P2X receptor subtype-specific modulation of excitatory and inhibitory synaptic inputs in the rat brainstem

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The role of P2 receptors in synaptic transmission to the rat medial nucleus of the trapezoid body (MNTB) was studied in an *in vitro* brain slice preparation. Whole-cell patch recordings were made and spontaneous synaptic responses studied under voltage clamp during application of P2X receptor agonists. ATPγS (100 μM) had no effect on holding current, but facilitated spontaneous excitatory postsynaptic current (sEPSC) frequency in 41% of recordings and facilitated spontaneous inhibitory postsynaptic currents (sIPSCs) in 20% of recordings. These were blocked by the P2 receptor antagonist suramin (100 μM). α,β-meATP also facilitated sEPSC and sIPSC frequency, while L-β,γ-meATP facilitated only sIPSCs. The sEPSC facilitation by ATPγS was blocked by TTX (but did not block facilitation of sIPSCs). sEPSC facilitation was blocked by PPADS (30 μM) and the selective $P2X_3$ receptor antagonist A-317491 (3 μM), suggesting that modulation of sEPSCs involves $P2X_3$ receptor subunits. α,β-meATP-facilitated sIPSCs were also recorded in wild-type mouse MNTB neurones, but were absent in the MNTB from $P2X_1$ receptor-deficient mice demonstrating a functional role for $P2X_1$ receptors in the CNS.

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ATP is released from neurones along with a range of classical neurotransmitters (Richardson & Brown, 1987; von Kugelgen *et al.* 1998; Jo & Schlichter, 1999), from glial cells and following tissue damage (Inoue, 1998; Queiroz *et al.* 1999), and acts via P2X and P2Y receptors. P2X receptors are ligand-gated non-selective cation channels with significant calcium permeability (Evans *et al.* 1996; Garcia-Guzman *et al.* 1997; Virginio *et al.* 1998*a*; Surprenant *et al.* 2000). Seven receptor subunits ($P2X_{1–7}$) have been identified at the molecular level which associate as homo- and hetero-trimeric channels (e.g. $P2X_{2/3}$, $P2X_{4/6}$, $P2X_{1/5}$) with a range of phenotypes (see review see North, 2002). Seven P2Y G-protein-coupled receptor genes have been identified ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$) with an array of signalling and pharmacological properties (Chang *et al.* 1995; Rice *et al.* 1995; Tokuyama *et al.* 1995; Communi *et al.* 1997; Bogdanov *et al.* 1998; Hollopeter *et al.* 2001; Zhang *et al.* 2002).

P2 receptors mediate a broad range of effects in the nervous system. For example, presynaptic P2X receptors regulate transmitter release (von Kugelgen *et al.* 1999; Nakatsuka & Gu, 2001; Smith *et al.* 2001) both through their depolarizing action and through direct calcium influx (Lalo & Kostyuk, 1998; Shibuya *et al.* 1999; Khakh & Henderson, 2000). Postsynaptic P2X receptors mediate fast excitatory transmission as well as having roles in sensory transduction and neuronal excitability (Jang *et al.* 2001; Vlaskovska *et al.* 2001). P2Y receptors exert both excitatory and inhibitory influences, regulating ion channels and transmitter release and mediating calcium waves in glial cells (Boehm *et al.* 1995; Harden *et al.* 1995; Ikeuchi & Nishizaki, 1995; Fam *et al.* 2000; Filippov *et al.* 2000).

The medial nucleus of the trapezoid body (MNTB) forms an inverting relay in the binaural auditory pathway (Barnes-Davies & Forsythe, 1995; Forsythe *et al.* 1998). It receives an excitatory glutamatergic input via the calyx of Held (Forsythe, 1994) and provides an inhibitory projection (Smith *et al.* 2000) to ipsilateral medial and lateral superior olives (MSO and LSO, respectively). MNTB neurones also receive excitatory glutamatergic inputs from non-calyceal terminals and glycinergic/GABAergic inhibitory inputs (Forsythe & Barnes-Davies, 1993; Hamann *et al.* 2003). Immunohistochemical, *in situ* and electrophysiological studies show that P2X receptors are expressed in the auditory system (Nikolic *et al.* 2001; Housley *et al.* 2002), hair

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cells (Glowatzki *et al.* 1997; Raybould & Housley, 1997; Housley *et al.* 1998), spiral ganglion (Salih *et al.* 1999) and brainstem, including the trapezoid nucleus and cochlear nucleus (Yao *et al.* 2000). There is also evidence for cochlea expression of P2Y receptors (Housley *et al.* 2002).

There are many regions in the CNS where P2 receptors have been localized but comparatively few specific functional roles have been identified. In this study, we have explored the role of P2 receptors in rat auditory brainstem. We have shown that ATP enhances excitatory and inhibitory transmission in the MNTB via distinct P2X receptor-ion channels and demonstrated a functional role for $P2X_1$ receptor subunits in the CNS.

Methods

Brain slice preparation

Transverse brainstem slices including the MNTB, were prepared as previously described (Barnes-Davies & Forsythe, 1995; Smith *et al.* 2000). In brief, 9- to 13-day-old Lister Hooded rats or mice (wild-type or $P2X_1$ receptor deficient as previously described; Mulryan *et al.* 2000) were killed by decapitation and the brainstem removed into cooled (0–4°C) low-Na^+, high-sucrose artificial cerebrospinal fluid (aCSF; see below). Transverse slices (120 μm thick) were cut sequentially in the rostral direction from the level of the 7th nerve. The slices were then incubated for 1 h at 37°C in normal aCSF (see below) bubbled with 95% O_2–5% CO_2, giving a pH of 7.4. Following incubation, the slice maintenance chamber was allowed to cool to room temperature.

For recording, one slice was transferred to a Peltier controlled environmental chamber mounted on the stage of an upright Axioskop microscope (Zeiss, Germany). The microscope was fitted with differential interference contrast (DIC) optics and individual cells were visualized with a × 40 water-immersion objective (Zeiss, NA 0.75). The environmental chamber (300–400 μl volume) was continuously superfused with normal aCSF (bubbled with 95% O_2–5% CO_2) at a rate of 0.7–1.0 ml min^{-1} using a peristaltic pump (Gilson, Minipuls 3), at a temperature of 27°C. Drugs were applied by switching between one of four perfusion lines, all of which entered directly into the recording chamber so as to minimize dead space.

Cell culture

Human embryonic kidney 293 (HEK-293) cells stably expressing the recombinant rat glycosilated $P2X_6$ receptor were a gift from Dr I. P. Chessell (GlaxoSmithKline, UK). Cells were maintained in Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids and 0.6 mg ml^{-1} Geneticine (Gibco BRL, UK) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. When required for study, cells were attached to glass coverslips (13 mm) and used for experiment the next day.

Electrophysiological study

Recordings were made using the whole-cell patch-clamp technique as previously described (Barnes-Davies & Forsythe, 1995; Smith *et al.* 2000). Patch pipettes were made with a two stage vertical pipette puller (PP-83, Narishige, Japan) from standard walled filamented borosilicate glass (Clark Electromedical, GC150F-7.5) Pipette resistances were around 5 MΩ when filled with intracellular solution (see below). Recordings were made from visually identified MNTB principal neurones. An Axopatch 200B patch-clamp amplifier (Axon Instruments, CA, USA) was used. Series resistances were under 20 MΩ and 70–80% compensation was used with 10 μs lag. Membrane currents were acquired by Digidata 1322A interface (Axon Instruments) with a PC computer using pCLAMP8 software (Axon Instruments). Data were filtered at 5 kHz with a low-pass Bessel filter and digitized at between 5 and 20 kHz. Spontaneous currents (sEPSCs and sIPSCs) were recorded at a holding potential of −70 mV. Analysis of sEPSCs and sIPSCs was conducted using the whole-cell analysis programs WinEDR and WinWCP (John Dempster, University of Strathclyde) with a detection amplitude threshold of 29.3 pA. For analysis of spontaneous currents we measured the number of events in 30 s intervals; control currents were measured 60–30 s before drug application, a test period was measured 30 s after the commencement of agonist perfusion and recovery was measured 5 min after drug washout. For experiments with the antagonist suramin the frequency of spontaneous events was measured 150 s after the start of suramin application. Pharmacological studies were conducted only on those MNTB neurones showing spontaneous IPSCs or EPSCs under control conditions. Resting average spontaneous rates could vary by up to 20%, hence only enhancements of over 2-fold and where the rate on washout returned to control levels were analysed further. Recordings from HEK-293 cells expressing recombinant rat $P2X_6$ receptors were made using whole-cell patch-clamp and agonists were applied using a U-tube (Evans & Kennedy, 1994). Cells were perfused with an E_{total} solution (see below). Whole-cell currents were recorded at holding potential of −60 mV.

Solution and drugs

The low-Na^+, high-sucrose aCSF used for slice preparation contained (mM): 250 sucrose, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 2 sodium pyruvate, 3 *myo*-inositol, 0.5 ascorbic acid, 4 $MgCl_2$ and 0.1 $CaCl_2$. The normal aCSF used for incubation and control perfusion media for slices contained (mM): 125 NaCl, 2.5 KCl, 10 glucose, 1.25 NaH_2PO_4, 2 sodium pyruvate, 3 *myo*-inositol, 0.5 ascorbic acid, 2 $CaCl_2$ and 1 $MgCl_2$. The normal aCSF solutions were bubbled with 95% O_2–5% CO_2, giving a pH of 7.4. The patch solution for slices, contained (mM): 110 CsCl, 40 Hepes, 10 TEA-Cl, 1 EGTA (pH 7.3 adjusted by CsOH). The external solution for the HEK-293 cell line contained (mM): 150 NaCl, 2.5 KCl, 10 Hepes, 2.5 $CaCl_2$ and 1 $MgCl_2$ (pH 7.3 adjusted by NaOH). The patch solution for the HEK-293 cell line contained (mM): 140 potassium gluconate, 5 NaCl, 10 Hepes, 10 EGTA (pH 7.3 adjusted by KOH).

ATP, UTP, adenosine-5′-O-(3-thiotriphosphate) (ATPγS), α,β-methyleneATP (α,β-meATP), adenosine, suramin, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonate (iso-PPADS) and tetrodotoxin (TTX) were purchased from Sigma (UK). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), strychnine and bicuculline were purchased from Tocris Cookson (St Louis, MO, USA). L-β,γ-Methylene ATP (L-β,γ-meATP) was purchased from RBI (UK). A-317491 was a gift from Abbott Laboratories, USA.

Statistical analyses

Statistical analyses were performed with the Dunnett multiple comparisons or Chi-squared tests, with $P < 0.05$ considered significant.

Results

Spontaneous excitatory and inhibitory synaptic currents

Two distinct types of spontaneous synaptic transient inward currents were recorded from MNTB neurones in the brain slice; (i) fast currents with a half-decay time of ≤ 3 ms and (ii) slow currents that decayed with a half-time of > 3 ms. Using a $CsCl_2$-based internal solution and holding potential of -70 mV, both excitatory and inhibitory synaptic inputs gave rise to inward currents. The MNTB expresses AMPA receptors of the GluRD 'flop' spliced-variant that have rapid kinetics and are calcium permeable (Geiger *et al.* 1995), giving fast EPSC decay time constants (Barnes-Davies & Forsythe, 1995;

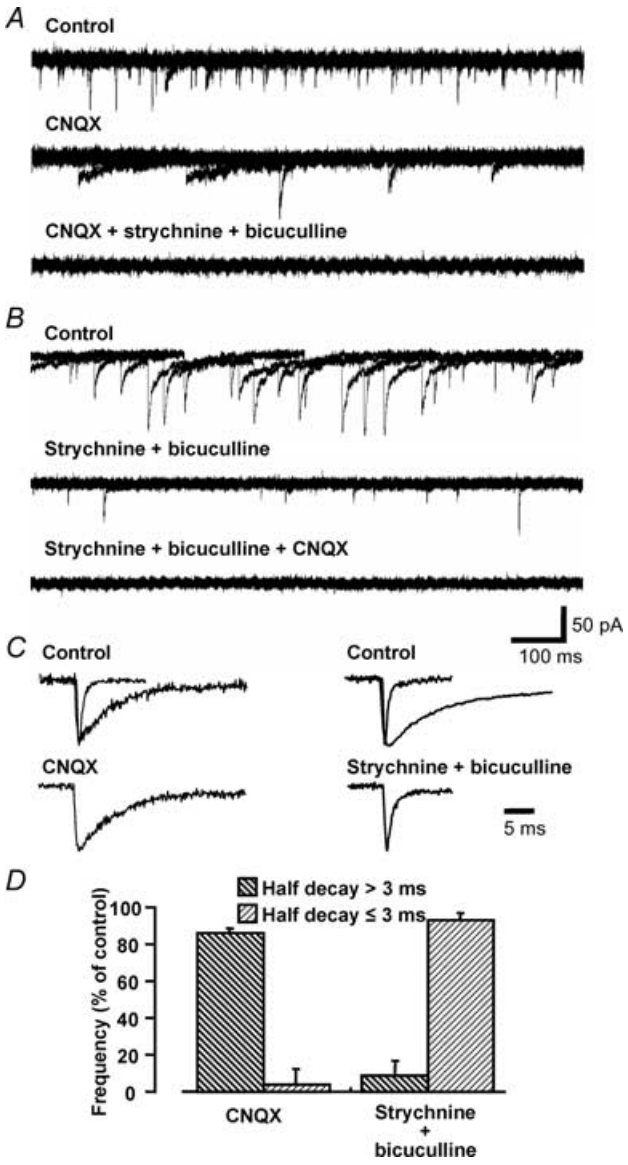

Figure 1. Characteristics of spontaneous excitatory and inhibitory synaptic currents in MNTB neurones
A, CNQX inhibited only spontaneous currents with a fast time course (half-decay ≤ 3 ms). Strychnine and bicuculline inhibited spontaneous currents with a slow time course (half-decay > 3 ms). Five overlaid traces are shown for control (upper), after application of 10 μM CNQX (middle) and after application of 1 μM strychnine and 10 μM bicuculline in the presence of 10 μM CNQX (bottom). *B*, in another cell the antagonists were applied in reverse order. All spontaneous activity was abolished in the presence of CNQX, strychnine and bicuculline. *C*, averaged and expanded traces of the two types of spontaneous currents showing their different decay times. The upper panel shows normalized averaged traces (10 events) of fast and slow events in control conditions. The lower panels show traces following the application of 10 μM CNQX (left panel, data from *A*) or 1 μM strychnine with 10 μM bicuculline (right panel, data from *B*). *D*, summary: CNQX reduced only the fast spontaneous currents (half-decay ≤ 3 ms) and strychnine with bicuculline reduced only slow spontaneous current (half-decay > 3 ms). Each column indicates the mean ± S.E.M. from 5 cells.

Table 1. Summarized data of effect of some P2 agonists and antagonists on sEPSC and sIPSC frequency

Agonist	sEPSC			sIPSC			No change		Total
(100 μM)	%	(*n*)	Fold	%	(*n*)	Fold	%	(*n*)	*n*
ATPγS	41	(70)	$10.7 \pm 3.7^{**}$	20	(33)	$4.0 \pm 1.0^{**}$	47	(79)	169
ATP	56	(9)	$9.9 \pm 5.4^{*}$	25	(4)	$4.3 \pm 1.7^{*}$	38	(6)	16
α,β-meATP	50	(7)	$13.3 \pm 7.0^{*}$	29	(4)	$4.5 \pm 1.7^{*}$	36	(5)	15
L-β,γ-meATP	0	—	—	19	(5)	$4.0 \pm 0.8^{**}$	81	(21)	26
UTP	0	—	—	0	—	—	100	(9)	9
UDP	0	—	—	0	—	—	100	(9)	9
ADP	0	—	—	0	—	—	100	(9)	9
ATPγS +									
TTX	0	—	—	24	(4)	$5.4 \pm 1.3^{**}$	76	(13)	17
TTX, low $[Ca^{2+}]_o$	0	—	—	0	—	—	100	(16)	16
Adenosine	60	(6)	$9.2 \pm 3.7^{*}$	30	(3)	$3.8 \pm 1.8^{*}$	30	(3)	10
Suramin	0	—	—	0	—	—	100	(11)	11
Suramin washout	55	(6)	$10.8 \pm 0.5^{*}$	27	(3)	$4.0 \pm 0.2^{*}$	36	(4)	11
PPADS	0	—	—	—	—	—	100	(5)	5
A-317491	0	—	—	—	—	—	100	(4)	4

Data show percentages and numbers (*n*) of neurones responding, and increases in frequency of sEPSCs and sIPSCs relative to control (Fold). Fold data shown as mean $\pm$ S.E.M. of effective cells only. No change means that change is less than 1.5-fold of control. (The data for sEPSC and sIPSC included the number of neurones in which both sEPSC and sIPSC frequency was increased). $^{**}P < 0.01$, $^{*}P < 0.05$, Dunnett's multiple test.

Taschenberger & von Gersdorff, 2000). AMPA receptor-mediated currents were blocked by the glutamate receptor antagonist CNQX (10 μM) in all cells tested (5/5) (Fig. 1*A*, *C* and *D*). The slow currents mediated by corelease of GABA and glycine (Jonas *et al.* 1998; Smith *et al.* 2000) were abolished by combined application of the glycine receptor antagonist strychnine (1 μM) and the $GABA_A$ receptor antagonist bicuculline (10 μM) in all cells tested (5/5) (Fig. 1*B*, *C* and *D*). TTX (0.5 μM) had no effect on basal rates of spontaneous IPSCs or EPSCs in all cells tested (17/17). Co-application of all three antagonists blocked all spontaneous synaptic currents (Fig. 1*A* and *B*). Therefore spontaneous synaptic currents in the MNTB arise from glutamatergic 'fast' sEPSCs and mixed glycine–GABAergic 'slow' sIPSCs.

ATPγS evoked transient facilitation of sEPSC and sIPSC frequency

We applied ATPγS, the metabolically stable ATP analogue, to examine the effects of P2 receptor activation on auditory brainstem activity. ATPγS (100 μM) had no effect on the holding current of MNTB neurones ($n = 169$), indicating that functional P2X receptors are not expressed on the cell body or dendrites of MNTB neurones. However, in 53% of neurones ATPγS showed a dramatic potentiation of spontaneous synaptic current frequency (see Table 1; 47% of neurones showed no change in spontaneous synaptic currents). The effect was transient and decayed during the continued application of ATPγS (over a time course of around 1–2 min). Analysis of this increased activity revealed three different patterns (Figs 2 and 3); (1) the most common response (34%, 57/169) was a transient increase in sEPSC frequency (Fig. 2); (2) 12% (20/169) of MNTB neurones showed a large increase in sIPSC frequency (Fig. 3*A*), and (3) 8% of neurones (13/169) showed a large increase of both sEPSC and sIPSC frequency (Fig. 3*B*). Overall, 41% of MNTB neurones showed increased sEPSC frequency and 20% showed increased sIPSC frequency (Table 1, Fig. 3*C*), however, ATPγS had no effect on the mean current amplitude of sEPSCs ($101 \pm 8\%$ of control) or sIPSCs ($94 \pm 7\%$ of control). Following 3 min pre-application of suramin, co-application with ATPγS had no effect on either sIPSC or sEPSC frequency (11/11 neurones) (Fig. 4). As a positive control following washout of suramin, ATPγS increased sIPSC and sEPSC frequency in 27% and 55% of neurones (3/11 and 6/11), respectively. These results indicate that ATPγS mediates the increase in spontaneous synaptic transmission through the activation of P2 receptors.

P2Y receptor and P1 adenosine receptor agonists have no effect on spontaneous activity

ATPγS is an effective agonist at P2X receptors and at some P2Y receptors. To investigate which P2 receptors are expressed in the auditory brainstem we used a range of nucleotide agonists showing some P2Y subtype specificity

and with action at P2X receptors. $P2Y_{1,}$ $P2Y_{11}$, $P2Y_{12}$ and $P2Y_{13}$ are ADP sensitive, $P2Y_2$ and $P2Y_4$ receptors are UTP sensitive, and the $P2Y_6$ receptor is UDP sensitive (Webb *et al.* 1993; Chen *et al.* 1996; Communi *et al.* 1996; Nicholas *et al.* 1996; Bogdanov *et al.* 1998; Hollopeter *et al.* 2001; Zhang *et al.* 2002).

UTP, UDP and ADP (100 μM) preferentially activate specific P2Y receptors but had no effect on the holding current of MNTB neurones or on the frequency of spontaneous synaptic currents (Fig. 5*A*, Table 1). As a positive control following the washout of ADP, UTP or UDP, ATPγS increased the frequency of spontaneous synaptic events (Fig. 5*A*). ATP is metabolically unstable and is degraded to ADP, AMP and adenosine by ecto-nucleotidases (Kegel *et al.* 1997; Cunha *et al.* 1998; Ohkubo *et al.* 2000). For example in the caudal regions of the rat nucleus tractus solitarii, ATP mediates excitatory transmission indirectly through breakdown to adenosine and activation of A_1 receptors (Cunha *et al.* 1998; Kato &

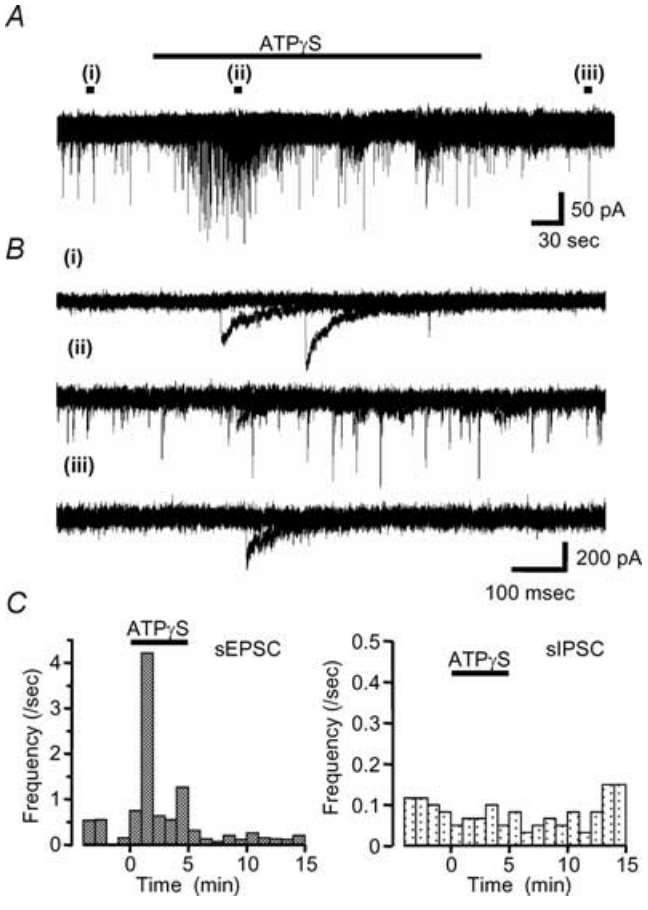

Figure 2. ATPγS evoked a transient facilitation of sEPSC frequency
In this cell ATPγS facilitated only spontaneous EPSC frequency with no effect on spontaneous IPSCs. *A*, ATPγS was applied during the period indicated by the horizontal bar. *B*, overlaid traces (five sweeps) during control (i), during the application of 100 μM ATPγS (ii) and after washout (iii) in the periods indicated by the filled bars in *A*. *C*, the time course of changes in sEPSC frequency are shown on the left, no changes were observed in sIPSC frequency (right).

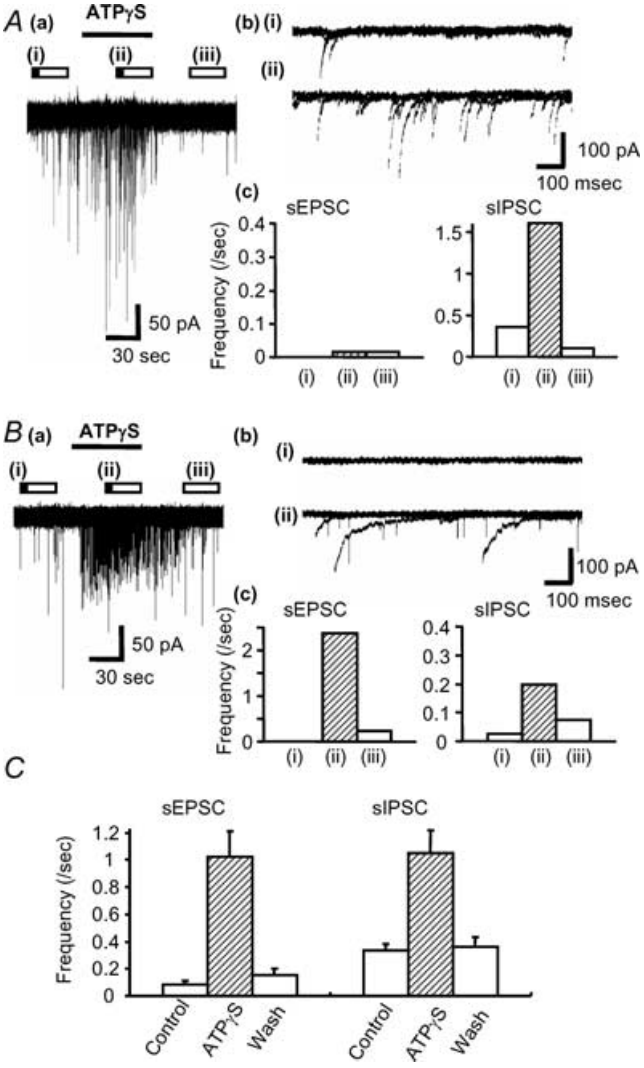

Figure 3. ATPγS can evoke transient facilitation of sEPSC- and sIPSC-frequency
A, ATPγS increased sIPSCs frequency only in this MNTB neurone. *Aa*, ATPγS was applied during the period indicated by the horizontal bar. *Ab*, overlaid traces (5 sweeps) are shown for control (i) and during the application of 100 μM ATPγS (ii). *Ac*, summary of ATPγS-mediated frequency changes for sEPSCs (left) and sIPSCs (right) in this cell. The bar graphs shows the frequency in control (i), and during the application of 100 μM ATPγS (ii) and after wash (iii) in the period indicated by the bars in *a*. *B*, ATPγS evoked facilitation of both sEPSC and sIPSCs frequency in this MNTB neurone. *Ba*, example traces showing the effect of ATPγS. *Bb*, overlaid traces (5 sweeps) are shown for control (i) and during the application of 100 μM ATPγS (ii). *Bc*, bar graphs show the change in sEPSC and sIPSC frequency in control (i), on application of 100 μM ATPγS (ii) and following wash (iii). *C*, summary: each column indicates the mean ± s.e.mean from 56 (sEPSC) and 24 cells (sIPSC). Open bars above the data trace indicate analysis epochs, with illustrated traces corresponding to times indicated by the filled portion of the bar in this and subsequent figures.

Shigetomi, 2001). In the present study adenosine (10 μM) had no effect on resting frequency or ATPγS-induced facilitation (Table 1). ATP (100 μM) had similar effects to ATPγS on sEPSC and sIPSC frequency (Table 1), indicating that it was acting directly through P2X receptors (ATP had no significant effect on the mean current amplitude of sEPSCs 92 $\pm$ 7% ($n = 9$) and sIPSCs 97 $\pm$ 3% of control ($n = 4$)). These results indicate that P2Y or P1 adenosine receptors do not regulate MNTB spontaneous activity and that ATPγS effects are most likely to be mediated through P2X receptors. The lack of expression of P2X receptors on acutely dissociated astrocytes suggests that ATPγS is acting directly at neuronal P2X receptors to modulate release of excitatory mediators, e.g. glutamate (Jeremic *et al.* 2001).

Subclassification of P2X receptor-mediated increases in synaptic activity

To further characterize the P2X receptor subtypes underlying the ATPγS-mediated responses we used

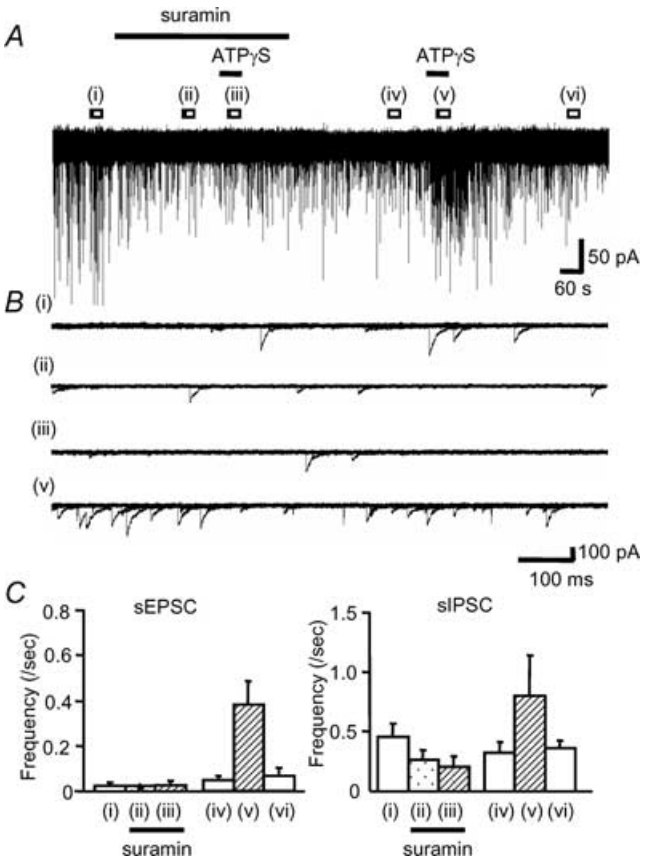

Figure 4. Suramin blocked the ATPγS-evoked facilitation of sEPSC and sIPSC frequency

A, suramin and ATPγS were applied as indicated by the horizontal bars. *B*, overlaid traces (5 sweeps) are shown for control (i), on application of 100 μM suramin (ii), during co-application of 100 μM ATPγS and suramin (iii), washout (iv), for 100 μM ATPγS applied following washout of suramin (v). The final wash (vi) data are shown in the averaged graphs. *C*, summary bar graphs showing the effect of ATPγS on the frequency of sEPSCs (left) and sIPSCs (right) in the presence (ii, iii) or absence of suramin. Each column indicates the mean $\pm$ S.E.M. from 6 (sEPSC) and 3 (sIPSC) cells (only those cells that responded to ATPγS following washout of suramin were included).

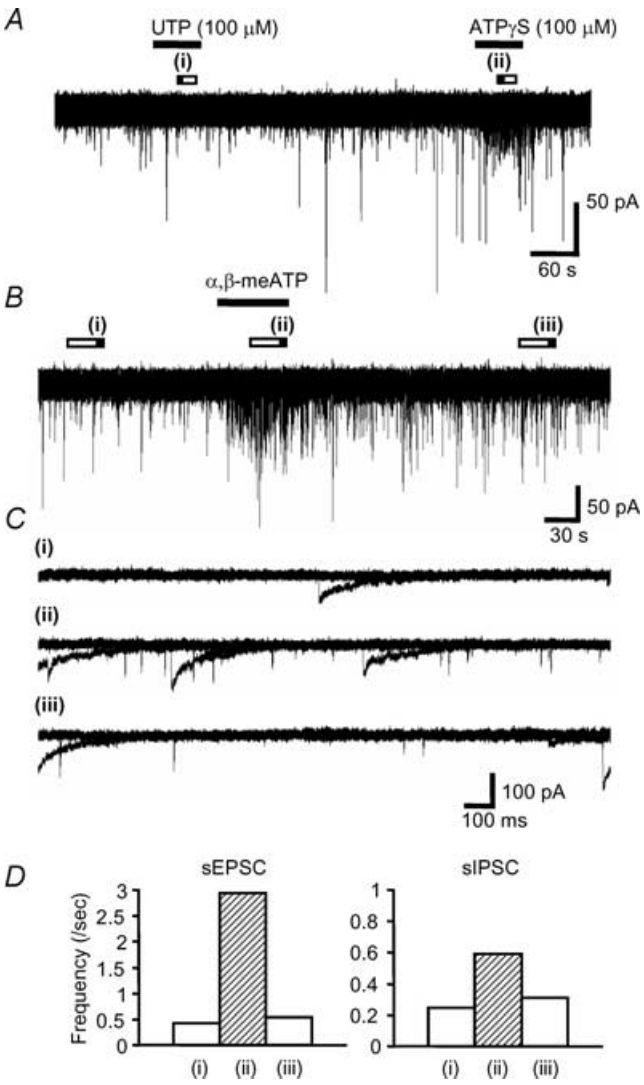

Figure 5. Effect of UTP and α,β-meATP on the spontaneous currents

A, UTP had no effect on the spontaneous currents; however, this cell could respond to ATPγS. *B*, α,β-meATP evoked transient facilitation of both sEPSC and sIPSC frequency. α,β-meATP was applied during the period indicated by the horizontal bars. *C*, overlaid traces (5 sweeps) are shown for control (i), during the application of 100 μM α,β-meATP (ii) and after wash (iii). *D*, summary data for the effect of α,β-meATP on the frequency of sEPSCs (left) and sIPSCs (right). Each column shows the frequency in control (i), on application of 100 μM α,β-meATP (ii) and after wash (iii). Each column shows the mean from 7 (sEPSC) and 4 (sIPSC) cells (including only those cells responding to α,β-meATP).

metabolically stable and subtype-selective agonists. α,β-meATP is an agonist at recombinant P2X receptors containing $P2X_1$, $P2X_3$ or $P2X_6$ receptor subunits (e.g. homomeric $P2X_3$ and heteromeric $P2X_{2/3}$ receptors; North, 2002; Jones *et al.* 2004 and current study) and evoked increases in sIPSC and sEPSC frequency similar to ATPγS (Table 1, Fig. 5*B–D*) with no effect on the mean current amplitudes (sEPSCs $100 \pm 10\%$ of control ($n = 7$) and sIPSCs $102 \pm 13\%$ control ($n = 4$)). The percentages of neurones that responded to ATPγS, ATP and α,β-meATP were similar, indicating no additional expression of ATPγS-sensitive but α,β-meATP-insensitive P2X receptors (Table 1). The relative contribution of individual subunits was investigated using L-β,γ-meATP, which acts at $P2X_1$ receptors, but not at $P2X_3$ receptors (Evans *et al.* 1995; Lewis *et al.* 1995; Grubb & Evans, 1999). The properties of recombinant $P2X_6$ homomeric receptors have been described recently; this receptor only forms functional channels when correctly glycosylated (Jones *et al.* 2004). ATP (100 μM), α,β-meATP (100 μM) and L-β,γ-meATP (100 μM) all evoked inward currents at $P2X_6$ receptors (9.9 ± 1.2, 12.0 ± 0.9 and 4.5 ± 0.4 pA pF^{-1}, respectively, Fig. 6*A*) expressed in HEK-293 cells.

In the MNTB slices L-β,γ-meATP (100 μM) evoked increases in sIPSC frequency (with no effect on the mean current amplitude $104 \pm 9\%$ of control ($n = 5$) but did not change sEPSC frequency (Fig. 6*B–D*, Table 1). The similar percentage of inhibitory neurones responding to ATPγS, ATP, α,β-meATP and L-β,γ-meATP indicates that there are no additional inhibitory neurones expressing ATPγS-sensitive but L-β,γ-meATP-insensitive P2X receptors. To determine whether the α,β-meATP-sensitive, but L-β,γ-meATP-insensitive increase in sEPSCs was mediated by receptors containing $P2X_3$ receptor subunits we used the selective $P2X_3$ receptor antagonist A-317491 (Jarvis *et al.* 2002). ATPγS-evoked increases in sEPSCs were abolished by A-317491 (3 μM) and iso-PPADS (30 μM) (Fig. 7).

The sensitivity to α,β-meATP and L-β,γ-meATP of the sIPSCs suggested the involvement of either $P2X_1$ or $P2X_6$ receptor subunits and is similar to that described recently in the somatosensory cortex (Pankratov *et al.* 2003). To determine the contribution of $P2X_1$ receptors we compared responses in MNTB neurones from wild-type and $P2X_1$ receptor-deficient

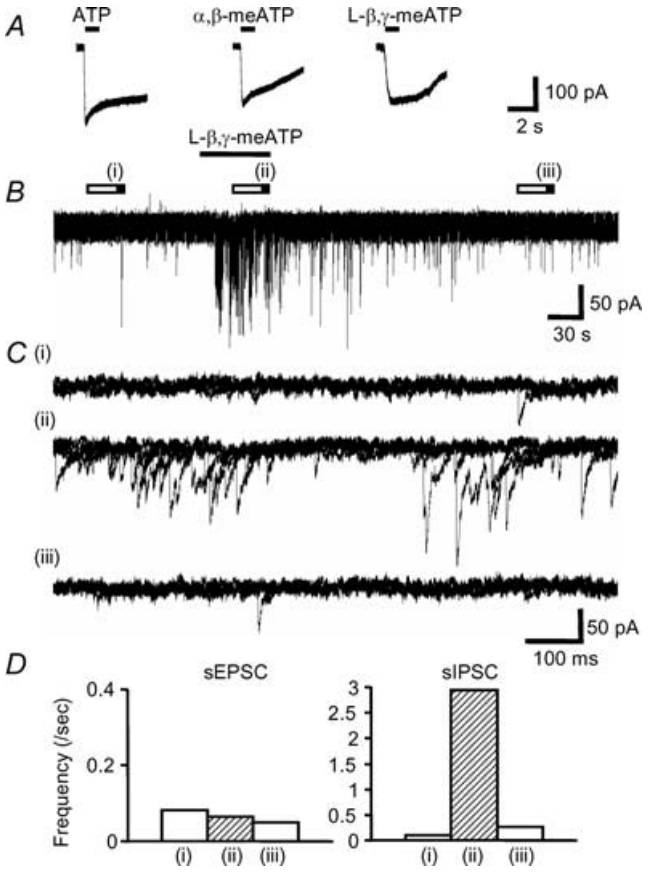

Figure 6. l-β,γ-meATP can induce $P2X_6$ receptor-mediated currents and evoked transient facilitation of sIPSC frequency only

A, ATP (100 μM), α,β-meATP (100 μM) and L-β,γ-meATP (100 μM) evoked inward currents via $P2X_6$ receptors expressed in HEK-293 cells. *B*, L-β,γ-meATP facilitated sIPSC frequency onto MNTB neurones. *C*, overlaid traces (5 sweeps) for control (i), during the application of 100 μM L-β,γ-meATP (ii) and after wash (iii). *D*, summary of the effect of L-β,γ-meATP on the frequency of sEPSCs (left) and sIPSCs (right). Each column shows the frequency in control (i), on application of 100 μM L-β,γ-meATP (ii) and after wash (iii). Data are the mean from 26 and 5 cells, respectively.

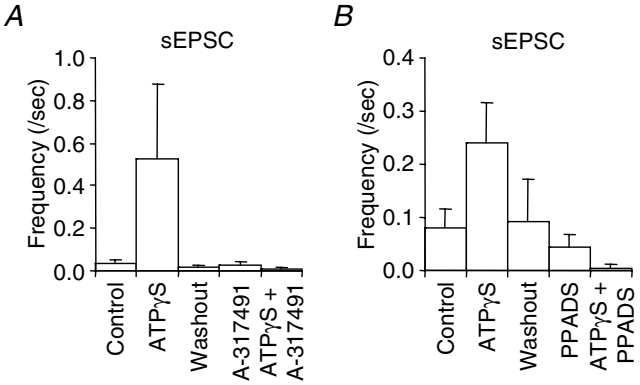

Figure 7. Summary of P2 antagonism of sEPSC frequency potentiation by ATPγS (100 μM)

A, the $P2X_3$-specific antagonist A-317491 (3 μM) caused a $87.5 \pm 12.5\%$ ($n = 4$) inhibition of the sEPSC potentiation generated by ATPγS. *B*, 30 μM PPADS reduced the ATPγS-induced potentiation of sEPSCs by $98.8 \pm 1.2\%$ ($n = 5$).

mice (Mulryan *et al.* 2000). The sIPSC frequency was potentiated by α,β-meATP in 8/8 MNTB neurones from wild-type mice, but α,β-meATP failed to evoke any change in sIPSC frequency recorded from $P2X_1$ receptor-deficient mouse MNTB neurones (0/5) (χ^2 $P < 0.01$). These results demonstrate that in the mouse and probably in the rat, $P2X_1$ receptors are expressed on the inhibitory projections to the MNTB.

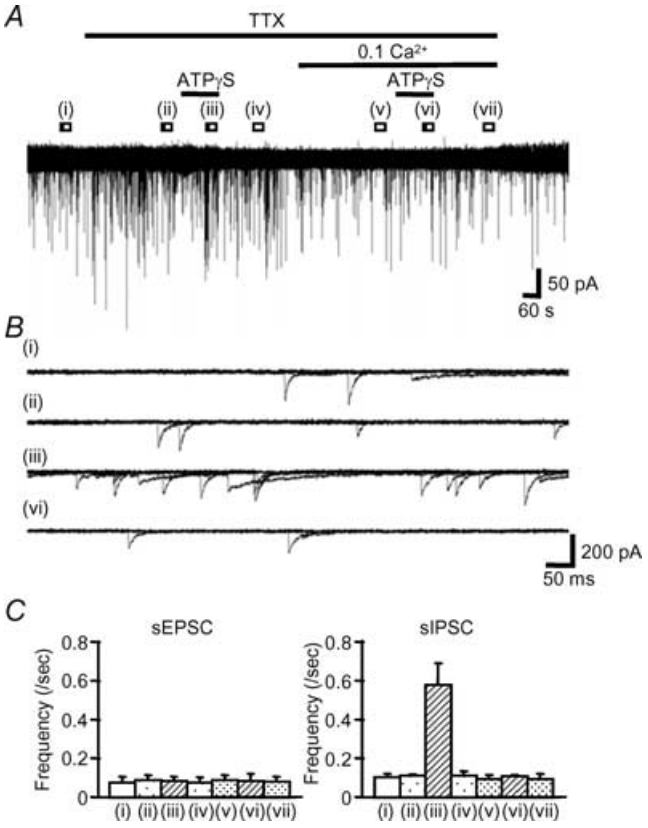

Figure 8. ATPγS applied in the presence of TTX and low $[Ca^{2+}]_o$ (0.1 mM)

A, ATPγS evoked facilitation of sIPSC-frequency but not sEPSC-frequency in the presence of 0.5 μM TTX. ATPγS failed to change sIPSC or sEPSC frequency in low Ca^{2+} conditions (0.1 mM). *B*, overlaid traces (5 sweeps) in control (i), 5 min following the application of 0.5 μM TTX (ii), during co-application of 100 μM ATPγS and 0.5 μM TTX (iii) and during application of 100 μM ATPγS in low $[Ca^{2+}]_o$ and in the presence of 0.5 μM TTX (vi). *C*, summary bar graphs showing frequency changes for sEPSCs (left) and sIPSCs (right). Each column shows control (i), application of 0.5 μM TTX (ii), co-application of 100 μM ATPγS and 0.5 μM TTX (iii), wash of ATPγS (iv) in TTX, 5 min after the application of low $[Ca^{2+}]_o$ (v), on application of 100 μM ATPγS in low $[Ca^{2+}]_o$ in the presence of 0.5 μM TTX (vi) and after wash ATPγS in low $[Ca^{2+}]_o$ conditions in TTX (iv). Each column indicates the mean ± S.E.M from 16 and 4 cells, respectively (all cells for sEPSCs and only those cells potentiated by ATPγS for sIPSCs).

Is action potential firing required for the modulatory actions of ATPγS on sEPSC and sIPSC frequency in MNTB neurones?

The P2X receptor effects may depend on the cellular localization of the receptors; for example in intrinsic sensory neurones P2X receptors are present on the cell body and their activation leads to depolarization and firing of action potentials (Bertrand & Bornstein, 2002). P2X receptors may also be present at the presynaptic nerve terminal and directly regulate transmitter release via calcium influx through the receptor ion channel (Rhee *et al.* 2000; Kato & Shigetomi, 2001; Nakatsuka & Gu, 2001). We used the voltage-gated sodium channel blocker tetrodotoxin (TTX) to determine whether action potential propagation was required for P2X receptor-mediated facilitation of spontaneous synaptic transmission. Figure 8 shows the typical effect of TTX (0.5 μM) on spontaneous currents and the ATPγS-evoked facilitation. TTX did not change the frequency of spontaneous currents in any neurones (17/17) and in the presence of TTX, ATPγS no longer facilitated sEPSC frequency (Table 1, Fig. 8*C*). However the proportion of cells responding to ATPγS with an increase in sIPSCs was unchanged (Table 1, Fig. 8*C*). These results suggest that P2X receptors are present predominantly on the cell body or fibre tracts in excitatory pathways, while for inhibitory pathways they are located on the presynaptic nerve terminals.

Is the facilitation of sIPSC frequency dependent on calcium influx?

In order to determine if the facilitation of sIPSC frequency was Ca^{2+} dependent we tested the effect of ATPγS in low external Ca^{2+} solution (low $[Ca^{2+}]_o$) and in the presence of TTX. As a positive control in 0.5 μM TTX, ATPγS facilitated sIPSC frequency in 24% (4/17) of cells (Table 1, Fig. 8). After 5 min perfusion of low $[Ca^{2+}]_o$, ATPγS no longer facilitated sIPSC frequency in any of the 16 cells tested (Table 1, Fig. 8), indicating that calcium influx via presynaptic P2X receptors mediates the increase in sIPSCs frequency.

Discussion

In this study we have shown that functional P2X receptor-ion channels are expressed in the auditory brainstem where they act to facilitate transmitter release in the superior olivary complex. Although ATP potentiates release at both excitatory and inhibitory synapses, it does so by different P2X receptor subtypes expressed at different

cellular locations: receptors comprising $P2X_3$ receptor subunits on cell bodies or axons of excitatory pathways and receptors comprising $P2X_1$ receptor subunits directly on the presynaptic terminals of inhibitory pathways (Fig. 9).

The application of ATPγS (or indeed any of a number of purinergic agonists) had no effect on the holding current of MNTB neurones, suggesting that functional P2X receptor-ion channels are not present on MNTB somas (or in their short dendritic tree). P2X receptors could be present on the axonal projections of the MNTB, but this remains to be determined. In contrast, ATPγS gave a profound increase in spontaneous transmitter release from both excitatory (11.5-fold) and inhibitory inputs (3.6-fold), indicating a high degree of purinergic regulation within the superior olivary complex. However not all inputs responded with an increase in activity (41% of excitatory and 20% of inhibitory inputs showed potentiation), suggesting that there is a heterogeneity in the neuronal input to the MNTB. ATPγS is an agonist at all P2X receptors and many P2Y receptors. We have shown that only P2X receptors were involved in the reported effects since a range of P2Y receptor agonists (selective for all currently identified P2Y receptor subtypes) had no effect on spontaneous synaptic currents in the MNTB. Using subtype-selective purinergic agonists we have demonstrated the presence of molecularly distinct P2X receptors on excitatory and inhibitory inputs.

MNTB neurones receive excitatory input from a single giant synapse (calyx of Held), which covers around half of the somatic surface area, as well as conventional glutamatergic synaptic terminals (Hamann *et al.* 2003). P2X receptor-mediated potentiation of sEPSCs was sensitive to TTX, indicating that the receptors were located predominantly on cell bodies or axon tracts of excitatory fibres innervating the MNTB (it is unlikely that a polysynaptic pathway is involved due to the orientation during cutting of the brain slice). The giant calyceal input is unlikely to be involved in P2X receptor stimulation as the increases in sEPSCs were blocked by TTX, hence they must require action potential propagation. Such spontaneous action potentials would generate giant sEPSCs at the calyx of Held, which were never observed. However, presynaptic P2X receptors have been observed at other giant synapses (Sun & Stanley, 1996). Previous reports have demonstrated a modest depression of the evoked EPSC at the calyx of Held/MNTB synapse by adenosine (Barnes-Davies & Forsythe, 1995) and in the present study adenosine had no effect on the frequency of sEPSCs. Thus the potentiation of spontaneous excitatory events is probably via non-calyceal high threshold excitatory inputs innervating the MNTB, as previously described (Hamann *et al.* 2003).

In addition to ATPγS stimulation, transmitter release from the excitatory terminals was potentiated by α,β-meATP (an agonist at recombinant receptors containing $P2X_1$, $P2X_3$ and $P2X_6$ receptor subunits). The potentiation of sEPSCs was abolished by the $P2X_3$ receptor-selective antagonist A-317491 (Jarvis *et al.* 2002) and insensitive to the $P2X_1$ and $P2X_6$ receptor subunit agonist L-β,γ-meATP (Evans *et al.* 1995; Buell *et al.* 1996). These results indicate that the high threshold excitatory neurones express $P2X_3$ receptor subunits. $P2X_3$ receptor subunits are thought to be expressed predominantly, if not exclusively, on sensory nerves, suggesting that either the MNTB receives a sensory afferent input or more likely, given the anatomy of the brainstem, that $P2X_3$ receptors are not expressed exclusively on sensory fibres. The

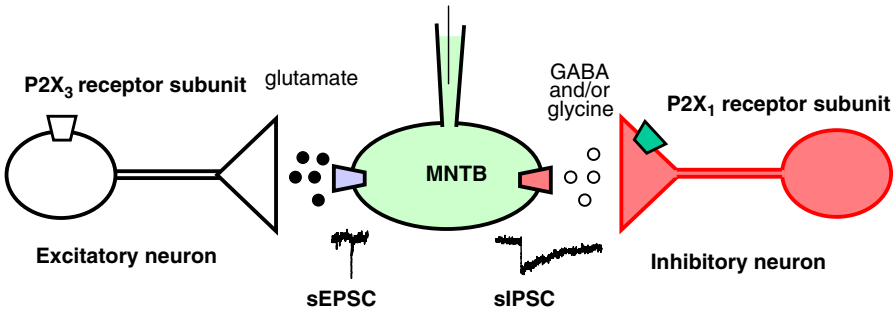

Figure 9. Schematic diagram of excitatory and inhibitory inputs innervating rat MNTB neurones and regulated by P2X receptors

The α,β-meATP-sensitive, L-β,γ-meATP-insensitive, TTX-sensitive excitatory input is consistent with mediation by channels containing $P2X_3$ receptor subunits located on the cell body. Activation of these P2X receptors increases spontaneous glutamate release at non-calyceal synapses. Mediation of the L-β,γ-meATP-sensitive inhibitory input is consistent with expression of channels containing $P2X_1$ receptor subunits located on the nerve terminal. Activation of these receptors leads to an increase in spontaneous IPSCs in a TTX-insensitive manner.

incorporation of $P2X_3$ receptor subunits into heteromeric channels can confer α,β-meATP sensitivity to the resulting channel, e.g. $P2X_{2/3}$ (where $P2X_2$ receptor homomeric channels are essentially insensitive to α,β-meATP) (Lewis *et al.* 1995; Le *et al.* 1998). The desensitizing nature of the response (1–2 min) is considerably slower than that for recombinant $P2X_3$ receptors (1–2 s) and suggests that the $P2X_3$ receptor subunit contributes α,β-meATP sensitivity to a heteromeric P2X receptor on excitatory neurones.

The MNTB receives inhibitory drive from recurrent inputs and from other brainstem nuclei of the superior olivary complex (Guinan & Stankovic, 1996; Kopp-Scheinpflug *et al.* 2002). The mixed GABA–glycine-mediated sIPSCs reported are similar to those characterized previously in the medial superior olive (Smith *et al.* 2000). The sensitivity of P2X receptors located on inhibitory inputs to L-β,γ-meATP ($P2X_1$ or $P2X_6$ receptor subunit selective) and the abolition of responses in $P2X_1$ receptor-deficient mice suggest that the P2X channel on these neurones expresses $P2X_1$ receptor subunits. This is consistent with *in situ* hybridization (Kidd *et al.* 1995; Collo *et al.* 1996) and immunohistochemical studies (Xiang *et al.* 1998; Yao *et al.* 2000; Rubio & Soto, 2001) that have shown these subunits to be expressed in the CNS and brainstem (Yao *et al.* 2000). However, it is unlikely that the response corresponds to homomeric $P2X_1$ receptors as these show rapid desensitization (1–2 s) (Evans *et al.* 1995). It seems more likely that $P2X_1$ receptor subunits contribute α,β-meATP and L-β,γ-meATP sensitivity to a heteromeric P2X receptor on the terminals of inhibitory neurones similar, for example, to the $P2X_{1/2}$ heteromeric channel in superior cervical ganglion neurones (Calvert & Evans, 2004).

The TTX insensitivity of the P2X agonist effects on inhibitory pathways demonstrates that action potential propagation is not involved and suggests that the P2X receptors are present on the presynaptic nerve terminal. This is consistent with previous studies demonstrating presynaptic P2X receptors regulating transmitter release (Sun & Stanley, 1996; Nakatsuka & Gu, 2001; Smith *et al.* 2001) and including inhibitory glycinergic (Rhee *et al.* 2000; Jang *et al.* 2001) and GABAergic neurones (Hugel & Schlichter, 2000).

In summary we have shown that ATP can regulate both excitatory and inhibitory inputs to MNTB neurones by the discrete localization of functional P2X receptor subtypes in the brainstem; one mechanism is via $P2X_3$-containing receptors located on excitatory neuronal cell bodies and a second is via presynaptic $P2X_1$-containing receptors located on inhibitory neurones. P2X receptors have been described in the primary sensory apparatus of the cochlea and this study demonstrates that these receptors can also play a functional role in the regulation of auditory processing at the level of the brainstem. This is the first time that a functional role of $P2X_1$ receptors has been demonstrated in the central nervous system.

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