) and therefore too high for reliably detecting low nanomolar concentrations of ATP (see Results section and Fig. 2a). However, we have previously shown that homomeric P2X2 receptors are strongly and selectively potentiated by the steroid/neurosteroid PROG {De Roo, 2010 #102}. In line with this finding, preincubation of the HEK293-P2X2 cells with PROG increased by sevenfold the sensitivity of the cells to ATP, shifting the EC_{50} for ATP to 0.1 μ M. Moreover, the high oil-water partition coefficient of steroids/neurosteroids such as PROG and PROG derivatives which is >50.000 {Akk, 2005 #65} provides an ideal situation in which these substances can rapidly gain access to the lipophilic compartment of the plasma membrane where they remain trapped for long periods of time. The residence/dwell time of these lipophilic substances within the membrane will be mainly determined by a combination of slow wash out (diffusion into the extracellular medium) and metabolism of the substances within the cell {Akk, 2007 #64}. Once in the hydrophobic part of the membrane bilayer, these steroids can

diffuse laterally and interact with integral membrane proteins to modulate their activity, as it has been shown for the positive allosteric modulation and direct activation of GABA_A receptors {Akk, 2005 #65; Hosie, 2006 #80; Inquimbert, 2008 #82; Shu, 2004 #79}. A similar effect is probably involved in the potentiation of P2X₂ receptors by PROG {De Roo, 2010 #102}. Whatever the mechanism involved, we noticed that when the HEK293-P2X₂ receptors were in contact with PROG, i.e. 5 μ M for 100 s or 200 nM for 1 h, we observed a robust potentiation of P2X₂ receptor activity that lasted for at least 3 hours after removing PROG from the extracellular solution. We took advantage of this situation and incubated the HEK293-P2X₂ cells alone with 200 nM PROG before plating them on top of the cultured DH glial cells, thus avoiding to expose the glial cells directly to PROG. This system allowed us to reliably detect ATP release from the DH glial cells (see below) but also from neurons such as primary sensory neurons (data not shown) (XX add suppl Fig showing ATP release from DRG after High K and absence of High K on HEK cells alone). More generally, we think that this detection system could be useful for the study of ATP release from virtually every cell type.

Vesicular ATP release from cultured DH glial cells and its modulation by NA

The improved sniffer cells system described above allowed us to clearly detect NA-evoked ATP release from DH glial cells. Application of NA or the specific α_1 adrenoceptor agonist PHE induced peak-like $[Ca^{2+}]_i$ transients in the detector HEK293-P2X₂-PROG cells. These transients were absent when the DH glial cells were loaded with the calcium chelator BAPTA-AM or when the extracellular medium contained PPADS, an antagonist of P2X₂ receptors. These results indicated that activation of α_1 adrenoceptors in DH glial cells induced calcium-dependent release of ATP that was detected by the HEK293-P2X₂-PROG cells. This calcium-dependent release apparently resembled that described for vesicular release of ATP, glutamate,

aspartate or D-serine from astrocytes in the CNS {Gundersen, 2015 #83;Harada, 2015 #84}. In line with these studies, ATP release induced by PHE from our cultured DH glial cells was completely blocked when the DH glial cells were pretreated with bafilomycin A1, an inhibitor of the proton pump present on the membrane of acidic secretory vesicles, preventing the loading of the vesicles by the transmitter {Bowman, 1988 #66;Yoshimori, 1991 #67}. Most interestingly, calcium-dependent ATP release induced by α_1 adrenoceptor stimulation was also blocked when DH glial cells were treated with GPN, a small peptide-like molecule that is selectively taken up by secretory lysosomes and leads to their selective functional perturbation {Haller, 1996 #100;Jadot, 1984 #70;Zhang, 2007 #68}. The complete suppression of PHE-induced ATP release by GPN suggested that α_1 adrenoceptor stimulation selectively recruited a pool of secretory lysosomes and induced their exocytosis. This is consistent with previous studies in which it was shown that ATP release from hippocampal and cortical astrocytes seems to involve mainly secretory lysosomes {Jaiswal, 2007 #87;Li, 2008 #69;Verderio, 2012 #85;Zhang, 2007 #68}.

However, we observed that a release of ATP independent of α_1 adrenoceptor stimulation was still possible after selectively affecting secretory lysosomes with GPN. Indeed, it has been shown that in astrocytes, application of a low K^+ containing solution induced an increase in $[Ca^{2+}]_i$ by activating inwardly rectifying Kir4.1 K^+ channels {Dallwig, 2000 #98;Hartel, 2007 #63}. In our DH glia cultures, application of a low K^+ solution increased $[Ca^{2+}]_i$ in the vast majority (87%) of cells and this led to the release of ATP that could be detected by the HEK293-P2X2-PROG sniffer cells (see Fig. 5). Like for the PHE effect, low K^+ -induced ATP release was blocked by BAPTA loading of the glial cells or by pretreating the cells with bafilomycin A1. Yet, pretreatment of the DH glia with GPN did not block the effect of low K^+ on ATP release, whereas ATP release induced by PHE was completely suppressed under these conditions. This clearly pointed to the existence of a second pool of vesicles mediating calcium-

dependent ATP release that was distinct from secretory lysosomes and that could be recruited by application of a low K^+ solution but neither by NA nor PHE. This pool could correspond to the small clear vesicle pool or the large dense-core vesicle pool that are present in astrocytes {Verderio, 2012 #85}.

Spontaneous release of ATP from DH glial cells

Transiently/temporarily interrupting the continuous superfusion of the cells with extracellular solution allowed us to reveal an increase in $[Ca^{2+}]_i$ in approximately half of the sniffer HEK293-P2X2-PROG cells seeded on top of the DH glial cells. This increase in $[Ca^{2+}]_i$ was inhibited by PPADS, preloading the glial cells with BAPTA or pretreating them with bafilomycin A1, indicating that this increase in $[Ca^{2+}]_i$ reflected the release of ATP from at least a subset of glial cells. The fact that only half of the detector cells displayed such an increase in $[Ca^{2+}]_i$ might also reflect that only a fraction of the sniffer cells was appropriately positioned (i.e. close to the release sites of ATP) to detect ATP release. The fraction of responsive cells might therefore be a lower estimate of the fraction of glial cells that are able to release ATP spontaneously. Most importantly, spontaneous ATP release was still present after blocking vesicular ATP release from acidic vesicles by bafilomycin A1 pretreatment but was suppressed when glial cells were preloaded with BAPTA. The study of the exact cellular mechanisms underlying spontaneous ATP release were beyond the main scope of our study and were not further investigated. However, in line with literature concerning ATP release from glial cells, the non-vesicular spontaneous release of ATP observed in our model might possibly involve release of ATP through ATP-permeable membrane channels, or membrane transporters {Blum, 2008 #103;Dubyak, 2012 #96;Verkhratsky, 2018 #73}.

Physiological considerations

ATP release from DH glia. Our results confirm that secretory lysosomes are important for ATP release from DH glial cells. Moreover, this lysosomal pool of ATP is apparently the selective target of NA-mediated regulation of ATP release from these cells. The stimulation of ATP release by NA involved $\alpha 1$ adrenoceptors that are coupled to phospholipase C activation {Verkhratsky, 2018 #73}. Lysosome-mediated ATP release has been shown to underlie propagation of calcium waves in glial networks and glia-to-neuron communication {Verderio, 2012 #85}. Interestingly, another vesicular but non-lysosomal pool of ATP could be released when $[Ca^{2+}]_i$ was increased by application of a low K^+ solution {Dallwig, 2000 #98;Hartel, 2007 #63}. In heterologous expression systems transfected with Kir4.1 channels, a decrease in extracellular K^+ concentration ($[K^+]_o$) to a final concentration of 2 mM was sufficient to trigger important increases in $[Ca^{2+}]_i$ in about 40% of the recorded cells {Hartel, 2007 #63}. Decreases in $[K^+]_o$ following neuronal activity (K^+ undershoot) have been observed in the cortex {Heinemann, 1975 #88}, the hippocampus {D'Ambrosio, 2002 #89} and the spinal cord {Jendelova, 1991 #90}. It remains to be established if the observed decreases in $[K^+]_o$ observed are sufficient to trigger rises in $[Ca^{2+}]_i$ under physiological conditions. Important reductions in $[K^+]_o$ might be achieved in very localized regions where the volume of extracellular space is particularly small. Finally, the spontaneous calcium-dependent and non-vesicular release of ATP is of particular interest. This release might serve homeostatic purposes by locally allowing the production of adenosine {Pascual, 2005 #91} but might also represent an important element for basal and activity-dependent signaling within the glial network and between the glial and neuronal networks.

ATP and NA systems in the DH. Stimulation of glial ATP release by NA contained in the descending axons of *Locus Coeruleus* neurons might participate in the control of nociception {Millan, 2002 #2;Pertovaara, 2006 #3;Seibt, 2015 #41}. ATP is an important extracellular

signaling molecule (REFXXBurnstock Physiol Rev 2007) that plays a role in sensory transmission (REFXX Nakatsuka PflugersArch 2006), in particular in nociceptive transmission (REFXXTsuda Brain Res Rev 2010). In the spinal cord, ATP can be released from neurons and glial cells (REFXX Jo NatNeurosci 1999, Scemes) and receptors for ATP are expressed by both cell types (REFXX REF pour neurons + Fields Burnstock NatRevNeurosci). Different P2X receptor subunits are expressed in the DH (REFXX Collo J Neurosci) and their expression appears to increase during the post-natal period, in particular in the deep DH of the spinal cord (REFXX XX Shiokawa J Physiol 2006). Glial cells play a role in setting basal threshold for mechanical nociception (REFXX Foley MolPain 2011) and ATP release participates in the development of neuropathic pain (REFXX Ji & Nedergaard Pain 2013, Masuda NatComm 2016 release neuronal ATP). Moreover, P2X receptors have been shown to participate in the processing of nociceptive signals in the DH and glial cells seem to be involved in this phenomenon (REFXX Aoyama Pain 2011).

The noradrenergic innervation of the DH is due to axons of neurons having their cell bodies in the Locus Coeruleus (REFXX Fritschy Brain Res 1987, Lyons J Neurosci 1989). In the rat, noradrenergic axons are observed in the DH at birth and display a high affinity uptake mechanism for NA that reaches its adult-like characteristics during the third postnatal week (REFXX Singer Brain Res 1980). The development of functional adrenoceptors follows a similar time course although the presence of noradrenergic fibres is apparently not necessary for the pattern of expression of the receptors (REFXX Singer Brain Res 1980).

In conclusion, our results show for the first time the coexistence of three distinguishable pools of ATP and mechanisms of ATP release from postnatal DH glial cells. In the DH of the spinal cord, this ATP release and its modulation by NA or other neurotransmitters, might play a pivotal role in the modulation of nociceptive signaling.

Compliance with ethical standards

Conflicts of interest The authors declare no conflict of interest

Ethical approval All applicable international, national and institutional guidelines for care and use of animals were followed.

References

Legends to figures

Fig. 1 Effect of noradrenaline (NA) and different adrenoceptor agonists on $[Ca^{2+}]_i$ in cultured fura-2-loaded DH glial cells. **a** Application of NA (20 μ M, 50 s) elicited three types of $[Ca^{2+}]_i$ responses that consisted of a peak and a plateau (type 1), oscillations in $[Ca^{2+}]_i$ (type 2) or a single initial peak (type 3). **b** Effect of different classical adrenoceptor agonists on $[Ca^{2+}]_i$ in DH glial cells. Phenylephrine (PHE 20 μ M, 50 s), an α_1 adrenoceptor agonist, reproduced the effect of NA, whereas isoproterenol (ISO, 10 μ M, 50 s), a β adrenoceptor agonist, or clonidine (CLO 20 μ M, 50 s), an α_2 adrenoceptor agonist, increased $[Ca^{2+}]_i$ in very small fractions of cells. The effects of the adrenoceptor agonists were significantly reduced by classical antagonists of these receptors, i.e. prazosin (Praz, 2 μ M), an α_1 adrenoceptor antagonist, propranolol (Prop, 20 μ M), a β adrenoceptor antagonist and yohimbine (Yoh, 1 μ M), an α_1 adrenoceptor antagonist. The effect of NA was potently reduced by a combination of all three antagonists. Fisher's exact test ****P < 0.0001, ***P < 0.001, *P < 0.05, n.s. not significant. In this and following figures, the ratios of numbers above the columns of the histograms indicate the proportion of responding cells. **c** Occurrence of type 1, 2 and 3 responses among DH glial cells that responded to NA (20 μ M), PHE (20 μ M), CLO (20 μ M) or ISO (10 μ M). The total number of cells sampled is given by "n" and indicated below the histograms for each agonist tested. In this and following figures, the single numbers above the columns of the histograms indicate the number of responding cells within the total sample.

Fig. 2 Characterization of the ATP biosensor detector system. **a** Proportion of HEK293 cells stably transfected with the rat P2X2 subunit (HEK293-P2X2 cells) responding to various concentrations of locally applied ATP. The dose-response relationship of ATP on HEK293-

P2X2 cells (open circles) indicated an apparent EC_{50} of $0.75 \pm 0.02 \mu\text{M}$. When cells were preincubated for 1 h with 200 nM PROG (HEK293-P2X2-PROG, filled circles), the dose-response curve was shifted to the left (EC_{50} of $0.10 \pm 0.01 \mu\text{M}$) reflecting an increased detection capacity at low ATP concentrations. Each point represents mean \pm s.e.m.

($32 \leq n \leq 142$ cells and $3 \leq n \leq 5$ experiments per data point). **b** A low concentration of ATP (200 nM) triggered small amplitude increases in $[\text{Ca}^{2+}]_i$ in a small fraction of HEK293-P2X2 cells. Application of PROG (5 μM) alone did not induce a change in $[\text{Ca}^{2+}]_i$. However, after PROG application, a larger fraction of HEK293-P2X2 displayed $[\text{Ca}^{2+}]_i$ responses following ATP (200 nM) application, indicating an increase in detection capacity of ATP by these cells. **c** The fraction of cells responding to 200 nM ATP was significantly increased after PROG (5 μM) application and persisted for at least 90 minutes after PROG wash-out without significant attenuation. Fisher's exact test, **** $P < 0.00001$, n.s. not significant. n.s. applies to each of the individual time points (30, 60, 90) compared to time point 0. **d** In cells preincubated with a low concentration of PROG (200 nM) for 1 h (HEK293-P2X2-PROG), a low concentration of ATP (200 nM) induced large increases in $[\text{Ca}^{2+}]_i$ that were completely and reversibly blocked by the P2X2 receptor antagonist PPADS (50 μM).

Fig. 3 Detection of ATP release from DH glial cells by HEK293-P2X2-PROG cells and its modulation by NA and PHE. HEK293-P2X2 cells preincubated with PROG (200 nM, 1 h) were plated on top of DH glial cells and served as detectors of ATP released from the glia. Only HEK293-P2X2-PROG cells were loaded with fura-2. **a1** Application of NA (20 μM , 150 s) induced an increase in $[\text{Ca}^{2+}]_i$ in a biosensor HEK293-P2X2-PROG cell. The response consisted of peak-like increases in the fura-2 fluorescence ratio (F_{350}/F_{380}) observed during NA application but also after the end of the application. **a2** Histogram showing the occurrence of fluorescence ratio peaks before, during and after application of NA (20 μM , 150 s). The

histogram was obtained by pooling the observations from 89 different biosensor cells. Note the very small number of peaks before NA application and the increase in the number of peaks during and following NA application. **b1** and **b2** Application of PHE (20 μ M, 150 s) mimicked the effect of NA. Histogram obtained by pooling the observations from 68 biosensor cells. **c** When DH glial cells were preloaded with the calcium chelator BAPTA-AM (10 μ M, 1 h) before adding the HEK293-P2X2-PROG cells on top of them, application of PHE (20 μ M) failed to induce any change in F_{350}/F_{380} fluorescence ratio, indicating that ATP was no longer released from DH glial cells under these conditions. **d** In the steady presence of PPADS (50 μ M) added to the extracellular medium, PHE did not induce changes in $[Ca^{2+}]_i$, demonstrating that the calcium signals induced by PHE were mediated by P2X2 receptors present on the detector HEK293-P2X2-PROG cells. **e** Quantification of ATP release from DH glial cells by calculating the area under the curve (AUC) of the calcium signal measured in the detector HEK293-P2X2-PROG cells. The AUC was determined over a period of 500 s before (baseline, BL) and after the application of PHE for individual cells under different experimental conditions: standard conditions (PHE), when DH glial cells were preloaded with BAPTA (PHE (BAPTA)) and in the steady presence of PPADS in the extracellular medium (PHE + PPADS). Statistical comparisons were made between PHE and corresponding BL for each experimental condition using a paired Student's t-test. **** $P < 0.0001$; n.s. not significant). Values of AUC are expressed in arbitrary units (a.u.). n values are between 68 and 87.

Fig. 4 Application of a low K^+ solution induced calcium-dependent ATP release from DH glial cells. **a** In fura-2 loaded DH glial cells, local application of an extracellular solution in which the concentration of K^+ was lowered to 0.2 mM (low K^+ solution) triggered an increase in $[Ca^{2+}]_i$ that lasted as long as the solution was applied and then rapidly returned to pre-application baseline levels. This effect of the low K^+ solution on $[Ca^{2+}]_i$ could be repeated at

least two times in the same cell. **b** The proportion of DH glial cells displaying an increase in $[Ca^{2+}]_i$ following the application of the low K^+ solution was not affected in the presence of PPADS (50 μ M) in the extracellular medium (88.6%, 39/44, Fisher's exact test, $P > 0.05$ n.s.) but strongly reduced when the glial cells were preloaded with the calcium chelator BAPTA (Fisher's exact test, **** $P < 0.0001$). **c** When fura-2 loaded HEK293-P2X2-PROG cells were plated on top of DH glial cells, application of a low K^+ solution triggered an increase in $[Ca^{2+}]_i$ indicative of ATP release from DH glial cells. **d** The mean AUC of the calcium signals measured in detector HEK293-P2X2-PROG cells following the application of the low K^+ solution was also significantly reduced when P2X2 receptors were blocked in the steady state presence of PPADS (50 μ M) (unpaired Student's t-test, *** $P < 0.001$) or when DH glial cells were preloaded with BAPTA-AM (unpaired Student's t-test, *** $P < 0.001$). Number of cells was between 37 and 131.

Fig. 5 Characterization of vesicular ATP release from DH glial cells and its modulation by PHE and low K^+ . **a** Comparison of calcium responses in detector HEK293-P2X2-PROG cells plated on top of DH cultured glial cells under different experimental conditions: no treatment of glial cells (**a1**), pretreatment of DH glial cell cultures with GPN (100 μ M, 20 min, **a2**) or with bafilomycin A1 (0.2 μ M, 90 min, **a3**). In each situation, we tested the application of PHE (20 μ M), of a low K^+ solution and of ATP (200 nM). Note that GPN or bafilomycin A1 pretreatment abolished ATP release induced by PHE, but that only bafilomycin A1 but not GPN prevented the release of ATP following low K^+ stimulation. In all cases, application of ATP (200 nM) increased $[Ca^{2+}]_i$ in the detector HEK293-P2X2-PROG cells indicating that they were able to detect low concentrations of ATP. **b, c** Quantification of the effects of pretreatment with GPN (**b**) or bafilomycin A1 (**c**) by determination of the area under the curve (AUC) of the recorded calcium signals. The bars indicate mean values and error bars represent s.e.m. Paired Student's

t-test, *** $P < 0.001$, n.s. not significant. Number of cells per bar was $n=80$ for Fig 5b and between 51 and 68 in Fig 5c. BL. Baseline. Values of AUC are expressed in arbitrary units (a.u.).

Fig. 6 Identification of a spontaneous calcium-dependent but non-vesicular release of ATP from cultured DH glial cells. In order to detect low levels of spontaneously occurring ATP release from DH glial cells, the continuous superfusion with extracellular medium (Flow condition) was interrupted (Stop condition) allowing a time-dependent local accumulation of extracellular ATP. **a** Stopping the general and local superfusion triggered a rapid and sustained increase in $[Ca^{2+}]_i$ measured in the HEK293-P2X2-PROG detector cells plated on top of cultured DH glial cells (top trace: Control). Note that the $[Ca^{2+}]_i$ value rapidly returned to baseline values once the superfusion was turned on again (Flow condition). The change in $[Ca^{2+}]_i$ triggered by stopping general superfusion was not observed when the extracellular medium contained PPADS (50 μM) to block P2X2 receptors (PPADS condition) or when DH glial cells were preloaded with the calcium chelator BAPTA-AM (BAPTA in glia condition), but persisted when glial cells were pretreated with bafilomycin A1 to suppress the release of acidic secretory vesicles (Baf. A1 in glia condition). **b** Quantification of the experiments illustrated in **a**. The percentage of cells responding with an increase in $[Ca^{2+}]_i$ when the general superfusion was stopped is displayed as a function the different experimental conditions. The numbers above the columns indicate the proportions. (Fisher's exact test, **** $P < 0.0001$, n.s. not significant)