Purinergic modulation of norepinephrine release and uptake in rat brain cortex: contribution of glial cells

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Pinho D, Quintas C, Sardo F, Cardoso TM, Queiroz G. Purinergic modulation of norepinephrine release and uptake in rat brain cortex: contribution of glial cells. J Neurophysiol 110: 2580–2591, 2013. First published September 11, 2013; doi:10.1152/jn.00708.2012.—The pathogenesis of psychiatric and neurodegenerative diseases is often associated with a deregulation of noradrenergic transmission. Considering the potential involvement of purinergic signaling in the modulation of noradrenergic transmission in the brain cortex, this study aimed to identify the P2Y receptor subtypes involved in the modulation of neuronal release and neuronal/glial uptake of norepinephrine. Electrical stimulation (100 pulses at 5 Hz) of rat cortical slices induced norepinephrine release that was inhibited by ATP and ADP (0.01–1 mM), adenosine 5′-O-(2-thiodiphosphate) (ADPβS, 0.03–0.3 mM), and UDP (0.1–1 mM). The effect of ADPβS was mediated by P2Y1 receptors and possibly by A1/P2Y1 heterodimers since it was attenuated by the A1 receptor antagonist DPCPX and by the P2Y1 receptor antagonist MRS 2578 but was resistant to the effect of adenosine deaminase (ADA). UDP inhibited norepinephrine release through activation of P2Y6 receptors, an effect that was abolished by the P2Y6 receptor antagonist MRS 2578 and by DPCPX, indicating that it depends on the formation and/or release of adenosine and activation of A1 receptors. Supporting this hypothesis, the inhibitory effect of UDP was also prevented by inhibition of ectonucleotidases, by ADA and was attenuated by the inhibitor of nucleoside transporter 6-[4-nitrobenzyl(1-thio)-9-β-D-ribofuranosyl]purine (NBTI). Additionally, the inhibitory effect of UDP was attenuated when norepinephrine uptake 1 or 2 was inhibited. In astroglial cultures, ADPβS and UDP increased norepinephrine uptake mainly by activation of P2Y1, and P2Y6 receptors, respectively. The results indicate that neuronal and glial P2Y1 and P2Y6 receptors may represent new targets of intervention to regulate noradrenergic transmission in CNS diseases.

adeno-sine receptors; P2Y receptors; glial norepinephrine uptake; glia-neuron communication

IN THE CENTRAL NERVOUS SYSTEM (CNS), ATP and other nucleotides are released from neurons (Burnstock 2004) or glial cells in response to neurotransmitters, such as glutamate (Queiroz et al. 1997) or norepinephrine (Gordon et al. 2005), and activate purinergic receptors mediating a bidirectional communication between astrocytes and neurons (Fields and Burnstock 2006).

Norepinephrine has relevant cognitive actions that include the regulation of attention, mood, and motivation (Sara 2009). The release of norepinephrine can be triggered by activation of neurons that project from the locus coeruleus (Kittner et al. 1999) to several brain regions, but it can also be locally induced. For example, in the brain cortex release of norepinephrine can be induced by activation of local NMDA receptors (Fink et al. 1989, 1990, 1992). The purinergic receptors, in particular the P2Y receptors, which include eight subtypes, P2Y1,2,4,6,11,12,13,14 (Abbracchio et al. 2006), have also been shown to play a relevant role in the regulation of synaptic transmission in several brain regions (Gonçalves and Queiroz 2008; Sperlágh et al. 2007), participating in the modulation of neuronal transmitter release. Cortical norepinephrine release evoked by electrical stimulation was shown to be modulated by P2Y receptors (von Kügelgen et al. 1994), but the subtypes of P2Y receptors have not been identified.

The identification of the P2Y receptor subtypes involved in the modulation of synaptic transmission has been difficult to achieve because endogenous nucleotides, the natural agonists of P2Y receptors, like ATP and ADP, are metabolically unstable, being rapidly converted by the ectonucleotidases (Zimmermann 2012) into active metabolites such as adenosine, which also modulates neurotransmission. This problem has been partially overcome by using agonists metabolically more stable and by blocking the A1 receptors, the main adenosine receptors involved in the modulation of transmitter release in the brain (Fredholm et al. 2005). Additionally, several P2Y and adenosine receptors such as P2Y1, P2Y2, P2Y12, P2Y13, A1, and A2A receptors may interact physically, forming heterooligomers between them but also with NTPDase1 (Nakata et al. 2005; Schicker et al. 2009), or may interact functionally (Quintas et al. 2009; Tonazzini et al. 2007), both mechanisms contributing to confound even more the identification of individual P2Y receptor subtypes involved in the regulation of synaptic transmission.

Furthermore, astrocytes that stand on synapses also play an important role in the modulation of synaptic transmission by expressing several transporters that remove neurotransmitters from the synaptic cleft (Yang and Rothstein 2009). Cortical astrocytes express norepinephrine transporters similar to those expressed by neurons (Inazu et al. 2003; Takeda et al. 2002), which contribute to terminate norepinephrine actions, influencing the efficacy (Gordon et al. 2005) and plasticity (Achour and Pascual 2010; Inoue et al. 2007) of synaptic transmission. Several psychoactive drugs exert their effects by blocking norepinephrine transporters (Axelrod et al. 1961; Perry 1996), but the role of P2Y receptors in the regulation of glial norepinephrine transporters and its implication in the regulation of noradrenergic transmission has never been investigated.
The pathogenesis of several mood disorders (Marien et al. 2004; Wiste et al. 2008) and neurodegenerative diseases (Carnevale et al. 2007) is associated with a deregulation of noradrenergic transmission that may be caused by changes in neuronal release or an impairment in the functionality of glial cells (Rajkowska and Miguel-Hidalgo 2007). Considering the potential involvement of purinergic receptors in the modulation of noradrenergic transmission in the brain cortex, we proposed to identify the subtypes of P2Y receptors involved in the modulation of the neuronal release of norepinephrine and neuronal/glial uptake of this neurotransmitter.

MATERIALS AND METHODS

Drugs used. The following drugs were used: levo-[ring-2,5,6-3H]-norepinephrine ([3H]NE), specific activity 1.65 TBq/nmol, was from DuPont NEN (Garal, Lisbon, Portugal); inosine, adenosine, adenosine 5'-diphosphate sodium salt (ADP), adenosine 5'-triphosphate disodium salt (ATP), adenosine 5'-O-(2-thiodiphosphate) trimethyl salt (ADPβS), adenosine deaminase type VI (ADA; EC 3.5.4.4.), t(=)-2-amino-5-phosphonopentanoic acid (t(=)-AP5), N6-cyclopentyladenosine (CPA), 2-p-(2-carboxyethyl) phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride (CSG 21680), corticosterone, 8-cyclopentyl-1,3-diphosphate sodium salt (ADP), adenosine 5'-triphosphate trisodium salt (ATR 67156), desipramine hydrochloride (D(MI), α,β-methyleneadenosine 5'-diphosphate lithium salt (AOPCP), 6-[(4-nitrobenzyl)thio]-9-β-d-ribofuranosylpurine (NB1T), 7-(2-phenylethyl)-5-amino-2-(2-furyl)-pyrazolo-[4,3-e]pyrimidine (SCH 58261), pargyline hydrochloride, tetrodotoxin (TTX), tropolone, uridine 5'-triphosphate trisodium salt (UTP), uridine 5'-diphosphate disodium salt (UDP), and yohimbine hydrochloride were from Sigma (Sintra, Portugal); N,N',N'-4-butenediylbis-N'-(3-isothiocyanatophenyl)thiourea (MRS 2578), 2-(2-chloro-5-nitrophenylazo)-5-hydroxy-6-methyl-3-[phosphonoxy]methyl]-4-pyridinecarboxaldehyde disodium salt (MRS 2211), 2-propyl(adenosine 5'-O-(β,β-difluoromethyl)pyrimidine phosphate tetrasodium salt (AR-C 66096), and (1R,2S),4-[2-iodo-6-(methylamino)-9H-purine-9-yl]-2-(phosphonyloxy) bicyclo[3.1.0]hexane-1-methanol dihydrogen phosphate ester tetraammonium salt (MRS 2500) were from Tocris (Bristol, UK). Stock solutions of drugs were prepared with distilled water or DMSO and kept at -20°C.

Solutions of drugs used in the experiments were prepared from aliquots of the stock solutions that were diluted in buffer immediately before use. The solvent was added to the superfusion medium in parallel control experiments.

Preparation of slices from rat brain cortex. Animal experiments were conducted under a license from RESEARCH PROJECTS/ANIMAL TESTING of the General Directorate of Veterinary Medicine (DGV) of Portugal, proving the existence of conditions for the realization of the project with the reference PTDC/SAU-TOX/115597/2009, which was approved and financed by the Department of Programs and Projects ICDT, Foundation for Science and Technology (FCT) of Portugal. Animal handling and experiments were conducted according to the guidelines of Directive 2010/63/EU of the European Parliament and the Council of the European Union. Adult male Wistar rats (Charles River, Barcelona, Spain) were kept at a constant temperature (21°C) and a regular light (06:30–19:30 h)-dark (19:30–06:30 h) cycle, with food and water ad libitum. Animals were decapitated, and the brain was quickly removed and chilled. Transverse slices were cut from the occipital-parietal cortex. The superficial slice was discarded, and round slices of 400 μm (Microtome Leica VT1000S, Nussloch, Germany) were incubated for 45 min in a warmed (37°C) and gassed (95% O2, 5% CO2) Krebs solution with the following composition (mM): 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 NaH2PO4, 25 NaHCO3, 11 glucose, 0.3 ascorbic acid, and 0.03 sodium EDTA.

Experiments of [3H]norepinephrine release. The procedures used to label the slices with [3H]norepinephrine ([3H]NE) and to estimate changes in electrically evoked tritium overflow as an indicator of neuronal norepinephrine release from cortical brain slices have been previously described in other studies (von Kügelgen et al. 1994). Briefly, occipital-parietal cortical brain slices were incubated in 2 ml of Krebs solution containing 0.1 μM [3H]NE (specific activity of 1.65 TBq/mmol) for 15 min. After the incubation period, the slices were washed for 10 min and transferred to superfusion chambers, where they were held by a polypropylene mesh between platinum electrodes placed 7 mm apart and then superfused with Krebs solution at a constant flow rate of 0.6 ml/min. A stimulator (type 215, Hugo Sachs Elektronik, Hugstetten, Germany), operating in the constant-current mode, was used for electrical field stimulation with square wave pulses (1-ms width; 50-mA current strength; voltage drop of 18 V per chamber). The stimulation periods consisted of 100 pulses at 5 Hz (100 pS/Hz). A primer stimulation period, applied at t = 30 min (S0), t = 0 min being the onset of superfusion, was not used for determination of tritium overflow. The following stimulation periods were applied at t = 60 (S1), t = 90 (S2), t = 120 (S3), and t = 150 (S4) min. In some experiments only S0, S1, and S2 were applied. Superfusate samples were collected at 5-min intervals, from t = 55 min onwards. At the end of the experiments, the tritium remaining in the slices was extracted with 0.2 M NaOH overnight at 4°C and the tritium content of superfusate samples and tissue extracts was determined by scintillation spectrometry (Beckman LS 6500, Beckman Instruments, Fullerton, CA). Agonists were added to the superfusion medium 10 min before S2, S3, and S4 at increasing concentrations and were kept until the end of the respective stimulation period. TTX, receptor antagonists, enzyme inhibitors, and the nucleoside uptake inhibitor NBTI, when present, were added 20 min before S3 and kept until the end of the experiment. Omission of Ca2+ from the medium with or without EDTA was performed 20 min before S3 and kept until the end of the experiment. In some experiments slices were superfused with a Krebs solution without Mg2+ from the beginning to the end of superfusion. In other experiments, DPCPX (the selective antagonist of A1 receptors), yohimbine (a selective antagonist of α2-adrenoceptors), DMI (an uptake 1 inhibitor), and corticosterone (an uptake 2 inhibitor) were present throughout superfusion.

Tritium overflow was estimated as a fraction of the slice tritium content at the onset of the respective collection period (fractional rate of overflow/min): b1 was the fractional rate of overflow in the 5-min period before S1 was the fractional rate of overflow in the 5-min period after S1 and before S2 was the fractional rate of overflow in the 5-min period before S2 was the fractional rate of overflow in the 5-min period before S3 and S4 were kept respectively. Drug effects on basal overflow were evaluated as ratios b0/b1. Tritium overflow evoked by electrical stimulation was estimated by subtracting basal overflow from total overflow observed during and in the 10-min period subsequent to each stimulation period, being expressed as percentage of the total tritium present in the slice at the onset of the respective stimulation period. Effects of drugs added after S1 were evaluated as ratios of the overflow elicited by S0, S1, S2, S3, and S4 and the overflow elicited by S1 (S2/S1). Drug effects on basal tritium overflow or evoked overflow were expressed as percentage of the mean ratio obtained in the respective control.

Separation and quantification of endogenous adenosine nucleosides. A high-performance liquid chromatography-diode array detector (HPLC-DAD) analytical method was used for detection and quantification of endogenous adenosine nucleosides (adenosine plus inosine) in the supernatants of astroglial cultures or in the samples collected from the superfusion of cortical brain slices. Quantitative analyses were performed on Finigan Thermo Fisher Scientific System LC/DAD, equipped with Accela Pump, Accela Autosampler, and diode array detector (Accela PDA). X-Calibur software chromatography manager (Thermo Fisher Scientific) was used to pilot the LC/DAD instrument and to process data (area integration,
calculation, plotting of chromatograms and UV spectra) throughout the method validation and sample analysis. A Hypersil GOLD C18 column (5 μm, 2.1 mm × 150 mm) with a Hypersil GOLD C18 guard column (5 μm, 2.1 mm × 1 mm) was used.

The separation was carried out with an elution gradient composed of ammonium acetate (5 mM, pH 6, adjusted with acetic acid) and methanol. During the procedure, the flow rate was 200 μl/min and the column temperature was maintained at 20°C. The auto sampler was set at 4°C, and 50 μl of standard or sample solution was injected, in duplicate, for each HPLC analysis. To obtain chromatograms and quantitative analysis with maximal sensibility, the diode array detection wavelength was set at 259 nm for adenosine and 248 nm for inosine.

Preparation of astroglial cultures. Primary astroglial cultures were prepared from cerebral hemispheres of newborn (P0–P2) Wistar rats as previously described (Queiroz et al. 1997), with minor modifications. Briefly, the brains were placed in ice-cold phosphate-buffered saline containing 0.2% glucose. The hemispheres were freed from meninges and blood vessels. After being washed twice with ice-cold phosphate-buffered saline, the hemispheres were cut into small pieces in culture medium, i.e., DMEM containing 1 g/l glucose buffered with 10 mMNaOH and 10 HEPES, and 1 mM pyruvate with ascorbic acid, adjusted to a pH of 7.4, containing 5% FBS and supplemented with 10% fetal bovine serum and 50 U/ml of penicillin plus 50 μg/ml of streptomycin. Tissue from two to four hemispheres was then dissociated by trituration in 10 ml of culture medium. The cell suspension was passed through a 40-μm pore nylon mesh and centrifuged at 200 g for 5 min. The pellet was suspended in medium, and the cell suspension was centrifuged again at 200 g for 5 min. Suspension and centrifugation were repeated once more, and the final pellet was suspended in 1 ml of medium. The number of cells was determined and adjusted to a density of 2 × 10^5 cells/ml. Five hundred microliters of this cell suspension was seeded in each well of a 24-well culture plate.

The cultures were incubated at 37°C in a humidified atmosphere of 95% air-5% CO₂. The culture medium was replaced 1 day after preparation and subsequently twice a week. Confluent 2-wk-old cultures were used in the experiments to evaluate the uptake of [³H]NE. These cultures were characterized by immunocytochemistry and contained ~90% astrocytes (Quintas et al. 2011).

Experiments on [³H]norepinephrine uptake. The uptake of [³H]NE by primary glial cultures and cortical brain slices was evaluated as previously described (Bönisch 1984), with minor modifications. Cells in culture were rinsed twice with physiological salt solution at 37°C, called the uptake buffer, with the following composition (mM): 125 NaCl, 5 KCl, 1.8 CaCl₂, 0.8 MgSO₄, 10 glucose, 10 HEPES, and 1 Na-pyruvate with ascorbic acid, adjusted to a pH of 7.4, containing 0.1 mM pargyline to inhibit monoamine oxidase and 0.1 mM tropolon to inhibit catechol-O-methyl transferase. The cells in each well were incubated with the uptake buffer for 45 min at 37°C in an atmosphere of 95% air-5% CO₂. After this period, the uptake buffer was discharged, being replaced by fresh buffer containing the agonists that were added for 15 min, followed by the addition of 30 nM [³H]NE for a further 15 min. When present, antagonists were added 30 min before agonists. Tritium uptake by glial cells was terminated by removing the buffer followed by three rapid washes with ice-cold buffer, and then cells were harvested with 0.1 M NaOH. The tritium taken up by the cells was quantified by scintillation spectrometry (Beckman LS 6500, Beckman Instruments). The specific [³H]NE uptake was calculated by subtracting the total uptake from the unspecific uptake evaluated in the presence of 0.3 mM DMI and normalized by the protein content determined by the Bradford method.

In experiments designed to evaluate the influence of UDP on [³H]NE uptake by brain slices, each slice was incubated in a warmed (37°C) and gassed (95% O₂-5% CO₂) Krebs solution for 20 min and then incubated with solvent or UDP for 10 min, followed by incubation with 10 nM [³H]NE for an additional 15 min. When tested, antagonists were added 20 min before the agonist. Tritium uptake was stopped by washing the slices several times with ice-cold Krebs solution. Tritum taken up by slices was extracted by incubation with 0.2 M NaOH overnight at 4°C and quantified by scintillation spectrometry. Tritium uptake by each slice was normalized by its weight. Drug effects on [³H]NE uptake in cultured glial cells and cortical brain slices were expressed as percentage of respective control.

Statistical analysis. Data are expressed as means ± SE from n experiments. Statistical analysis of the effect of drugs on basal tritium overflow and evoked tritium overflow was carried out with the unpaired Student’s t-test or one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. P values < 0.05 were considered to indicate significant differences.

RESULTS

Characterization of electrically evoked tritium overflow from cortical brain slices. In rat brain slices, the fractional release of basal tritium overflow before S₁ (b₁) was 0.283 ± 0.006 Bq/min and the evoked overflow by trains of 100 p/s Hz (S₂) was 1.579 ± 0.063% of total tritium content, corresponding to 106.1 ± 4.6 Bq (n = 145). In the absence of any drugs added after S₁, the ratio b₂/b₁ was 0.95 ± 0.01 and the ratio S₂/S₁ was 0.98 ± 0.01 (n = 145). When five periods of electrical stimulation were applied (S₀–S₄) basal tritium overflow and tritium overflow remained constant throughout superfusion, with b₂/b₁ and S₂/S₁ close to unity (not shown). The drugs used to block norepinephrine uptake 1 and 2, DMI (1 μM) and corticosterone (10 μM), respectively, the selective A₁ receptor antagonist DPCPX (0.1 μM), and the P₂ receptor antagonists or the enzyme inhibitors tested did not change the basal tritium outflow or the evoked overflow from cortical brain slices, except for the selective α₂-antagonist yohimbine (3 μM), which increased tritium overflow by 297 ± 27% (n = 4; P < 0.05), indicating the presence of a high inhibitory tonus mediated by α₂-adrenoceptors. The superfusion of slices with Ca²⁺-free buffer and the blockade of voltage-sensitive Na⁺ channels with TTX (0.3 μM) abolished tritium overflow (Fig. 1A). Stimulation of brain slices with trains of 100 p/s Hz has been shown to increase glutamate release (Di Iorio et al. 1996; Savage et al. 2001), which, acting on ionotropic glutamate receptors mainly of the NMDA subtype, may contribute to norepinephrine release (Fink et al. 1989, 1992). However, under the experimental conditions used, no contribution of ionotropic glutamate receptors could be detected, since neither the AMPA and kainate receptor antagonist DNQX (10 μM) nor the selective NMDA receptor antagonist d-AP5 (100 μM) changed tritium overflow (Fig. 1A). In the absence of Mg²⁺, tritium overflow evoked by 100 p/s Hz increased to 191.2 ± 35.1 Bq (n = 20; P < 0.01). This effect was Ca²⁺ dependent, since it was attenuated by Ca²⁺ withdrawal and was abolished with further addition of 1 mM EDTA (Fig. 1B).

In Mg²⁺-free conditions tritium overflow was not changed by DNQX (10 μM) but was attenuated by the selective NMDA receptor antagonist d-AP5 (100 μM, Fig. 1B). The results indicate that, in the presence of physiological concentrations of Mg²⁺, the ionotropic glutamate receptors do not contribute to tritium overflow evoked by 100 p/s Hz.

Effects of nucleotides in [³H]NE release. The effect of nucleotides with different selectivity for P₂Y receptor subtypes, UTP, which preferentially activates P₂Y, receptor subtypes, UTP, which preferentially activates P₂Y₂ receptor subtypes, and UDP, which is selective for the P₂Y₆ receptors (Abbracchio et al. 2006).
Electrical stimulation with trains of 100 p/5 Hz caused an overflow of tritium that was inhibited in a concentration-dependent manner by the adenine nucleotides ATP (0.01–1 mM), ADP (0.01–1 mM), and its more stable analog ADPβS (0.03–0.3 mM; Fig. 2A). The higher potency of the endogenous nucleotide ADP compared with ADPβS can be explained by the higher susceptibility of ADP to metabolism by ectonucleotidases and its conversion into adenosine that activates A1 receptors and inhibits norepinephrine release (von Kügelgen et al. 1994). Since ADPβS is less prone to metabolism, its effect results mainly from activation of P2Y receptors. The uracil nucleotides UTP and UDP also inhibited tritium overflow, but the effects were of lower magnitude and were only observed at very high concentrations (0.1–1 mM; Fig. 2B).

**P2Y receptor subtypes activated by adenine nucleotides.** The effects of ADP and its stable analog ADPβS were characterized in more detail, to verify which of the P2Y receptor subtypes were involved in the inhibition of tritium overflow caused by these adenine nucleotides.

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**Fig. 1.** Characterization of tritium overflow evoked by electrical stimulation from cortical brain slices and the contribution of ionotropic glutamate receptors in the presence (A) and in the absence (B) of Mg2+. Slices were electrically stimulated with 3 trains of 100 pulses at 5 Hz (EFS) (S0–S2). Ca2+ was omitted from the superfusion medium 20 min before S2 until the end of superfusion. TTX, EDTA, and the glutamate receptor antagonists DNQX and D-AP5 were added to the superfusion medium 20 min before S2 and kept until the end of the experiment. y-Axes, tritium overflow expressed as % respective control. Values are means ± SE from 5 experiments. Significant differences: *P < 0.05 from control (solvent); †P < 0.05 from the effect observed in the absence of Ca2+ alone.

**Fig. 2.** Effect of adenine nucleotides (A) and uracil nucleotides (B) on tritium overflow from cortical brain slices electrically stimulated with 5 trains of 100 pulses at 5 Hz (S0–S4). The nucleotides were added 10 min before S0 (S0–S4) at increasing concentrations and kept until the end of the respective stimulation period. y-Axes, tritium overflow expressed as % respective control; x-axes, concentration of nucleotides. Values are means ± SE from 6 experiments. Significant differences: *P < 0.05 from control (solvent); †P < 0.05 from the effect of ADP. ADPβS, adenosine 5′-O-(2-thiodiphosphate).
The inhibitory effect caused by ADPβS (0.3 mM) was not influenced by ADA (2 U/ml), the enzyme that metabolizes adenosine, or by the NTPDase inhibitor AR-L 67156 (0.1 mM), but was attenuated by the selective A1 receptor antagonist DPCPX (0.1 μM) and by the selective P2Y1 receptor antagonist MRS 2500 (1 μM), being abolished by the combination of DPCPX plus MRS 2500 (Fig. 3A). ADPβS may also activate the P2Y12 and P2Y13 receptor subtypes, but neither the selective P2Y12 receptor antagonist AR-C 66096 (10 μM) nor the selective P2Y13 receptor antagonist MRS 2211 (10 μM) changed its effect (Fig. 3A). Similar results were obtained when ADP (0.3 mM) was tested (Fig. 3B). Like ADPβS, it caused an inhibition of tritium overflow that was attenuated by DPCPX (0.1 μM) and by MRS 2500 (1 μM) and was abolished by the combination of DPCPX plus MRS 2500 (Fig. 3B). Unlike the effect of ADPβS (0.3 mM), the effect of ADP (0.3 mM) was attenuated by ADA (2 U/ml; Fig. 3B). The participation of A1 receptors in the inhibition of tritium overflow was confirmed by the effect of the selective A1 receptor agonist CPA (0.001–10 μM), which caused a concentration-dependent inhibition up to 49 ± 3% (n = 7; P < 0.05), an effect that was prevented by DPCPX (0.1 μM) to 90 ± 1% (n = 4; P < 0.05). These results indicate that despite the contribution of P2Y1 receptors the A1 receptors are also involved in the inhibition of tritium overflow mediated by ADPβS and ADP. However, the contribution of A1 receptors to the effect of ADPβS seems to be independent of adenosine formation.

To explain the participation of A1 receptors in the inhibitory effect of ADPβS, we investigated whether an interaction between A1 and P2Y1 receptors could contribute. CPA (10 μM) and ADPβS (0.3 mM) caused an inhibition of tritium overflow of similar magnitude, 56 ± 2% (n = 5; P < 0.05) and 62 ± 3% (n = 5; P < 0.05), respectively. When both agonists were tested simultaneously, the effect was similar to that observed with each agonist alone, 65 ± 3% (n = 5). The absence of additive effects may suggest that A1 and P2Y1 receptors form A1/P2Y1 heterooligomers that are being maximally activated by both agonists or may be explained by the occurrence of a functional interaction between A1 and P2Y1 receptors at the level of intracellular signaling pathway. Inhibitory G protein-coupled receptors commonly interact when they share the same intracellular signaling pathways, which may occur under these experimental conditions since the α2-adrenoceptors are active. Considering this phenomenon, tonic activation of α2-adrenoceptors could be masking the additive effects of additional inhibitory receptors. Therefore, the effect of CPA plus ADPβS was tested in the presence of the α2-antagonist yohimbine (3 μM), but again there was no additive effect; CPA (10 μM) plus ADPβS (0.3 mM) inhibited tritium overflow to 64 ± 3% (n = 5).

P2Y receptor subtypes activated by uracil nucleotides. Despite the higher affinity of UTP for P2Y2,4 receptor subtypes, it caused a very small inhibition of tritium overflow at a concentration of 1 mM that was abolished by the NTPDase inhibitor AR-L 67156 (0.1 mM) and by the selective antagonist of P2Y6 receptors MRS 2578 (1 μM), indicating that the effect of this uracil nucleotide depends on its conversion into UDP and activation of P2Y6 receptors (Fig. 4A). Interestingly, the effect of UTP (1 mM) was also attenuated by DPCPX (0.1 μM; Fig. 4A). Corroborating the results obtained with UTP, the UDP effect was also prevented by MRS 2578 (1 μM) and DPCPX (0.1 μM; Fig. 4B). Since the formation of adenosine from UDP metabolism is highly unlikely, and the contamination of UDP...
with adenosine has been excluded by HPLC-UV analysis, these findings suggest that activation of P2Y$_6$ receptors may cause the production and/or release of adenosine that activates A$_1$ receptors. This hypothesis is supported by the effect of several compounds that may reduce adenosine accumulation. The effect of UDP (1 mM) was abolished by the inhibitor of NTPDases ARL 67156 (0.1 mM), by the 5’-nucleotidase (5’-NT) inhibitor AOPCP (0.1 mM), and by the enzyme that metabolizes adenosine, ADA (2 U/ml; Fig. 4B). Additionally, it was also attenuated by the inhibitor of equilibrative adenosine transporters NBTL (10 µM; Fig. 4B). To further confirm the role of adenosine in the inhibitory effects mediated by the P2Y$_6$ receptors, the concentration of adenosine present in the supernatants of primary astroglial cultures and in superfusate samples collected during the superfusion of cortical brain slices, treated with UDP or solvent, was evaluated by HPLC-DAD. Chromatograms of samples treated with UDP revealed the presence of a very small peak, whose retention time and UV spectra were coincident with those of the adenosine standard; however, it was not possible to make a reliable quantification of the adenosine present in the samples because its levels were below the lower limit of quantification of the method. This peak was absent in chromatograms of samples treated with solvent.

Modulation of [3H]NE uptake by purinergic receptors. Despite the presynaptic modulation of transmitter release, noradrenergic transmission may also be regulated by mechanisms involved in the removal of norepinephrine from the synaptic cleft. Neuronal uptake of norepinephrine is clearly established and has been widely demonstrated (Schömig et al. 1989; Vizi et al. 2004). Astrocytes also express norepinephrine transporters (Inazu et al. 2003; Russ et al. 1996), as well as several subtypes of purinergic receptors that may participate in the regulation of glial uptake of norepinephrine, but their contribution was never investigated.

To evaluate whether the subtypes of purinergic receptors that were involved in the modulation of tritium overflow were also involved in the modulation of its uptake, we investigated the effect of ADP$_{B}$S and UDP on norepinephrine release evoked by 100 µS Hz in the presence of the uptake 1 and 2 inhibitors DMI and corticosterone, respectively. The influence of ADP$_{B}$S, UDP, and the selective agonists of A$_1$ and A$_{2A}$ receptors CPA and CGS 21680, respectively, on the uptake of norepinephrine by primary astroglial cultures prepared from the brain cortex of newborn rats was also evaluated.

ADP$_{B}$S and UDP inhibited tritium overflow; however, the effect of ADP$_{B}$S was not changed by the norepinephrine uptake inhibitors DMI (1 µM) and corticosterone (10 µM; Fig. 5A) whereas the effect of UDP was attenuated by both uptake inhibitors (Fig. 5B).

In primary astroglial cultures, ADP$_{B}$S (Fig. 6A) increased [3H]NE uptake, an effect that was not modified by the P2Y$_{13}$ receptor antagonist MRS 2211 but was attenuated by the P2Y$_1$ receptor antagonist MRS 2500 and to a lesser extent by the P2Y$_{12}$ receptor antagonist AR-C 66096 (Fig. 6A). CPA (10 µM), which inhibited tritium overflow to 49 ± 3% (n = 7, P < 0.05), did not change glial norepinephrine uptake (not shown), but the selective A$_{2A}$ agonist CGS 21680 (0.1 µM), despite not changing tritium overflow from cortical brain slices (98 ± 6%, n = 3; see von Kügelgen et al. 1994), increased [3H]NE uptake by astroglial cultures (Fig. 6B). The effect of CGS 21680 (0.1 µM) was not modified by the A$_1$ receptor antagonist DPCPX but was abolished by the selective A$_{2A}$ receptor antagonist SCH 58261 (Fig. 6B). UDP increased

Fig. 4. Pharmacological characterization of purinergic receptors activated by UTP (A) and UDP (B) involved in the modulation of tritium overflow from cortical brain slices electrically stimulated with 3 trains of 100 pulses at 5 Hz (S$_1$–S$_2$). UTP and UDP were added 10 min before S$_2$ and kept until the end of the respective stimulation period. The selective A$_1$ receptor antagonist DPCPX, the selective P2Y$_6$ receptor antagonist MRS 2578, the NTPDase inhibitor ARL 67156, the 5’-nucleotidase (5’-NT) inhibitor AOPCP, the adenosine-metabolizing enzyme ADA, and the equilibrative adenosine transporter inhibitor NBTL were all added 20 min before S$_2$ and kept until the end of the experiment; γ-Axes, tritium overflow expressed as % respective control. Values are means ± SE from 6–14 experiments. Significant differences: *P < 0.05 from control (solvent); †P < 0.05 from the effect of UTP or UDP alone.
In previous studies, it was shown that stimulation of brain tissue with trains of 100 p/5 Hz triggers the release of glutamate (Di Iorio et al. 1996; Savage et al. 2001) and that in rat brain cortex activation of ionotropic glutamate receptors, mostly of NMDA receptor subtypes, evokes the release of norepinephrine (Fink et al. 1992). The results obtained did not support any contribution of ionotropic glutamate receptors to the release of norepinephrine evoked by 100 p/5 Hz, since neither the antagonist of AMPA and kainate receptors DNQX (Sheardown et al. 1990) nor the selective antagonist of NMDA receptors d-AP5 (Evans et al. 1982) changed tritium overflow. A contribution of NMDA receptors was only detected when cortical brain slices were superfused with medium without Mg^{2+}, indicating that this pattern of stimulation is insufficient to prevent the voltage-dependent blockade of NMDA receptors caused by physiological concentrations of Mg^{2+} (Nowak and Wright 1992).

The involvement of adenosine receptors, namely, the A<sub>1</sub> and A<sub>2A</sub> subtypes, in the modulation of transmitter release has been known for a long time and has been described in several brain regions (Fredholm et al. 2005; Sebastião and Ribeiro 2000). However, the identity of the P2Y receptor subtypes that modulate transmitter release in the brain is just beginning to be unveiled.

In this study, all adenine (ATP, ADP, and ADPβS) and uracil (UTP and UDP) nucleotides tested inhibited norepinephrine release. Most of these nucleotides are metabolically un-

**Discussion**

In the present study we investigated the contribution of adenosine and P2Y receptors to the modulation of two important mechanisms involved in the regulation of noradrenergic transmission: 1) the neuronal release of norepinephrine evoked by 100 p/5 Hz and 2) the norepinephrine uptake by cortical brain slices and cultured astrocytes.

The contribution of purinergic receptors to the modulation of neuronal release of norepinephrine evoked by electrical stimulation was evaluated in slices incubated with [³H]NE, with tritium overflow as an indicator of the action potential-evoked release of endogenous norepinephrine (Starke et al. 1989). To individualize the effects mediated by nucleotides acting on P2Y receptors from those mediated by adenosine, we evaluated the influence of inhibitors of the NTPDases and 5'-NT, which are the main enzymes involved in the metabolism of nucleotides (Fields and Burnstock 2006).

The experiments designed to characterize the release of norepinephrine evoked by trains of 100 p/5 Hz showed that it was dependent on the propagation of action potentials and on the exocytosis of vesicles containing the neurotransmitter, as indicated by the inhibition of tritium overflow caused by TTX and Ca^{2+} withdrawal from the medium, respectively. The release of norepinephrine was under a tonic influence of α<sub>2</sub>-autoreceptors, since tritium overflow increased in the presence of the α<sub>2</sub>-adrenoceptor antagonist yohimbine.

![Figure 5](http://jn.physiology.org/)

**Fig. 5.** Effect of ADPβS (A) and UDP (B) on the modulation of tritium overflow from cortical brain slices electrically stimulated with 3 trains of 100 pulses at 5 Hz (S<sub>0</sub>–S<sub>2</sub>) in the absence and in the presence of the norepinephrine uptake 1 and 2 inhibitors desipramine (DMI) and corticosterone, respectively. UDP and ADPβS were added 10 min before S<sub>0</sub> and kept until the end of the respective stimulation period, whereas the uptake inhibitors were present throughout superfusion. γ-Axis, tritium overflow expressed as % respective control. Values are means ± SE. Significant differences: *P < 0.05 from control (solvent); + P < 0.05 from the effect of UDP alone.

[³H]NE uptake, in a concentration-dependent manner, in astroglial cultures (Fig. 7A) and in cortical brain slices (Fig. 7B), an effect that was antagonized by the selective P2Y<sub>6</sub> receptor antagonist MRS 2578 (Fig. 7, A and B).

**Fig. 6.** Effect of ADPβS (A) and the A<sub>2A</sub> receptor agonist CGS 21680 (B) on the uptake of [³H]NE by cultured glial cells. Cell cultures prepared from cortex of newborn rats were treated with ADPβS or CGS 21680 for 15 min and incubated with 30 nM [³H]NE for an additional 15 min. The selective antagonists of P2Y<sub>1</sub>, P2Y<sub>12</sub>, and P2Y<sub>13</sub> receptors MRS 2500, AR-C 66096, and MRS 2211, respectively, and the selective antagonists of A<sub>1</sub> and A<sub>2A</sub> receptors DPCPX and SCH 58261, respectively, were added to the incubation medium 30 min before the agonists and were kept until the end of the experiment. γ-Axis, uptake of [³H]NE expressed as % respective control. Values are means ± SE from 5 experiments with different culture preparations. Significant differences: *P < 0.05 from control (solvent); + P < 0.05 from the effect of ADPβS or CGS 21680 alone.
stable, and adenine nucleotides may be degraded into adenosine, which activates the main inhibitory receptors in the brain, the A1 receptors (Cunha et al. 1998; Fredholm et al. 2005). These receptors have been shown to inhibit norepinephrine release in rat cortical brain slices (von Kügelgen et al. 1994), an effect that is corroborated in this study by the effect of the selective A1 receptor agonist CPA.

To overcome adenosine interference and clarify the identity of the P2Y receptor subtypes involved in the inhibition of norepinephrine release mediated by adenine nucleotides, ADPβS, which is metabolically more stable (Gendaszewska-Darmach et al. 2003) and less susceptible to produce adenosine, was tested in parallel with ADP, its naturally occurring analog. However, the A1 receptors still partially contributed to the effect of both agonists since it was attenuated by the selective antagonist of A1 receptors DPCPX (Lohse et al. 1987) and in the case of ADP it was also attenuated by ADA, which metabolizes adenosine into its inactive metabolite inosine. P2Y receptors also contributed to the inhibitory effect of ADPβS and ADP, which are potent agonists at the P2Y1,12,13 receptor subtypes, but only the P2Y1 receptor contributed to their inhibitory effects on norepinephrine release, a conclusion supported by the antagonism caused by the selective P2Y1 receptor antagonist MRS 2500 (Hechler et al. 2006). Neither the selective P2Y1,12,13 receptor antagonist AR-C 66096 (Ingall et al. 1999) nor the selective P2Y1,13 receptor antagonist MRS 2211 (Jacobson et al. 2009) changed the effect of ADPβS.

In the peripheral sympathetic nerve terminals, P2Y1 and P2Y12 receptors are the main P2Y receptor subtypes involved in the modulation of norepinephrine release, where they may operate as autoreceptors (Lechner et al. 2004; Quintas et al. 2009). In the CNS, the P2Y1, P2Y12, and/or P2Y13 subtypes have been shown to modulate the release of norepinephrine from slices of hippocampus (Csóllé et al. 2008) and spinal cord (Heinrich et al. 2008) and the P2Y1 receptors also inhibited the release of glutamate from hippocampal synaptosomes (Roddigues et al. 2005). The results obtained in this study indicate that P2Y1 receptors are also involved in the modulation of norepinephrine release from cortical brain slices and any contribution of these receptors to this effect cannot be attributed to a modulation of NMDA receptor channels, as previously described (Luthardt et al. 2003), since NMDA receptors did not contribute to the release of norepinephrine evoked by the stimulation conditions used (100 pS/Hz).

Interestingly, the effect of ADPβS was attenuated by DPCPX, supporting the involvement of A1 receptors, but was not changed by inhibition of NTPDases and was resistant to ADA, suggesting that this component of the ADPβS effect is independent of the formation of metabolites that activate A1 receptors, such as AMP (Rittiner et al. 2012) or adenosine. An interaction between A1 and P2Y1 receptors may explain the attenuation of the ADPβS inhibitory effect by DPCPX. This interaction may result from the formation of A1/P2Y1 heterooligomers (Nakata et al. 2005; Schicker et al. 2009), which combine pharmacological properties of the A1 and P2Y1 receptors and have been shown to be expressed in the rat brain cortex, hippocampus, and cerebellum (Nakata et al. 2005). In functional assays, activation of A1/P2Y1 heterodimers by CPA or ADPβS caused an inhibition of adenylyl cyclase activity, which was prevented by the A1 receptor antagonist DPCPX but not by the P2Y1 receptor antagonist MRS 2179 (Yoshioka and Nakata 2004). Therefore, it is possible that part of the inhibitory effect of ADPβS that is blocked by DPCPX may be mediated by A1/P2Y1 heterooligomers, whereas the P2Y1 receptors mediate the inhibitory component that is antagonized by MRS 2500. Another possibility is that the A1 and P2Y1 receptors interact at a functional level by sharing the same or a common step of the intracellular signaling pathway they activate. This type of interaction has been frequently demonstrated in several tissues (Quintas et al. 2009; Schlicker and Göthert 1998). The A1 (Munshi et al. 1991) and P2Y1 (Filippov et al. 2000) receptors may both couple to Gi/o proteins that activate the same intracellular signaling pathway, thus preventing the observation of additive effects when these receptors are

**Fig. 7. Effect of UDP on the uptake of [3H]NE by cultured glial cells (A) and by cortical brain slices (B).** Glial cultures were incubated with UDP for 15 min and with 30 nM [3H]NE for an additional 15 min. Slices of rat brain cortex were incubated with UDP for 10 min and with 10 nM [3H]NE for an additional 15 min. The selective P2Y1 receptor antagonist MRS 2578 was added to the incubation medium 20 min before the agonists and kept until the end of the experiment. *P < 0.05 from the effect of UDP alone.
activated at the same time by high concentrations of the respective agonists, CPA and ADPβS.

The uracil nucleotides, UTP and UDP, have different selectivity for P2Y receptors: UTP activates mainly P2Y<sub>2,4</sub> subtypes, having low affinity for P2Y<sub>6</sub> receptors, whereas UDP is selective for P2Y<sub>6</sub> receptors (von Kügelgen 2006). However, both uracil nucleotides inhibited norepinephrine release by activation of P2Y<sub>6</sub> receptors, as indicated by the antagonism of the selective P2Y<sub>6</sub> receptor antagonist MRS 2578 (Mamedova et al. 2004). This result is supported by the attenuation of UTP's effect when NTPDases were inhibited with ARL 67156, indicating that UTP needs to be metabolized into UDP to inhibit norepinephrine release. The effects of UTP and UDP were also prevented by the A<sub>1</sub> receptor antagonist DPCPX, suggesting that these inhibitory effects may be indirect, involving the formation and/or release of endogenous adenosine and activation of neuronal A<sub>1</sub> receptors. This hypothesis is supported by the following results: the inhibitor of NTPDases ARL 67156, which prevents the metabolism of nucleotides, the inhibitor of 5'-NT AOPCP, which prevents the conversion of AMP into adenosine, and ADA, which metabolizes adenosine, all prevented the inhibitory effect of UDP. Moreover, blocking the equilibrative adenosine transporters with NBPT also attenuated the inhibitory effect of UDP. Although adenosine has been detected in the supernatants of astroglial cultures and in the superfuse samples of cortical slices treated with UDP, it was not possible to accurately quantify it. Adenosine has been described to have a rapid modulatory role, and consequently changes in its concentration were shown to be very transient and very variable depending on the brain region stimulated. In the cortex, adenosine has a half-life of 1 s (Pajski and Venton 2013), a factor that may contribute to prevent its accumulation and quantification.

In previous studies it was shown that UTP failed to activate locus coeruleus neurons (Khakhpay et al. 2010), but this does not exclude the possibility that receptors activated by this nucleotide or its active metabolite UDP may modulate norepinephrine release from the nerve terminals of noradrenergic neurons that project from locus coeruleus to the brain cortex. Other studies reported that P2Y<sub>6</sub> receptors may stimulate norepinephrine release from cultured sympathetic neurons; however, this effect was dependent on action potential propagation, suggesting that the P2Y<sub>6</sub> receptors involved were localized at the cell body (Vartian et al. 2001). Our study strongly suggests that P2Y<sub>6</sub> receptors contribute indirectly to the modulation of norepinephrine release from rat brain slices. Since it is unlikely that UDP may be converted into adenosine or activate A<sub>1</sub> receptors, the most likely explanation for the indirect effects of UDP is based on a dialogue between glial cells and noradrenergic neurons. The expression of P2Y<sub>6</sub> receptors by the noradrenergic nerve terminals present in the cerebral cortex is unknown, but these receptors are highly expressed in the rat cerebral cortex (Bennett et al. 2003), mostly in astrocytes and microglia (Bianco et al. 2005; Di Virgilio et al. 2009), and when activated may modify the neuronal response by stimulating the release of messengers (Carnevale et al. 2007). On the basis of the results obtained, the following mechanism is proposed to explain this dialogue: activation of P2Y<sub>6</sub> receptors expressed by glial cells may lead to the release of adenosine or ATP (Abdipranoto et al. 2003; Harden and Lazarowski 1999) that can be converted by NTPDases and 5'-NT into adenosine. The adenosine formed or released by glial cells upon activation of P2Y<sub>6</sub> receptors may activate A<sub>1</sub> receptors present on noradrenergic nerve terminals, causing the inhibition of transmitter release (Fig. 8). Alternatively, P2Y<sub>6</sub> receptors may also inhibit norepinephrine accumulation by another mechanism involving the regulation of neuronal norepinephrine uptake.

Although astroglial cultures have some limitations as a representative functional model of astrocytes in situ, which...
implies some caution in the analysis of the results when using this model (Kimelberg et al. 2000), many properties and relevant discoveries concerning the functions of astrocytes were first made in cultured astrocytes. Astrocytes may participate in the presynaptic modulation of norepinephrine release by releasing nucleotides (Abdipranoto et al. 2003; Queiroz et al. 1997) that activate purinergic receptors present in nerve terminals (Gordon et al. 2005; Zhang et al. 2003), but they also can play an important role in regulating noradrenergic transmission through the removal of this neurotransmitter from the synaptic cleft by specific transporters (Russ et al. 1996; Schömgig et al. 1998), some with properties similar to those expressed by neurons (Inazu et al. 2003; Yang and Rothstein 2009). In the hippocampus it has been shown that neuronal norepinephrine transporters are regulated by P2X receptors that trigger the release of norepinephrine by causing the inversion of the transporter function (Papp et al. 2004). Astrocytes express several purinergic receptors (Abbracchio et al. 2009) that modulate glutamate (Lo et al. 2008; Zeng et al. 2009) and GABA (Cristóvão-Ferreira et al. 2011) transporters, but the influence of purinergic receptors in the modulation of norepinephrine transport by astrocytes has never been investigated.

The influence of adenosine and P2Y receptors, previously identified to modulate norepinephrine release from rat brain cortex, was studied in the uptake of [3H]NE by primary cortical astrocytes and brain slices. The results obtained indicated that the P2Y12 and A2A receptors that did not change the norepinephrine release increased its uptake by astroglial cultures, whereas the A1 receptors, which inhibited the norepinephrine release, did not change its uptake by astrocytes. The P2Y1 receptors and the P2Y6 receptors increased the uptake of norepinephrine, an effect that in the case of P2Y6 receptors was concentration dependent, both in astroglial cultures and in cortical brain slices. Furthermore, inhibition of the uptake 1 or uptake 2 in cortical brain slices, with DMI and corticosterone, respectively, attenuated the inhibitory effect of UDP on norepinephrine release, but not that caused by ADPβS. These findings support an important role of P2Y6 receptors in the modulation of both neuronal and glial uptake of norepinephrine and suggest that uptake induced by P2Y6 receptors located at perisynaptic glia contributes to the overall effects of uracil nucleotides on noradrenergic transmission. The P2Y1, P2Y12, and A2A receptors modulate norepinephrine uptake by glial cells that may be located in astrocyte processes distant from the presynaptic noradrenergic terminals, therefore having a less influence on the amount of norepinephrine that is released into the synaptic cleft (Reichenbach et al. 2010).

Norepinephrine is involved in the regulation of cognitive and behavioral functions, and deficits in the noradrenergic system have been described in neurodegenerative disorders such as Alzheimer and Parkinson diseases (Marien et al. 2004) as well as in psychiatric disorders such as depression and mood disorders (Ressler and Nemeroff 1999).

This study shows that in rat brain cortex several purinergic receptors, acting by different mechanisms and in different cells, may play an important role in the modulation of noradrenergic transmission (Fig. 8). The A1 receptors and possibly A1/P2Y1 heterodimers are involved in the inhibition of norepinephrine release. The P2Y1 receptor was identified as the main P2Y receptor subtype involved in the modulation of neuronal release of norepinephrine. Additionally, the P2Y1, P2Y12, and A2A receptors contribute to norepinephrine uptake by glial cells. The P2Y6 receptors seem to contribute to the regulation of noradrenergic transmission, most likely through a dialogue between neurons and perisynaptic glia that may involve the release and/or formation of adenosine and an increase in norepinephrine uptake by neurons and glial cells. All purinergic receptors identified contribute, by different mechanisms, to an inhibition of noradrenergic transmission.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: D.P., C.Q., and G.Q. edited and revised manuscript. D.P., C.Q., and G.Q. drafted manuscript. C.Q. and G.Q. prepared figures; G.Q. conception and design of research; G.Q. interpreted results of experiments; D.P., C.Q., F.S., T.M.C., and G.Q. analyzed data; D.P., C.Q., F.S., T.M.C., and G.Q. interpreted results of experiments; D.P., C.Q., and G.Q. drafted manuscript; D.P., C.Q., F.S., T.M.C., and G.Q. approved final version of manuscript; C.Q. and G.Q. prepared figures; G.Q. conception and design of research; G.Q. edited and revised manuscript.

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