Evidence for the existence of pyrimidinergic transmission in rat brain

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ABSTRACT

The uridine nucleotides uridine-5′-triphosphate (UTP) and uridine-5′-diphosphate (UDP) have previously been identified in media from cultured cells. However, no study to date has demonstrated their presence in brain extracellular fluid (ECF) obtained in vivo. Using a novel method, we now show that UTP and UDP, as well as uridine, are detectable in dialysates of striatal ECF obtained from freely-moving rats. Intraperitoneal (i.p.) administration of uridine or exposure of striatum to depolarizing concentrations of potassium chloride increases extracellular uridine, UTP and UDP, while tetrodotoxin (TTX) decreases their ECF levels. Uridine administration also enhances cholinergic neurotransmission which is accompanied by enhanced brain levels of diacylglycerol (DAG) and inositol trisphosphate (IP_3) and blocked by suramin, but not by PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid) or MRS2578 suggesting a possible mediation of P2Y2 receptors activated by UTP. These observations suggest that uridine, UTP and UDP may function as pyrimidinergic neurotransmitters, and that enhancement of such neurotransmission underlies pharmacologic effects of exogenous uridine on the brain.

1. Introduction

Uridine, the principal circulating pyrimidine nucleoside in humans (Wurtman et al., 2000) and its nucleotide products uridine-5′-diphosphate (UDP), uridine-5′-triphosphate (UTP), UDP-glucose, UDP-galactose (Lecca and Ceruti, 2008) and cytidine-5′-triphosphate (CTP) (Cansev et al., 2005) affect numerous physiological functions, including the syntheses of RNA (Lecca and Ceruti, 2008) and membrane phosphatides (Ulus et al., 2006; Wurtman et al., 2006; Cansev and Wurtman, 2007; Sakamoto et al., 2007), and the differentiation of neuron-related cells (e.g. neurite outgrowth) (Pooler et al., 2005). Uridine's effects on phosphatide synthesis are mediated by CTP, an intermediate in the Kennedy Cycle (Kennedy and Weiss, 1956), while its stimulation of neuronal differentiation (Pooler et al., 2005) involves the activation by UTP of brain P2Y2 receptors (Lustig et al., 1993; Pooler et al., 2005).

The uridine nucleotides UTP and UDP are ligands to several P2Y receptors (Abbracchio et al., 2006). UTP activates P2Y2 (Lustig et al., 1993) and P2Y4 (Communi et al., 1995) receptors while UDP activates P2Y6 (Communi et al., 1996) and P2Y14 receptors (Carter et al., 2009). Activation of these cell surface receptors in the brain requires that UTP and UDP are released from cells into the extracellular fluid (ECF) (Cansev, 2007; Lecca and Ceruti, 2008). Therefore, intensive work has been done to identify uridine nucleotides in brain ECF. Although previous in vitro studies reported the detection of UTP in the culture medias of a variety of cells in low nanomolar ranges (1 ± 10 nM in 0.5 ml medium bathing 2.5 cm^2 dish) (Lazarowski et al., 1997; Lazarowski and Harden, 1999), to date, only uridine (Dobolyi et al., 1998), but not UTP or UDP, has been identified in brain ECF in vivo.

Therefore, the aim of the present study was to detect and quantify uridine and its nucleotides UTP and UDP in brain ECF by in vivo microdialysis. We initially aimed to solve the possible

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problem of rapid hydrolysis of extracellular nucleotides by membrane-bound ecto-nucleotidase enzymes (Zimmermann, 1996) which might have been the reason for prior failure to detect uridine nucleotides in brain ECF in vivo. We have thus developed a method — based on blocking the ecto-nucleotidase enzymes that would destroy the UTP and UDP in ECF dialysates — for quantifying these nucleotides in brain ECF.

Using that method, we confirmed their presence in rat brain ECF in vivo and observed increases in their levels following intraperitoneal (i.p.) administration of uridine in doses known to raise (Cansev et al., 2005) plasma and brain uridine levels and brain levels of UTP and UDP. We further observed that uridine, UTP and UDP are released from excitable cells in experimental designs including potassium chloride-induced neuronal depolarization and tetrodotoxin (TTX) blockade of action potentials. The enhanced release of these pyrimidines subsequently enhanced cholinergic neurotransmission, manifested by increased acetylcholine release; this effect was blocked by suramin, a non-selective antagonist of P2Y2 and P2Y6 receptors, but not by PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid), a non-selective antagonist of P2Y4 and P2Y6 receptors or MRS2578, a selective antagonist of P2Y6 receptors.

To the best of our knowledge, our data provide the first comprehensive evidence for a pyrimidinergic neurotransmission, the enhancement of which enhances cholinergic neurotransmission via, probably, P2Y2 receptors.

2. Material and methods

2.1. Animals

Adult male Sprague–Dawley rats (300–350 g; Experimental Animals Breeding and Research Center, Uludag University Medical School, Bursa, Turkey) were housed in groups of four in a temperature controlled room with free access to standard rat chow and water under a 12 h light/dark cycle. The experimental protocol was approved by the Animal Care and Use Committee of Uludag University, Bursa, Turkey (Approval ID: 2008-12/7), and all experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available.

2.2. Surgical procedures

Experiments were carried out in two settings; in vivo microdialysis, for obtaining brain ECF, and, in vivo freezing for obtaining samples of blood and of brain tissue.

For in vivo microdialysis experiments, skulls of rats which had been anesthetized with Ketamine and Xylazine (80 mg/kg and 10 mg/kg, respectively) and then placed in a stereotaxic frame were exposed, and a small hole was drilled over the left striatum, using the bregma as the reference point (coordinates were: AP 1.0 mm; ML 2.8 mm; V 6.0 mm). A hand-made probe (molecular weight cutoff of dialysis membrane was 13,000 Da and length was 1 mm) was implanted and then fixed to the skull, using acrylic cement. After surgery the rats were placed in individual cages and allowed to recover from anesthesia for 24 h. During this period, they remained calm and showed no evidence of pain.

For in vivo freezing experiments, rats were anesthetized with Ketamine and Xylazine (80 mg/kg and 10 mg/kg, respectively) and their skulls were exposed. Blood sampling was performed by heart puncture immediately before the rats' heads were dipped into liquid nitrogen. Blood sampling lasted for less than 10 s within which period the heart puncture probably resulted in a cessation of circulation such that the rats were technically dead.

2.3. In vivo microdialysis study

In vivo microdialysis experiments were carried out 24 h after surgery to allow rats to recover from the effects of anesthesia and surgery. The dialysis probe was perfused at a rate of 2 μl/min with artificial cerebrospinal fluid (CSF; pH = 7.4) of the following composition: 148 mM NaCl, 3.0 mM KCl, 1.4 mM CaCl₂, 0.8 mM MgCl₂, 1.3 mM NaH₂PO₄, 0.2 mM Na₂HPO₄. The artificial CSF also contained PSB069 (Tocris Bioscience, Bristol, UK), a non-selective nucleoside triphosphate diphosphohydrolase (NTPDase) inhibitor, which reportedly inhibits rat NTPDases 1, 2 and 3 with similar potencies (Baqi et al., 2009). Addition of PSB069 to the perfusion fluid protected extracellular uridine nucleotides from hydrolysis, and enabled us to measure these nucleotides in extracellular fluid by in vivo microdialysis technique.

In experiments that we tested the effect of uridine (1 mmol/kg; i.p.) on acetylcholine and choline release, 10 μM of physostigmine was included in the standard artificial CSF in order to block acetylcholinesterase and thus detect acetylcholine, as reported previously (Westerink, 2000). In follow-up experiments that we investigated the mediation of P2Y receptor stimulation on enhanced acetylcholine release, an intracerebroventricular (i.c.v.) cannula directed to the lateral ventricle was implanted along with the microdialysis probe one day prior to the experiments. The next day 10 μl of artificial CSF containing suramin (1 μmol; Sigma, St. Louis, MO, USA), a non-selective antagonist of P2Y2 and P2Y6 receptors, PPADS (0.1 μmol; Sigma, St. Louis, MO, USA), a non-selective antagonist of P2Y4 and P2Y6 receptors, or MRS2578 (1 μmol; Sigma, St. Louis, MO, USA), a selective antagonist of P2Y6 receptors was slowly injected 15 min prior to uridine (1 mmol/kg; i.p.) administration (Suramin at 5 and 10 μmol doses and PPADS at 0.5 and 1 μmol doses caused convulsions in the rat).

In separate experiments we tested the effect of high potassium stimulation on the release of uridine and its nucleotides into brain ECF. The concentration of KCl (Sigma, St. Louis, MO, USA) in the perfusion fluid was 52 mM and equimolar amount of NaCl (Sigma, St. Louis, MO, USA) was removed from the perfusion fluid during high potassium stimulation. Perfusion with 52 mM KCl was continued for 2 h and the medium was then replaced with the regular artificial CSF.

In additional experiments the effect of TTX perfusion on the release of uridine and its nucleotides into brain ECF was investigated. The concentration of TTX in the perfusion fluid was 1 μM. Perfusion with TTX was continued for 2 h and the medium was then replaced with the regular artificial CSF.

Dialysate samples of approximately 60 μl were collected at 30-min intervals for up to 5.5 h after the i.p. injection of uridine (0.1–1.0 mmol/kg) or perfusions of high KCL or TTX. The dialysis probes were perfused with artificial cerebrospinal fluid for an initial 60-min stabilization period, and then three consecutive basal samples were collected. The uridine, UTP and UDP levels in the three basal samples did not differ by more than 10%

2.4. Blood collection and brain tissue sampling by in vivo freezing method

A separate set of experiments was performed to determine serum uridine concentrations, and brain uridine and uridine nucleotide levels, after i.p. uridine administration. For analyses of serum uridine concentrations 200 μl blood samples were obtained by heart puncture using a 22G injector needle before (at time zero) and at 1, 2, 4, and 8 h after i.p. injection of uridine (0.1–1 mmol/kg). In vivo brain freezing was performed at the same time points immediately after collection of the blood samples. Bloods were centrifuged immediately at 10,000 g and 4 °C to obtain serum samples which were kept frozen until analysis for uridine.

In order to prevent rapid degradation of nucleotides after decapitation, we utilized an in vivo brain freezing method (Wiegand et al., 1999; Cansev et al., 2005) to measure brain concentrations of uridine and its nucleotides. Immediately after blood collection by heart puncture, as described above, heads of anesthetized rats were dipped into liquid nitrogen for 45 s at the same time points as those used for blood collection (i.e., before or 1, 2, 4 and 8 h after i.p. uridine administration). Samples of brain tissue were then obtained using a tissue homogenizer (Polytron PT 10–35, Kinematica AG, Lucerne, Switzerland). The homogenizer has two rotating knives, 1.1 cm apart, which extend 4 mm beyond its shaft vertically; when operated at high speed, it cuts the frozen skull enabling removal of a circular 1 cm-diameter piece of frozen cortex. This freezing method minimized the breakdown of brain nucleotides, as confirmed by the fact that several-fold greater nucleotide levels were found after in vivo freezing than after conventional brain dissection (data not shown) in which the brain is removed from the skull after decapitation and samples for assay are excised within minutes.

Brain tissues were homogenized using the same tissue homogenizer (Polytron PT 10–35, Kinematica AG, Lucerne, Switzerland), in 50 volumes of ice-cold deionized water also containing the PSB069 (10 μM); aliquots were kept frozen for analysis of brain uridine and the uridine nucleotides. Serum and brain samples were extracted using 80% methanol; dried under vacuum; and reconstituted with deionized water before analysis of uridine and uridine nucleotides. Tissue levels of uridine and its nucleotides were expressed as picomoles per mg tissue.

2.5. Analyses of uridine and uridine nucleotides in serum and brain tissue samples

Serum and brain tissue samples were analyzed for uridine and uridine nucleotides using High Performance Liquid Chromatography (HPLC), as described previously (Cansev et al., 2005). The HPLC (Hitachi, Tokyo, Japan) was attached to a diode-array detector on a reversed-phase column (Thermo Scientific Hypersil ODS, 5 μm, 150 mm × 4.6 mm I.D.) which was connected to a guard cartridge (Supelco Discovery HS C18 Supelguard, Sigma–Aldrich, St. Louis, MO, USA). Uridine in serum and brain samples was eluted using 4 mM potassium phosphate buffer containing 0.1% methanol at pH 5.5. Individual peaks were detected by UV absorption at 260 nm and their identities confirmed by comparison with the positions of authentic standards.

Uridine nucleotides (UTP and UDP) in brain were analyzed by HPLC (Hitachi, Tokyo, Japan) using a strongly basic anion-exchange column (Macherey-Nagel Nucleosil SB, 5 μm, 150 mm × 4.6 mm I.D.) attached to a guard cartridge (Partisil SAX 7.5 × 4.6 mm, Alltech, Deerfield, IL, USA) with an isocratic buffer containing 200 mM NaH₂PO₄ at pH 2.8 flowing at 1 ml/min. Individual peaks were detected by UV

absorption at 260 nm, and were identified by comparisons with the positions of authentic standards.

2.6. Analyses of uridine and uridine nucleotides in microdialysates

Since brain extracellular fluid has been described as containing uridine concentrations within low micromolar ranges (Dobolyi et al., 1998) and extracellular concentrations of uridine nucleotide concentrations were assumed to be even lower, we amplified the uridine and uridine-nucleotide peaks in some samples by adding known amounts of authentic standards to the microdialysates. Each microdialysis sample (60 μl) was divided into four aliquots of 15 μl: two of the aliquots lacking the standards were injected to the HPLC for uridine and uridine nucleotide analyses, and the other two aliquots were injected after addition of uridine and uridine nucleotide standards. This method enabled us to confirm elution times for uridine and uridine nucleotide peaks reliably.

Supplementary Fig. S1 panel A, showing a typical HPLC analysis of brain extracellular UTP and UDP, includes a chromatogram of a basal ECF sample (colored in blue); a chromatogram of sample plus standards (1 μM each; colored in green); and a chromatogram of standards alone (10 μM each; colored in turquoise). Standard solution contained standards for UDP and UTP, as well as the standard for cytidine-5′-diphosphate (CDP), the retention time of which is very close to that of UDP, in order to distinguish between UDP and CDP. Diode array detection showed that UTP and UDP peaks were not confounded by other compounds in a wide range of wavelengths confirming the accuracy of the analysis (Fig. S1, Panel B).

In order to validate the assay procedure, we tested the effects of three different (1, 10 and 100 μM) concentrations of PSB069 in the perfusion fluid. We observed that uridine nucleotides were not eluted in the presence of 1 μM of PSB, and that 100 μM of PSB created too much background in the HPLC chromatogram, however 10 μM PSB was the optimum concentration (Fig. S1, Panel C). In addition, we failed to detect extracellular UTP and UDP when we included ARL67156 (10 μM; Tocris Bioscience, Bristol, UK), a selective inhibitor of ecto-ATPase (Crack et al., 1995) in the perfusion

medium, thus confirming previous observations (Lévesque et al., 2007) that ARL67156 is not effective in suppressing the degradation of uridine nucleotides (Fig. S1, Panel C).

The detection limit of our analysis system was 0.01 pmol/10 μl (which corresponds to 1 nM) for uridine, UTP or UDP. Average recovery of our microdialysis probes, calculated by dialysis of known amounts of authentic standards for uridine and its nucleotides, was 15%. Uridine concentrations in microdialysates were expressed as picomoles per 30 min, and those of UTP and UDP were expressed as femtomoles per 30 min.

2.7. Confirmation of UTP and UDP's extracellular existences by LC-MS/MS

The structures of UTP and UDP detected by HPLC analyses were further confirmed using a gold standard method, LC-MS/MS.

We first injected the standards for UTP and UDP to the LC-MS/MS using an LC-20AXR UFLC system (Shimadzu, Kyoto, Japan) coupled to a mass spectrometer. LC conditions included autosampler temperature at 4 °C and a mobile phase consisting of 50:50 methanol:water at a solvent flow rate of 0.2 ml/min. This injection allowed us to obtain the mass spectra for UTP (Fig. S2A) and UDP (Fig. S2B) standards. The expected molecular ions for UTP and UDP were observed at m/z 527 and 447 Da, respectively. In order to distinguish between UTP and UDP, one characteristic peak that is uncommon in the two spectra (241 m/z for UTP and 360 m/z for UDP) was selected to match with the UTP and UDP in samples, respectively. Next, fragment scans for m/z 241 Da (for UTP; Fig. S2C) and m/z 360 Da (for UDP; Fig. S2D) were performed. Conditions (Table S1) and patterns (Fig. S3) for molecular fragmentations have been presented in supplementary files.

We then collected microdialysis samples containing only the UTP and UDP by injecting the microdialysates obtained at baseline conditions to the HPLC and collecting approximately 1-ml aliquots at the retention times of the UTP and UDP peaks. This collection only covered the time period that UTP (from 15.3 to 15.9 min) and UDP (from 7.00 to 7.5 min) peaks were eluted in order to avoid collection of
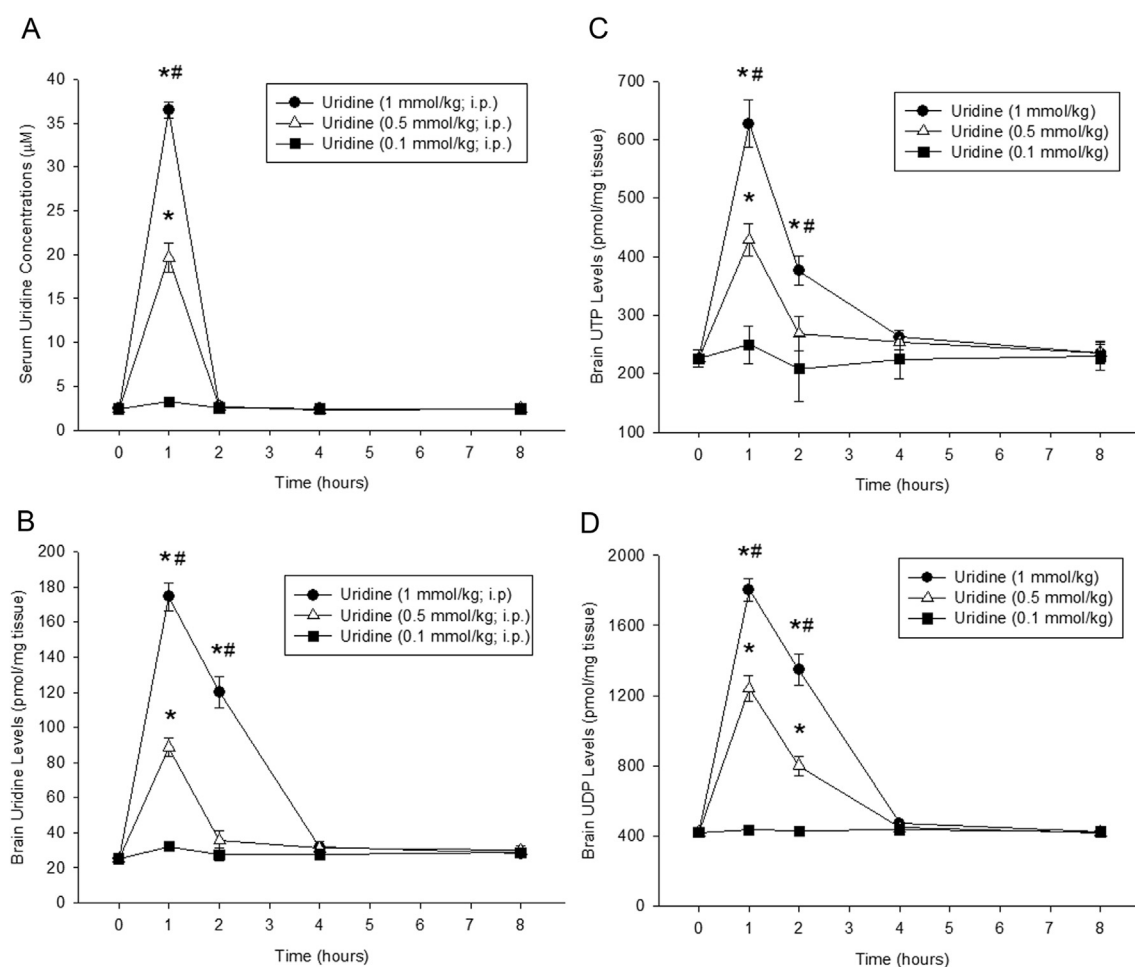

Fig. 1. Serum uridine concentrations (A) and levels of brain uridine (B), brain UTP (C) and brain UDP (D) under basal conditions and following i.p. uridine (0.1–1 mmol/kg) injection. Data are expressed as mean ± standard error of means (SEM). Statistical analyses were performed using Two-Way ANOVA followed by post-hoc Tukey test. *p < 0.001, compared with the baseline measurement within the same treatment; #p < 0.001, compared with values obtained following i.p. injection of 0.5 mmol/kg uridine. n = 6 for each data point.

other compounds within these samples. The aliquots were then lyophilized and reconstituted with 100 μl of deionized water prior to injection to mass spectrometry. These samples served the purpose of making comparisons with the authentic standards for UTP and UDP by direct injection to the mass spectrometer.

Mass spectrometric analyses were performed on a Shimadzu LCMS-8050 Ultra Fast Triple Quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). Electrospray ionization spray voltage was 4.5 kV in positive mode. Nitrogen was used as nebulizing gas at 2 L/min and as the drying gas at 10 L/min, and argon as the collision gas at 230 psi, with the block heater temperature at 400 °C.

Fragmentations of the selected ions (241 for UTP and 360 for UDP) yielded two peaks each for UTP (m/z 159 and 111 Da; Fig. S2E) and UDP (m/z 342 and 177 Da; Fig. S2F) in the microdialysis samples. These peaks coincided with the peaks eluted in the fragmentation for UTP (Fig. S2C) and UDP (Fig. S2D) standards. In addition, the proportions of intensities for m/z 111/241 (40%) and m/z 159/241 (96%) in UTP fragmentation and those for m/z 342/360 (37.5%) and m/z 177/360 (87.5%) in UDP fragmentation were identical in standards and the samples (Fig. S2C–F) (It is worthwhile to note that additional peaks observed in UTP and UDP standard fragmentation have probably arisen from the ionization matrix of these standards). Therefore, the data derived from LC-MS/MS analyses confirm that the compounds analyzed by HPLC were exactly the UTP and UDP.

2.8. Analyses of acetylcholine and choline in microdialysates

Acetylcholine and choline in striatal extracellular microdialysates were analyzed using HPLC equipped with an enzymatic column and electrochemical detector (Hewlett Packard, Palo Alto, CA, USA). The mobile phase consisted of 0.5 M NaH$_2$PO$_4$ (pH 8.5).

2.9. Analyses of brain DAG and IP$_3$ levels

DAG and IP$_3$ levels were analyzed in brain tissue homogenates obtained by in vivo freezing method using commercially available rat ELISA kits (Cusabio, Hubei Province, China) according to the manufacturer's instructions. Spectrophotometric absorbance in each well at 450 nm was quantified by comparison with the standard curve. DAG and IP$_3$ levels in brain tissue samples were represented as ng/ml and pg/ml, respectively.

2.10. Statistical analyses

Statistical analyses were performed using Sigma Plot software version 12.0. Data were expressed as means ± standard errors of means (SEM). Differences between or within groups were analyzed using One Way RM Analysis of Variance (ANOVA) or Two Way ANOVA followed by post-hoc Tukey test. The significance of an effect within a treatment group was evaluated by statistical comparison of any value obtained after uridine injection with the mean value of three baseline samples. A p value of less than 0.05 was considered significant.

3. Results

3.1. Effects of exogenous uridine on serum and brain uridine, and brain UTP and UDP levels

In initial experiments, the ability of various i.p. doses of uridine to raise serum uridine concentrations in rats was investigated. Mean basal serum uridine concentrations analyzed at zero time points were 2.4 ± 0.3 μM. These concentrations were increased significantly 1 h after i.p. injection of 1 or 0.5 mmol/kg uridine, to 36.5 ± 0.9 μM (15.2 fold) or 19.7 ± 1.7 μM (8.2 fold) respectively ($p < 0.001$ for each), but not after i.p. injection of 0.1 mmol/kg uridine (Fig. 1A). These increases were no longer observed at 2 h, and uridine levels remained stable thereafter (Fig. 1A). Two-Way ANOVA revealed significant effects of dose-[$F(2,45) = 170.957$; $p < 0.001$] and time-[$F(4,45) = 551.505$; $p < 0.001$] and a significant dose × time interaction [$F(8,45) = 168.565$; $p < 0.001$].

Intraperitoneal uridine injection also enhanced brain uridine levels. Mean basal brain uridine levels analyzed at zero time points were 25.1 ± 1.6 pmol/mg tissue. These levels were increased significantly 1 h after i.p. injection of 1 or 0.5 mmol/kg uridine, to 174.5 ± 8 pmol/mg tissue (7 fold) or 88.6 ± 5 pmol/mg tissue (3.5 fold) respectively ($p < 0.001$ for each), but not after i.p. injection of 0.1 mmol/kg uridine (Fig. 1B). These increases in brain uridine levels remained significant ($p < 0.001$) at 2 h after 1 mmol/kg uridine administration, but not thereafter (Fig. 1B). Two-Way ANOVA revealed significant effects of dose-[$F(2,45) = 176.116$;

$p < 0.001$] and time-[$F(4,45) = 171.047$; $p < 0.001$], and a significant dose × time interaction [$F(8,45) = 69.342$; $p < 0.001$].

The increases in serum concentrations and brain levels of uridine were followed by enhanced brain levels of uridine-5′-
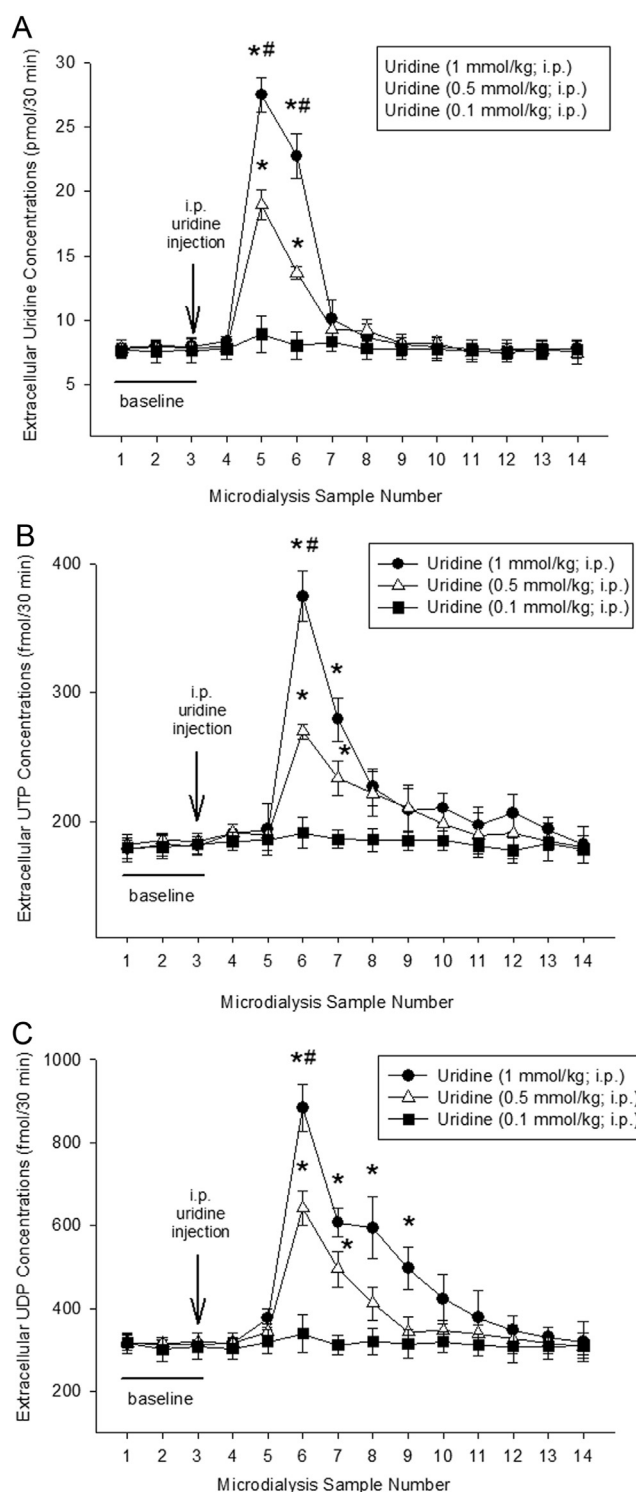

Fig. 2. Striatal extracellular uridine (A), UTP (B) and UDP (C) concentrations under basal conditions and following i.p. uridine (0.1–1 mmol/kg) injection. Data are expressed as mean ± SEM. Statistical analyses were performed using Two-Way ANOVA followed by post-hoc Tukey test. *$p < 0.001$, compared with the average of three baseline measurements within the same treatment; #$p < 0.001$, compared with values obtained following i.p. injection of 0.5 mmol/kg uridine. n = 6 for each data point.

triphosphate (UTP) and uridine-5′-diphosphate (UDP). Mean basal brain UTP levels analyzed at the zero time point were 225 ± 14.6 pmol/mg tissue. These levels were increased significantly 1 h after i.p. injection of 1 or 0.5 mmol/kg uridine, to 627 ± 41 pmol/mg tissue (2.8 fold) or 429 ± 28 pmol/mg tissue (1.9 fold), respectively (p < 0.001 for each), but not after 0.1 mmol/kg uridine (Fig. 1C). Compared with basal levels, brain UTP was still

significantly (p < 0.001) elevated 2 h after i.p. administration of 1 mmol/kg uridine (Fig. 1C). Two-Way ANOVA revealed significant effects of dose-[$F_{(2,45)}$ = 22.665; p < 0.001] and time-[$F_{(4,45)}$ = 29.58; p < 0.001] and a significant dose × time interaction [$F_{(8,45)}$ = 8.511; p < 0.001].

Mean basal brain UDP levels of rats analyzed at the zero time point were 421 ± 29 pmol/mg tissue. Peak increases in brain UDP
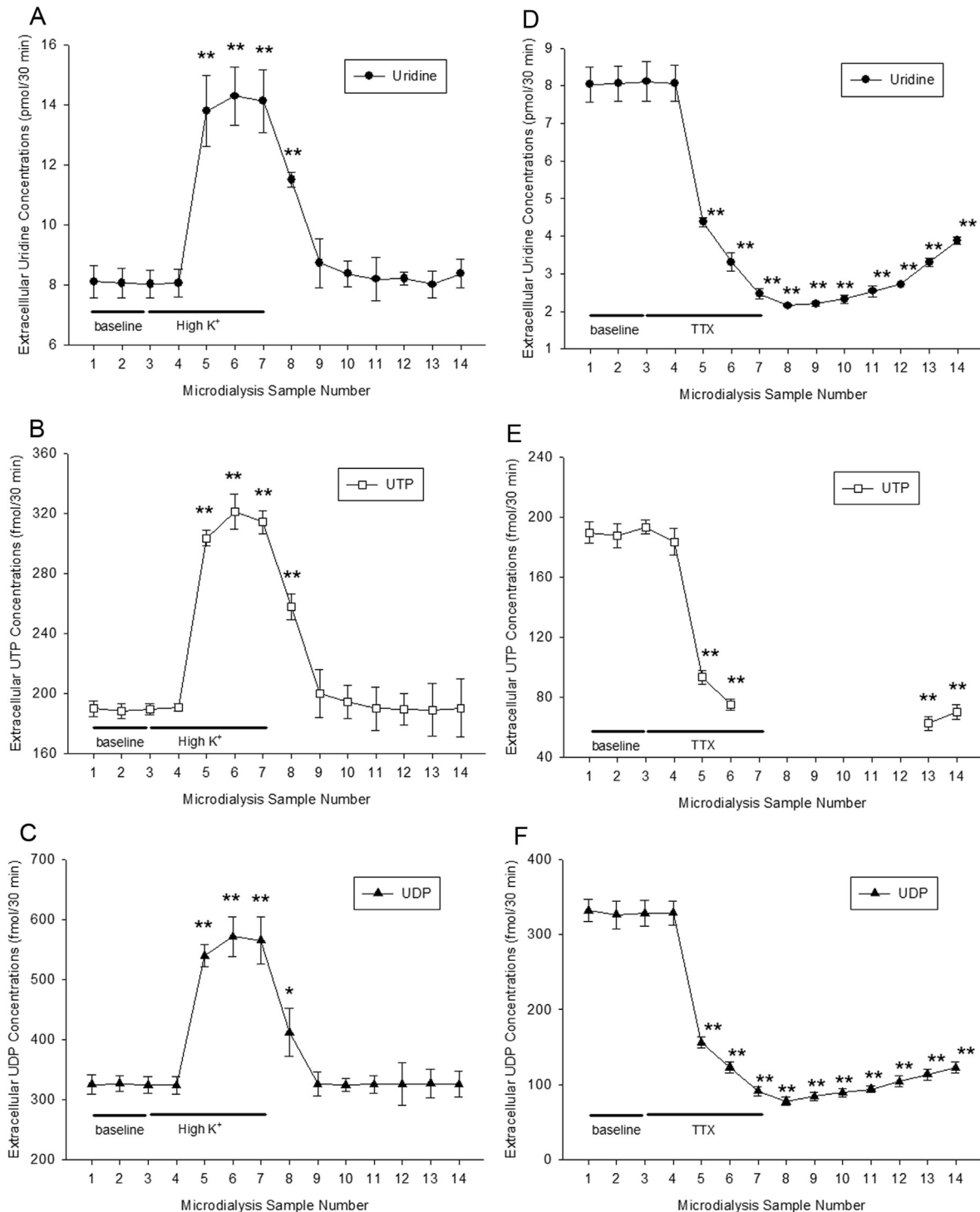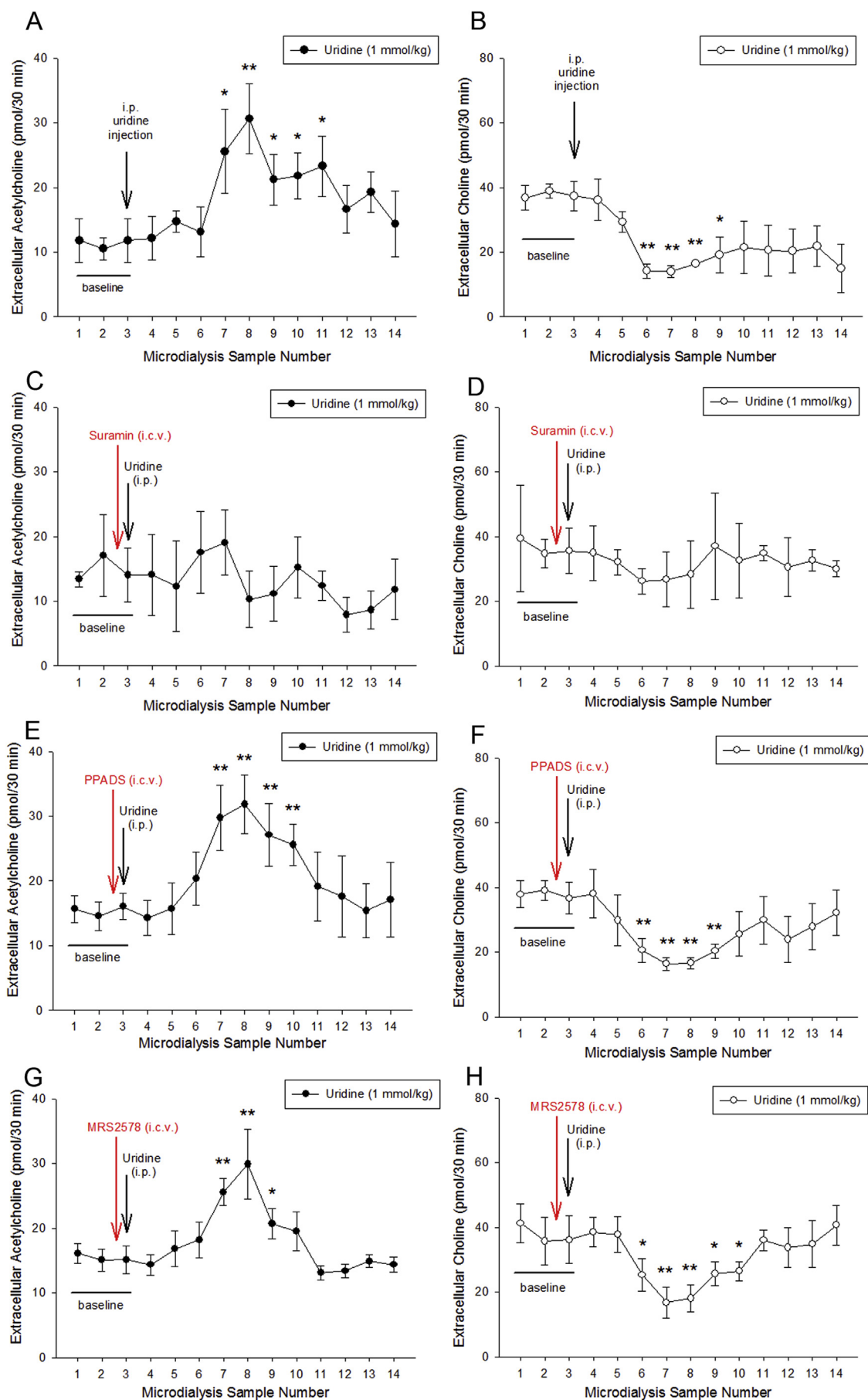

Fig. 3. Striatal extracellular uridine, UTP and UDP concentrations following infusion of high potassium (A–C) and TTX (D–E). Data are expressed as mean ± SEM. Statistical analyses were performed using One-Way RM ANOVA followed by post-hoc Tukey test. *p < 0.05 and **p < 0.001, compared with the average of three baseline measurements. n = 6 for each data point.

levels, up to 1803 ± 66 pmol/mg tissue (4.3 fold) or 1239 ± 75 pmol/mg tissue (2.9 fold) were observed 1 h after i.p. injection of 1 or 0.5 mmol/kg uridine, respectively ($p < 0.001$ for each); i.p. injection of 0.1 mmol/kg uridine did not affect brain UDP levels (Fig. 1D). Brain UDP remained significantly ($p < 0.001$) elevated 2 h after i.p. administration of 1 or 0.5 mmol/kg uridine, but returned to baseline thereafter (Fig. 1D). Two-Way ANOVA revealed significant effects of dose-[$F(2,45) = 146.43$; $p < 0.001$] and time-[$F(4,45) = 181.644$; $p < 0.001$] and a significant dose × time interaction [$F(8,45) = 56.783$; $p < 0.001$].

3.2. Effects of exogenous uridine on brain extracellular uridine, UTP and UDP concentrations

We first investigated uridine's existence in ECF under basal conditions. Basal uridine levels in striatal microdialysates, as calculated by averaging mean values of three baseline samples, were 7.79 (range: 7.76–7.83) pmol/30 min. These levels increased to 27.5 ± 1.4 pmol/30 min (3.5 fold) or 18.9 ± 1.1 pmol/30 min (2.4 fold) 1 h after i.p. injection of 1 or 0.5 mmol/kg of uridine, respectively ($p < 0.001$ for each), but not after 0.1 mmol/kg uridine (Fig. 2A). Extracellular uridine remained significantly ($p < 0.001$) elevated 1.5 h following i.p. administration of the 1 or 0.5 mmol/kg uridine doses but returned to baseline values thereafter (Fig. 2A). Two-Way ANOVA revealed significant effects of dose-[$F(2,210) = 37.098$; $p < 0.001$] and time-[$F(13,210) = 44.741$; $p < 0.001$] and a significant dose × time interaction [$F(26,210) = 12.684$; $p < 0.001$].

We then analyzed extracellular UTP and UDP levels in the same striatal ECF samples. Basal UTP levels in striatal microdialysates, as calculated by averaging mean values of three baseline samples, were 181 (range: 178–183) fmol/30 min. These levels increased to 375 ± 20 fmol/30 min (2.1 fold) or 270 ± 6 fmol/30 min (1.5 fold), 1.5 h after i.p. injections of 1 or 0.5 mmol/kg uridine, respectively ($p < 0.001$ for each), but not after 0.1 mmol/kg uridine (Fig. 2B). Extracellular UTP was still significantly ($p < 0.001$) elevated 2 h after administration of 1 or 0.5 mmol/kg uridine (Fig. 2B). Two-Way ANOVA revealed significant effects of dose-[$F(2,210) = 25.735$; $p < 0.001$] and time-[$F(13,210) = 16.148$; $p < 0.001$] and a significant dose × time interaction [$F(26,210) = 4.828$; $p < 0.001$].

Basal UDP levels in striatal microdialysates, as calculated by averaging mean values of three baseline samples, were 312 (range: 310–315) fmol/30 min. These levels increased to 884 ± 57 fmol/30 min (2.8 fold) or 642 ± 41 fmol/30 min (2.1 fold) 1.5 h after i.p. injection of 1 or 0.5 mmol/kg uridine, respectively ($p < 0.001$ for each), but not after i.p. injection of 0.1 mmol/kg uridine (Fig. 2C). Extracellular UDP was still significantly ($p < 0.001$) elevated 3 h and 2 h after i.p. administration of 1 or 0.5 mmol/kg uridine (Fig. 2C), respectively. Two-Way ANOVA revealed significant effects of dose-[$F(2,210) = 38.918$; $p < 0.001$] and time-[$F(13,210) = 19.334$; $p < 0.001$] and a significant dose × time interaction [$F(26,210) = 5.362$; $p < 0.001$].

3.3. Effects of high potassium stimulation or TTX blockade on brain extracellular concentrations of uridine, UTP and UDP

Stimulation by the high-potassium fluid significantly increased extracellular uridine (by 1.8 fold [Fig. 3A]) starting by 1 h and continuing for 2.5 h. One-Way RM ANOVA revealed a significant

effect [$F(5,55) = 16.902$; $p < 0.001$] of high-potassium stimulation. In addition, stimulation by the high-potassium fluid significantly increased extracellular UTP (by 1.7 fold [Fig. 3B]) and UDP (by 1.8 fold [Fig. 3C]) starting by 1 h and continuing for 2.5 h. One-Way RM ANOVA revealed a significant effect of high-potassium stimulation on the releases of UTP [$F(5,55) = 23.751$; $p < 0.001$] and UDP [$F(5,55) = 16.370$; $p < 0.001$]. The concentrations of uridine, UTP and UDP all returned to baseline by 1 h after resuming perfusion with the normal CSF that contained 3 mM KCl.

Inhibition of neuronal firing by TTX reduced striatal extracellular uridine (by 73.4%; from 8.07 ± 0.5 pmol/30 min to 2.15 ± 0.02 pmol/30 min, [Fig. 3D]) and UDP (by 76.4%; from 328.6 ± 16.9 fmol/30 min to 77.8 ± 5.4 fmol/30 min, [Fig. 3F]) concentrations. Baseline UTP concentrations were also reduced from 190 ± 6.5 fmol/30 min to 75.2 ± 3.6 fmol/30 min (by 61.5%, [Fig. 3E]) at 1.5 h following initiation of TTX perfusion. However, UTP fell below our detection limit which is around 1 nM (equivalent to 60 fmol/30 min) for 3 h between 7th and 13th microdialysate samples while it became detectable for the last 1 h of sample collection (Fig. 3E).

One-Way RM ANOVA revealed a significant effect of TTX blockade on uridine [$F(5,55) = 78.914$; $p < 0.001$], UTP [$F(5,55) = 94.57$; $p < 0.001$] and UDP [$F(5,55) = 118.932$; $p < 0.001$] release. In accordance with previous observations with regard to the time-course of TTX at 1 μM concentration (van Duuren et al., 2007), the effect of TTX on the releases of uridine, UTP and UDP was greatest at 2.5 h after the initiation of its perfusion. Uridine, UTP or UDP release was still significantly lower than their basal levels 3.5 h after the completion of TTX perfusion (Fig. 3D–F).

3.4. Effects of exogenous uridine on brain extracellular acetylcholine and choline concentrations

The enhanced releases of UTP and UDP following i.p. uridine were accompanied by increased acetylcholine release (Fig. 4A), while a simultaneous decrease was observed in extracellular choline concentrations (Fig. 4B). One-Way RM ANOVA revealed a significant effect of uridine injection on enhanced acetylcholine release [$F(3,33) = 3.492$; $p = 0.003$] and on the decrease in extracellular choline concentrations [$F(3,33) = 2.564$; $p = 0.018$].

3.5. Effects of P2Y receptor blockade on brain extracellular acetylcholine and choline concentrations

The increase in acetylcholine and the decrease in choline concentrations in the ECF following uridine administration were diminished by a pre-treatment with i.c.v. suramin 15 min prior to i.p. uridine injection (Fig. 4C and D), but not by a pre-treatment with i.c.v. PPADS (Fig. 4E and F) or i.c.v. MRS2578 (Fig. 4G and H). One-Way RM ANOVA revealed no effect of uridine injection on acetylcholine release [$F(3,33) = 0.578$; $p = 0.834$] and on extracellular choline [$F(3,33) = 0.184$; $p = 0.997$] in rats pre-treated with i.c.v. suramin. On the other hand, One-Way RM ANOVA revealed a significant effect of uridine injection on enhanced acetylcholine release [$F(4,44) = 3.769$; $p = 0.009$] or [$F(3,33) = 4.828$; $p < 0.001$] and on the decrease in extracellular choline concentrations [$F(4,44) = 3.705$; $p = 0.004$] or [$F(3,33) = 4.416$; $p < 0.001$] in rats pre-treated with i.c.v. PPADS or i.c.v. MRS2578, respectively.

Fig. 4. Striatal extracellular acetylcholine and choline concentrations following i.p. uridine (1 mmol/kg) injection (Panels A and B) alone and, after pre-treatment with suramin (Panels C and D), PPADS (Panels E and F) and MRS2578 (Panels G and H). Data are expressed as mean ± SEM. Statistical analyses were performed using One-Way RM ANOVA followed by post-hoc Tukey test. *$p < 0.05$ and **$p < 0.001$, compared with the average of three baseline measurements. n = 4 rats for each data point in uridine alone, suramin and MRS2578 experiments and n = 5 rats for each data point in PPADS experiments.

3.6. Brain levels of DAG and IP₃ following exogenous uridine administration

Baseline DAG levels (0.41 ± 0.02 nmol/ml) were increased by 1.9 fold (to 0.79 ± 0.08 nmol/ml; $p < 0.001$) at 2 h following i.p. uridine injection (Table 1). Similarly, baseline IP3 levels (24 ± 1.8 pg/ml) were increased by 2.1 fold (to 50.4 ± 4.7 pg/ml; $p < 0.001$) at the same time (Table 1).

4. Discussion

These data provide the first evidence that the uridine nucleotides UTP and UDP exist in brain ECF under baseline conditions and that their concentrations can be enhanced by administering uridine systemically. Stimulation of excitable brain cells by potassium chloride enhances the release of uridine, UTP and UDP, while on the other hand, blocking neuronal depolarization by TTX reduces their extracellular concentrations. Hence, the three pyrimidines are secreted constitutively and, to a greater extent, upon cellular depolarization.

The enhanced releases of uridine, UTP and UDP following systemic uridine are accompanied by increased ECF concentrations of acetylcholine and the effect is blocked by suramin, but not by PPADS or MRS2578. These data reveal, for the first time, that enhanced pyrimidinergic neurotransmission has a pharmacological consequence; it leads to enhanced cholinergic neurotransmission mediated by P2Y receptors, probably the P2Y2 subtype.

The present data and previously-published observations thus support the following formulation regarding the natural history of brain uridine: Uridine enters the blood principally, in adults, from hepatic secretion (Gasser et al., 1981); some of it then crosses the blood–brain barrier and enters brain cells (Cansev, 2006) where it can be phosphorylated to form UDP and UTP (Cansev et al., 2005); some of this UTP is further transformed to cytidine-5′-triphosphate (CTP) (Cansev et al., 2005), levels of which can be limiting in the rate at which membrane phosphatides are synthesized via the Kennedy Cycle (Kennedy and Weiss, 1956); and some of the UDP and UTP, as now shown, is released into brain ECF where they can activate cell-surface P2Y receptors (Abbracchio et al., 2006).

Existence of uridine in brain ECF in vivo was first demonstrated in rat thalamus, the authors describing baseline concentrations of 0.76 μM (Dobolyi et al., 1998). The present data confirm this concentration range: baseline extracellular uridine concentration in rat striatum was found to be 7.79 pmol/30 min (which corresponds to 0.87 μM calculated by the perfusion rate [2 μl/min], sample volume [60 μl], and recovery of our probes [15%]).

Uridine nucleotides are ligands to several P2Y receptors; P2Y2, P2Y4, P2Y6 and P2Y14. P2Y2 (Lustig et al., 1993) and P2Y4 (Communi et al., 1995) receptors are activated by UTP; the P2Y6 receptor is activated by UDP (Communi et al., 1996), and P2Y14 receptor is stimulated by both UDP and UDP-sugars (Chambers et al., 2000; Carter et al., 2009). Since these receptors are localized to cell surfaces (Abbracchio et al., 2006), in order for UTP and

UDP to activate them, these nucleotides presumably would have to be secreted into the extracellular space (Cansev, 2007; Lecca and Ceruti, 2008). UTP in low nanomolar ranges (1 ± 10 nM in 0.5 ml medium bathing 2.5 cm² dish) has been detected in the culture media of a variety of cells including platelets and leukocytes, primary airway epithelial cells and rat astrocytes by an assay based on the UTP-dependent formation of [14C]-UDP-glucose from [14C]-glucose-1P (Lazarowski et al., 1997; Lazarowski and Harden, 1999). However, no studies to date have described UTP and UDP in brain extracellular space possibly because the extracellular nucleotides are rapid hydrolyzed by membrane-bound ecto-nucleotidase enzymes (Zimmermann, 1996).

To identify and quantify these nucleotides in brain ECF we have developed a new method which involves including in the dialysate medium PSB069, a non-selective nucleoside triphosphate diphosphohydrolase (NTPDase) inhibitor that inhibits rat NTPDases 1, 2 and 3 with similar potencies (Baqi et al., 2009). Addition of PSB069 into the perfusion fluid enabled us to detect uridine nucleotides in the extracellular fluid reliably. Using this novel method, our study shows for the first time that both UTP and UDP exist in ECF in rat striatum and that uridine administration enhances their extracellular concentrations. These data show that uridine promotes not only the syntheses but the releases of UTP and UDP from brain cells.

We next investigated the mechanism by which the release of uridine and uridine nucleotides from brain cells is modulated, i.e. whether it's constitutive or activity-dependent. It was previously observed that ECF concentrations of uridine rose to $142.8 \pm 9.6\%$ of control following high-potassium stimulation (Dobolyi et al., 2000). Therefore, it is possible that neuronal activity plays a role in releasing the uridine nucleotides UTP and UDP. Confirming this hypothesis, stimulation by the high-potassium fluid significantly increased extracellular uridine, UTP and UDP. These observations were confirmed by the observation that TTX, a potent neurotoxin which inhibits the firing of action potentials by binding to the voltage-gated sodium channels in nerve cell membranes and blocking the passage of sodium ions (responsible for the rising phase of an action potential) (Lee and Ruben, 2008) decreased extracellular concentrations of uridine, UTP and UDP.

These data provide the first comprehensive evidence that uridine, UTP and UDP are all released into the brain ECF both constitutively and, to a greater extent, in an activity-dependent manner. Therefore, it is logical to suggest that pyrimidines, like the purine compounds adenosine and ATP (Burnstock et al., 2011), could well be postulated as signaling molecules, in other words, neurotransmitters. With the identification of pyrimidine-stimulated receptors P2Y2, P2Y4, P2Y6 and P2Y14, this suggestion becomes stronger.

ATP acts as a co-transmitter which is released from nerve cells along with glutamate, noradrenaline, GABA, acetylcholine and dopamine (Burnstock et al., 2011). It has been shown to act as a fast excitatory neurotransmitter and a neuromodulator and has potent long-term (trophic) roles in cell proliferation, differentiation and death in development and regeneration, as well as in disease (Abbracchio and Burnstock, 1998; Zimmermann, 2006; Burnstock and Verkhratsky, 2010). Evidence have been provided for exocytotic neuronal vesicular release (Pankratov et al., 2007) and a vesicular release of ATP from astrocytes (Lalo et al., 2014). Our present data show, for the first time, that not only purine compounds like ATP but the pyrimidines uridine, UTP and UDP are all released from brain cells. Whereas no receptor has been identified to be stimulated by uridine so far, a variety of P2Y receptors including P2Y2, P2Y4, P2Y6 and P2Y14 are activated by UTP and UDP. Therefore, our data provide the first in vivo evidence for a pyrimidinergic neurotransmission.

Pharmacological consequence of enhanced pyrimidinergic neurotransmission was next tested with regard to cholinergic

Table 1
DAG and IP3 levels under baseline conditions and following uridine (1 mmol/kg; i.p.) injection in brain tissue homogenates obtained by in vivo freezing method. Data are expressed as mean ± SEM. Statistical analyses were performed using One-Way ANOVA followed by post-hoc Tukey test. *$p < 0.001$, compared with baseline measurements. n = 6 in each group.

Second messenger	Baseline	Uridine (1 mmol/kg; i.p.)			
		1 h	2 h	4 h	8 h
DAG (nmol/ml)	0.41 ± 0.02	0.45 ± 0.03	0.79 ± 0.08*	0.49 ± 0.04	0.40 ± 0.02
IP₃ (pg/ml)	24 ± 1.8	23 ± 1.3	50.4 ± 4.7*	28 ± 2.2	25 ± 1.9

neurotransmission. We showed that the enhanced releases of UTP and UDP following i.p. uridine administration is accompanied by increased acetylcholine release while a simultaneous decrease was observed in extracellular choline concentrations which might be associated with rapid uptake of choline to be utilized for cellular acetylcholine synthesis, in order to meet the requirement for its enhanced release.

It is highly possible that the enhancement in cholinergic neurotransmission is mediated by the stimulation of uridine-nucleotide-recognizing P2Y receptors. An initial question arises as to whether the enhancements in extracellular UTP and/or UDP concentrations are adequate to stimulate their receptors in vivo. Striatal extracellular UTP or UDP concentration raised from 181 fmol/30 min to 375 ± 20 fmol/30 min (from 20.1 nM to 42.2 nM) or from 312 fmol/30 min to 884 ± 57 fmol/30 min (from 34.7 nM to 97.2 nM) with the highest i.p. uridine dose (1 mmol/kg) used in our study, respectively. The Michaelis−Menten kinetics (Km's) for UTP to stimulate P2Y4 receptors and for UDP to stimulate P2Y6 receptors were reported as 0.8 ± 0.2 μM and 0.19 ± 0.04 μM in human 1321N1 astrocytoma cells, respectively (Nicholas et al., 1996). In addition, cloning and expression studies of mouse neuroblastoma cells showed UTP's high potency (EC50 = 1.1 μM) in releasing $^{45}Ca^{+2}$ by stimulating P2Y2 receptors (Lustig et al., 1993). Although the highest extracellular UTP and UDP concentrations achieved in our study fall below the reported in vitro Km values, our findings suggest that P2Y receptor stimulation by enhanced UTP and UDP release is highly possible under the present in vivo conditions.

The mechanism by which acetylcholine is released following i.p. uridine injection, i.e., the P2Y receptor mediation, was tested by blocking the uridine-nucleotide-stimulated P2Y receptors using i.c.v. injection of suramin, a non-selective antagonist of P2Y2 and P2Y6 receptors, PPADS, a non-selective antagonist of P2Y4 and P2Y6 receptors or MRS2578, a selective antagonist of P2Y6 receptors (Abbracchio et al., 2006). We observed that the increase in acetylcholine and the decrease in choline concentrations in the ECF following uridine administration were diminished by a pre-treatment with i.c.v. suramin, but not by a pre-treatment with i.c.v. PPADS or i.c.v. MRS2578. Since no selective antagonist for the uridine-nucleotide-recognizing receptors (P2Y2, P2Y4 and P2Y14) is presently available in a commercial manner, it is safer to speculate, rather than conclude, that cholinergic neurotransmission is probably enhanced through P2Y2 receptor activation by enhanced release of UTP following peripheral uridine injection. Further evidence was provided by showing the enhanced brain levels of diacylglycerol (DAG) and inositol trisphosphate (IP$_3$), the second messengers for P2Y receptors (Albert et al., 1997; Arslan et al., 2000).

Enhancement of the pyrimidinergic neurotransmission might have significant impact on physiological and pathological processes in the brain. Pyrimidinergic signaling has been shown to be involved in a number of brain functions (Cansev, 2007; Lecca and Ceruti, 2008) including enhancement of the proliferation of adult multipotent neural stem cells (Mishra et al., 2006) and neurite outgrowth (Pooler et al., 2005), promotion of long-term potentiation (Price et al., 2003) and neuroprotection (Weisman et al., 2012). In fact, UTP and/or UDP, by activating P2Y receptors, reportedly exhibit neuroprotective effects via induction of microglial convergence (Davalos et al., 2005) and reactive astrogliosis (Wang et al., 2005).

While previous reports indicate involvement of pyrimidinergic signaling in various neuronal functions, the rapid enhancement of cholinergic neurotransmission associated with enhanced pyrimidinergic neurotransmission is the principal impact of our present findings. Considering the limited number of medications, most of which include acetylcholine esterase inhibitors, treatments enhancing pyrimidinergic neurotransmission may serve as an alternative approach and confer benefit in treatment of Alzheimer's disease (AD).

Our present data also may enhance our understanding of the pathologic processes in Alzheimer's disease. Previous in vitro studies showed that activation of P2Y2 receptors enhanced α-secretase-mediated amyloid precursor protein (APP) processing (Camden et al., 2005). In addition, P2Y14 receptor activation was shown to suppress the expression of matrix metalloproteinase-9 (MMP-9) (Kinoshita et al., 2013), a secreted protease which is found at higher levels in AD patients (Lorenzl et al., 2003). Involvement of pyrimidinergic neurotransmission in AD pathology is further supported by (i) a report that parietal lobe P2Y2 receptors were selectively deficient in brains of patients with AD (Lai et al., 2008); (ii) a recent study reporting the loss of P2Y2 nucleotide receptors enhances early pathology in the TgCRND8 mouse model of AD (Ajit et al., 2014); and (iii) by the demonstration of a correlation between low uridine levels in cerebrospinal fluids and cognitive deficiency in patients with mild AD (Czech et al., 2012).

5. Conclusion

In conclusion, our data show, for the first time, that pyrimidinergic neurotransmission occurs in brain, mediated by the basal and depolarization-induced release of uridine, UTP and UDP into the extracellular space, and by probable subsequent activation of P2Y2 receptors by UTP to mediate enhanced cholinergic neurotransmission. These data open new avenues for pyrimidine research and may contribute to our understanding of pathogenic mechanisms underlying Alzheimer's disease.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2014.12.019.

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