

# Presynaptic cross-talk of $\beta$ -adrenoreceptor and 5-hydroxytryptamine receptor signalling in the modulation of glutamate release from cerebrocortical nerve terminals

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**1** The presynaptic interactions between facilitatory  $\beta$ -adrenoreceptors and inhibitory 5-hydroxytryptamine (5-HT) receptors modulating glutamate release from cerebrocortical nerve terminals were examined.

**2** 4-Aminopyridine (4-AP, 1 mM)-evoked glutamate release was facilitated by the membrane permeant cyclic-3',5'-adenosine monophosphate (cAMP) analogue, 8-bromo-cAMP (8-Br-cAMP), used to directly activate cAMP-dependent protein kinase (PKA).

**3** The  $\beta$ -adrenoreceptor agonist, isoprenaline (ISO), effected a concentration-dependent potentiation of 4-AP-evoked glutamate release which was abolished by the  $\beta$ -adrenoreceptor antagonist, propranolol, and the PKA inhibitor, Rp-cyclic-3',5'-adenosine-monophosphothioate (Rp-cAMPS).

**4** 5-HT receptor activation by 100  $\mu$ M 5-HT produced an inhibition of 4-AP-evoked glutamate release in nerve terminals. The inhibitory effect of 5-HT could be mimicked by the selective 5-HT<sub>1A</sub> receptor agonist, 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) and antagonized by 1-(2-methoxyphenyl)-4-(4-phthalimidobutyl)piperazine (NAN-190).

**5** When 5-HT (or 8-OH-DPAT) was used in conjunction with ISO or 8-Br-cAMP, the  $\beta$ -adrenoreceptor- and PKA-mediated potentiation of glutamate release was abrogated.

**6** The inhibitory crosstalk of 5-HT<sub>1A</sub> receptors to  $\beta$ -adrenoreceptor-mediated facilitation of glutamate release was abolished in the presence of NAN-190.

**7** Examination of voltage-dependent Ca<sup>2+</sup> influx revealed that, while ISO and 5-HT alone caused a respective potentiation and diminution of the 4-AP-evoked increase in [Ca<sup>2+</sup>]<sub>i</sub>, the co-presence of 5-HT abolished the ISO mediated potentiation of Ca<sup>2+</sup> influx.

**8** Together, these results suggest that  $\beta$ -adrenoreceptors and 5-HT<sub>1A</sub> receptors coexist on the cerebrocortical nerve terminals and that the cross-talk between the two receptor signalling pathways occurs at a locus downstream from cAMP production, possibly at the level of voltage-dependent Ca<sup>2+</sup> influx.

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**Keywords:** Adenylyl cyclase; calcium; glutamate release; 5-hydroxytryptamine; isoprenaline; cAMP-dependent protein kinase (PKA); presynaptic G-protein-coupled receptors; phosphorylation; synaptosomes; voltage-dependent calcium channel

**Abbreviations:** AC, Adenylyl cyclase; 4-AP, 4-aminopyridine; 8-Br-cAMP, 8-bromo-cyclic-3',5'-adenosine monophosphate; cAMP, cyclic-3',5'-adenosine monophosphate; ISO, isoprenaline; 5-HT, 5-hydroxytryptamine; GPCR, G-protein-coupled receptors (GPCRs); 8-OH-DPAT, 8-hydroxy-dipropylaminotetralin; IBMX, 3-isobutyl-1-methylxanthine; NAN-190, 1-(2-methoxyphenyl)-4-(4-phthalimidobutyl)piperazine hydrobromide; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; Rp-cAMPS, Rp-cyclic-3',5'-adenosine-monophosphothioate; VDCC, voltage-dependent Ca<sup>2+</sup> channels

## Introduction

Metabotropic signalling cascades initiated by extracellular ligands or first messengers typically involve the posttranslational modification of proteins by phosphorylation/dephosphorylation and represent fundamental means for the regulation of cellular function. In the mammalian central nervous system, a number of neurotransmitters act *via* heterotrimeric G-protein-coupled receptors (GPCRs) to affect excitatory and inhibitory synaptic transmission by both

phosphorylation-dependent and independent mechanisms. Presynaptically, together with ionotropic receptors (MacDermott *et al.*, 1999), coincidence and interaction of GPCR-mediated signalling cascades potentially form the basis for modulation of neurotransmitter release underlying synaptic plasticity. With respect to glutamate release, facilitatory and inhibitory auto- and hetero-receptor GPCRs have been shown to produce opposing effects on release, as well as cross-regulating each other's function within the same terminal (Herrero *et al.*, 1992; Coffey *et al.*, 1994; Vazquez *et al.*, 1995; Budd & Nicholls, 1995). Perhaps the most compelling evidence for the role of GPCR signalling cascades

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in synaptic plasticity has been obtained for GPCRs that activate adenylyl cyclase (AC) to produce the prototypic second-messenger, cyclic-3',5'-adenosine monophosphate (cAMP). Apart from the demonstration that mouse knock-outs of AC lead to impairment of synaptic potentiation (Storm *et al.*, 1998), the target for cAMP, cAMP-dependent protein kinase (PKA), and its pre- and postsynaptic protein phosphorylation substrates, have been implicated in a number of models addressing synaptic plasticity. Thus, activation of  $\beta$ -adrenoreceptors, that are positively coupled to AC through a  $G_s$  protein, has been shown to enhance synaptic transmission in slice preparations (Gereau & Conn, 1994; Huang *et al.*, 1996). In direct support of a presynaptic mechanism of action of  $\beta$ -adrenoreceptor agonists, a  $\beta$ -adrenoreceptor/cAMP/cAMP-dependent protein kinase (PKA)-dependent pathway has been ascribed a facilitatory role in studies looking at glutamate release from cerebrocortical nerve terminals (Herrero & Sanchez-Prieto, 1996). Along the lines of a reciprocating GPCR signalling paradigm, 5-hydroxytryptamine (5-HT), has emerged as an important inhibitory neurotransmitter involved in central neuromodulation. Indeed, 5-HT<sub>1A</sub> receptors, that have been shown to be negatively coupled to AC through a pertussis toxin-sensitive  $G_i$  protein, depress synaptic transmission in the locus coeruleus and basolateral amygdala (Bobker & Williams, 1989; Cheng *et al.*, 1998). Consistent with a presynaptic mechanism of actions, data from *in vitro* release experiments as well as *in vivo* microdialysis studies have shown that 5-HT receptor activation can mediate the downregulation of the release of a number of neurotransmitters (Raiteri *et al.*, 1986; Srkalovic *et al.*, 1994; Maura & Raiteri, 1996; Maura *et al.*, 1998). Consequently, presynaptic coincidence of  $\beta$ -adrenoreceptor and 5-HT receptor signalling has been inferred in the PKA-dependent modulation of synaptic enhancement observed in slice preparations of the basolateral amygdala (Wang *et al.*, 1999). In the current study we therefore sought to determine whether we could obtain direct evidence for this type of cross-talk within nerve terminals.

The isolated cerebrocortical nerve terminal preparation (synaptosomes) represents a well established model for the specific study of presynaptic regulatory pathways and their possible cross-talk, in the absence of any complications of interpretation produced by concomitant postsynaptic effects (Nicholls, 1993; Sanchez-Prieto *et al.*, 1996). Using this model, we have addressed the question whether facilitatory  $\beta$ -adrenoreceptors and inhibitory 5-HT<sub>1A</sub> receptors coexist in the same glutamatergic nerve terminals and whether the two signal transduction pathways initiated by these, respectively  $G_s$ - and  $G_i$ -linked, receptors can cross-talk in their modulation of neurotransmitter glutamate release. We examined cAMP/PKA-dependent regulation of 4-AP-evoked glutamate release from cerebrocortical synaptosomes using exogenous cAMP or the  $\beta$ -adrenoreceptor agonist, isoprenaline (ISO), to stimulate AC and produce endogenous cAMP. Furthermore, we investigated the effect of 5-HT receptor activation on the PKA-mediated modulation of glutamate release. Finally, we addressed the mechanism of the  $\beta$ -adrenoreceptor and 5-HT receptor interaction by looking at the effects of the agonists on voltage-dependent  $Ca^{2+}$  entry into nerve terminals. We propose that the interaction between the intracellular signalling pathways activated by  $\beta$ -adrenoreceptors and 5-HT<sub>1A</sub> receptors occurs at a locus downstream from cAMP

production, at the level of nerve terminal excitability and/or voltage-dependent  $Ca^{2+}$  channels (VDCCs). Our findings contribute fundamental clues to the understanding of the presynaptic changes occurring during synaptic plasticity mediated with coincident facilitatory and inhibitory inputs, respectively coupled by  $G_s$  and  $G_i$ , to provide opposing modulation of neurotransmitter glutamate release.

## Methods

### *Preparation of synaptosomes*

Synaptosomes were prepared from the cerebral cortices of 2-month-old male Sprague–Dawley rats as described previously (Sihra, 1997). The final synaptosomal fraction was resuspended in approximately 2–3 ml HEPES-buffered incubation medium (HBM (mM): NaCl 140, KCl 5, NaHCO<sub>3</sub> 5, MgCl<sub>2</sub>·6H<sub>2</sub>O 1, Na<sub>2</sub>HPO<sub>4</sub> 1.2, glucose 10, HEPES 20, pH 7.4) and protein concentration determined using the Bradford assay. Synaptosomes were centrifuged in the final wash to obtain synaptosomal pellets with 0.3 mg protein. Synaptosomal pellets were stored on ice and used within 2–3 h.

### *Glutamate release assay*

Glutamate release was assayed by on-line fluorimetry (Nicholls & Sihra, 1986). Pelleted synaptosomes were resuspended at a protein concentration of 0.3 mg ml<sup>-1</sup> in HBM containing 16  $\mu$ M bovine serum albumin (BSA) and incubated in a stirred and thermostatted cuvette at 37°C in a Perkin-Elmer LS-3B spectrofluorimeter. NADP<sup>+</sup> (1 mM), glutamate dehydrogenase (50 units ml<sup>-1</sup>) and CaCl<sub>2</sub> (1 mM) were added after 3 min. After a further 7 min of incubation, 1 mM 4-AP was added to stimulate glutamate release. The oxidative decarboxylation of released glutamate leading to the reduction of NADP<sup>+</sup> was monitored by measuring NADPH fluorescence at excitation and emission wavelengths of 340 and 460 nm, respectively. Data were accumulated at 2-s intervals. A standard of exogenous glutamate (5 nmol) was added at the end of each experiment and the fluorescence change produced by the standard addition used to calculate the released glutamate as nmol glutamate released per mg synaptosomal protein (nmol mg<sup>-1</sup>). Release traces are shifted vertically to align the point of depolarization as zero release. Unless otherwise indicated, release values quoted in the text are levels attained at 'steady-state' after 5 min of depolarization (nmol mg<sup>-1</sup> 5 min<sup>-1</sup>). Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin. Statistical analysis was performed by two-tailed Student's *t*-tests.

### *Cytosolic Ca<sup>2+</sup> measurements using Fura-2*

Synaptosomes (0.3 mg ml<sup>-1</sup>) were preincubated in HBM containing 5  $\mu$ M Fura-2-acetoxymethyl ester, 0.1 mM CaCl<sub>2</sub>, and 16  $\mu$ M BSA for 25 min at 37°C in a stirred test-tube. After Fura-2 loading, synaptosomes were centrifuged in a microfuge for 15-s, the pellet resuspended in HBM at 37°C and the synaptosomal suspension stirred in a thermostatted cuvette in a Perkin-Elmer LS-3B spectrofluorimeter. CaCl<sub>2</sub> (1 mM) was added after 3 min and further additions were

made after an additional 5 min, as described in the legends to the figures. Fluorescence data were accumulated at excitation wavelengths of 340 and 380 nm (emission wavelength 505 nm) at 3.5-s intervals. Cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ , nM) was calculated following calibration procedures (Sihra *et al.*, 1993), using 0.1% sodium dodecyl sulphate to obtain the maximal fluorescence with Fura-2 saturation with  $\text{Ca}^{2+}$ , followed by 10 mM EGTA (Tris buffered) to obtain minimum fluorescence in the absence of any Fura-2/ $\text{Ca}^{2+}$  complex. Cytosolic free  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_c$ , nM) was evaluated using 340/380 fluorescence ratios as described (Grynkiewicz *et al.*, 1985). Cumulative data were analysed using Lotus 1-2-3 and MicroCal Origin. Statistical analysis was performed by two-tailed Student's *t*-tests.

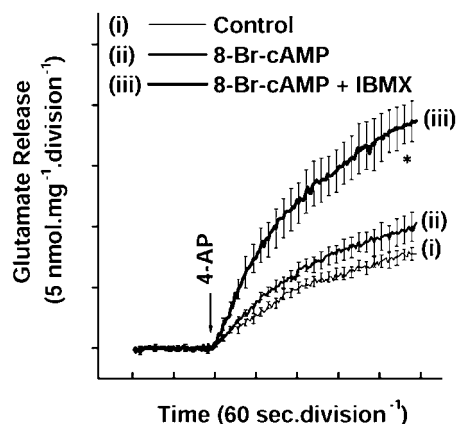
### Materials

8-OH-DPAT, 8-Br-cAMP, IBMX, Rp-cAMPS and propranolol were from Research Biochemicals International (Natick, MA, U.S.A.). Fura-2 acetoxymethyl ester (fura-2-AM) was from Molecular Probes (Eugene, OR, U.S.A.). Isoprenaline, 5-HT and all other reagents were from Sigma (Poole, U.K.) or Merck (Poole, U.K.).

### Results

Glutamate release from purified cerebrocortical synaptosomes was monitored on-line, using an assay employing exogenous glutamate dehydrogenase and  $\text{NADP}^+$  to couple the oxidative decarboxylation of the released glutamate to the generation of NADPH detected fluorometrically (Nicholls & Sihra, 1986). We first established the modulatory influence of cAMP-dependent protein kinase (PKA) on 4-AP-evoked glutamate release by activating the protein kinase using a cAMP analogue, 8-bromo-cAMP (8-Br-cAMP), to mimic the endogenous stimulation of AC. Under control conditions, 4-AP (1 mM) evoked a glutamate release of  $7.76 \pm 0.54$  nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$  from synaptosomes incubated in the presence of 1 mM  $\text{CaCl}_2$  (Figure 1(i)). Application of 8-Br-cAMP (150  $\mu\text{M}$ ) alone produced only slight potentiation of 4-AP-evoked glutamate release to  $9.98 \pm 1.22$  nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$  (Figure 1(ii)). The limited extent of this facilitation was possibly due to phosphodiesterase activity as well as the reported inhibitory action of 'leaked' endogenous adenosine acting at adenosine  $A_1$  receptors (Pockett *et al.*, 1993). Accordingly, when 8-Br-cAMP was coapplied with 3-isobutyl-1-methylxanthine (IBMX, 50  $\mu\text{M}$ ), which acts both as a phosphodiesterase inhibitor and an adenosine  $A_1$  receptor antagonist, 4-AP-evoked glutamate release was strongly potentiated by the cyclic nucleotide analogue to  $18.74 \pm 1.66$  nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$  (Figure 1(iii)).

We next examined whether stimulation of endogenous cAMP production through  $\beta$ -adrenoreceptor stimulation of AC could cause a facilitation of transmitter release similar to that seen with the cAMP analogue. Under control conditions, 4-AP (1 mM) evoked a glutamate release of  $8.32 \pm 0.79$  nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$  from synaptosomes incubated in the presence of 1 mM  $\text{CaCl}_2$  (Figure 2(i)). Preincubation with the  $\beta$ -adrenoreceptor agonist, isoprenaline (100  $\mu\text{M}$ ; ISO), significantly potentiated the 4-AP-evoked release to  $23.32 \pm 0.74$  nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$  (Figure 2a(ii)). The ISO-

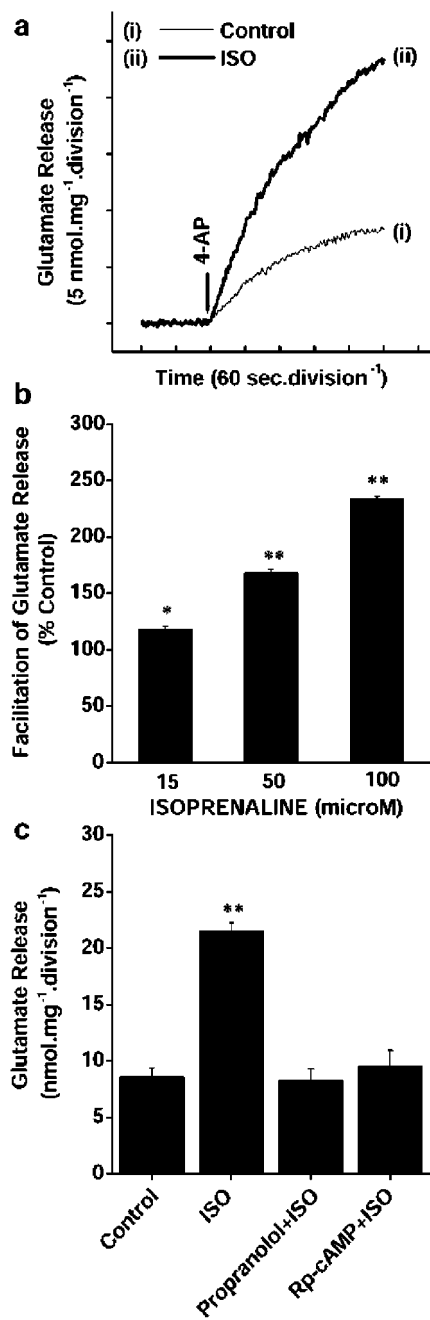


**Figure 1** Facilitation of 4-AP-evoked glutamate release by the cAMP analogue 8-Br-cAMP. Glutamate release was evoked by the addition of 1 mM 4-AP in (i) the absence (control) and (ii) the presence of 100  $\mu\text{M}$  8-Br-cAMP, or (iii) 100  $\mu\text{M}$  8-Br-cAMP + 100  $\mu\text{M}$  IBMX, added 5 min before depolarization. Results are means  $\pm$  s.e.means of three to five independent experiments. Means and s.e.means were calculated at each time-point (2-s), but error bars are only shown every 10-s for clarity. \* $P < 0.05$ ; different from control.

mediated potentiation of glutamate release was concentration-dependent, with 15, 50, and 100  $\mu\text{M}$  ISO increasing glutamate release to  $118.3 \pm 2.2\%$ ,  $168.5 \pm 2.6\%$ , and  $233.6 \pm 2.4\%$  of control values respectively (Figure 2b).

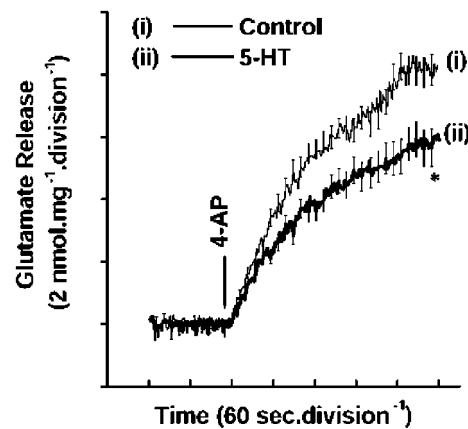
The ISO-mediated potentiation of glutamate release displayed classic  $\beta$ -adrenoreceptor pharmacology in that the facilitation of 4-AP (1 mM)-evoked release by 50  $\mu\text{M}$  ISO was effectively blocked by the  $\beta$ -adrenoreceptor antagonist propranolol (5  $\mu\text{M}$ ) (nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$ ): control 4-AP,  $8.59 \pm 0.79$ ; ISO (50  $\mu\text{M}$ ) + 4-AP,  $21.5 \pm 0.74$ ; propranolol + ISO (50  $\mu\text{M}$ ) + 4-AP,  $8.3 \pm 0.99$  (Figure 2c). To confirm that the  $\beta$ -adrenoreceptor-mediated potentiation of 4-AP-evoked glutamate release occurred as a consequence of the activation of PKA by cyclic nucleotide, we used the non-hydrolysable cAMP analogue, Rp-cyclic-3',5'-adenosine-monophosphothioate (Rp-cAMPS), which acts to specifically inhibit PKA through competitive binding to the cAMP-binding sites on the regulatory subunits of the kinase. The potentiation of control 4-AP (1 mM)-evoked release by 50  $\mu\text{M}$  ISO was effectively prevented by the application of 100  $\mu\text{M}$  Rp-cAMPS ( $9.62 \pm 1.43$  nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$ ) (Figure 2c). These results suggest that the presynaptic  $\beta$ -adrenoreceptor coupling to the facilitation of glutamate release from cerebrocortical nerve terminals is mediated by a cAMP/PKA signalling pathway.

In contrast to the facilitatory effect of  $\beta$ -adrenoreceptor activation on glutamate release, presynaptic 5-HT receptor stimulation produced an inhibition of neurotransmitter release. Control 4-AP (1 mM)-evoked release of  $8.28 \pm 0.94$  nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$  (Figure 3(i)) was attenuated by 5-HT (100  $\mu\text{M}$ ) to  $5.84 \pm 0.4$  nmol  $\text{mg}^{-1}$   $5 \text{ min}^{-1}$  (Figure 3(ii)). Looking for an interplay between facilitatory and inhibitory aminergic signalling pathways modulating glutamate release hitherto described, we next investigated the influence of 5-HT on the potentiation of 4-AP-evoked glutamate release obtained with ISO. Figure 4a compares glutamate release under control conditions (i), in the presence of 5-HT (ii) or ISO (iii) alone, and in the presence of the two



**Figure 2** ISO-mediated potentiation of 4-AP-evoked release of glutamate: Concentration-dependence and sensitivity to  $\beta$ -adreno-receptor and PKA antagonism. (a) Glutamate release was evoked by the addition of 1 mM 4-AP in the absence (control) and in the presence of 50  $\mu$ M ISO, added 5 min before depolarization. (b) Concentration-dependent potentiation of 4-AP-evoked glutamate release by ISO. Results are means  $\pm$  s.e.means of four independent experiments. (c) 4-AP-evoked  $\text{Ca}^{2+}$ -dependent glutamate release in control conditions (control) or in the presence of 50  $\mu$ M ISO, 5  $\mu$ M propranolol + 50  $\mu$ M ISO or 100  $\mu$ M Rp-cAMP + 50  $\mu$ M ISO, added 5 min before 4-AP addition. Columns are the means  $\pm$  s.e.means of four independent experiments. \* $P < 0.05$ ; \*\* $P < 0.01$ ; different from control.

receptor agonists together (iv). Pretreatment with 5-HT severely attenuated the potentiation of 4-AP-evoked glutamate release by ISO, with the magnitude of facilitated release



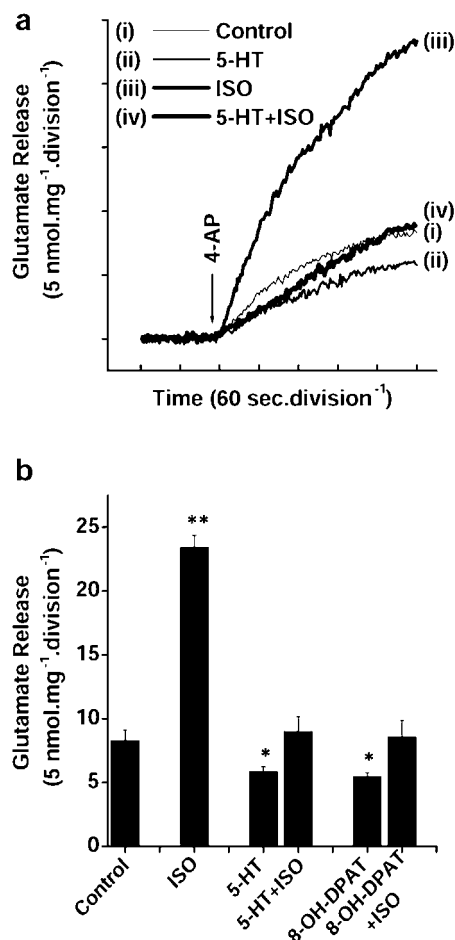
**Figure 3** 5-HT-mediated inhibition of 4-AP-evoked glutamate release. Glutamate release was evoked by the addition of 1 mM 4-AP in (i) the absence (control) and (ii) in the presence of 100  $\mu$ M 5-HT. Results are means  $\pm$  s.e.means of three independent experiments. Means and s.e.means were calculated at each time-point (2-s), but error bars are only shown every 10-s for clarity. \* $P < 0.05$ ; different from control.

decreasing from  $23.4 \pm 0.94$  nmol  $\text{mg}^{-1}$  5  $\text{min}^{-1}$  with ISO alone, back to levels ( $8.98 \pm 1.26$  nmol  $\text{mg}^{-1}$  5  $\text{min}^{-1}$ ) resembling controls when ISO was applied in the presence of 5-HT (Figure 4b).

To determine which 5-HT receptor subtype was involved in the ISO-mediated potentiation of 4-AP-evoked glutamate release, the effect of the selective 5-HT<sub>1A</sub> receptor agonist 8-hydroxy-dipropylaminotetralin (8-OH-DPAT; 10  $\mu$ M) was also examined in parallel experiments. Control 4-AP-evoked release of  $7.40 \pm 1.00$  nmol  $\text{mg}^{-1}$  5  $\text{min}^{-1}$  was attenuated by 8-OH-DPAT to  $4.33 \pm 0.88$  nmol  $\text{mg}^{-1}$  5  $\text{min}^{-1}$  in similar manner to 5-HT (Figure 4b). Also as with 5-HT, in the presence of 8-OH-DPAT, ISO again failed to potentiate the 4-AP-evoked release of glutamate ( $8.57 \pm 1.4$  nmol  $\text{mg}^{-1}$  5  $\text{min}^{-1}$ ) (Figure 4b), suggesting that the effect of 5-HT may be mediated via 5-HT<sub>1A</sub> receptor activation.

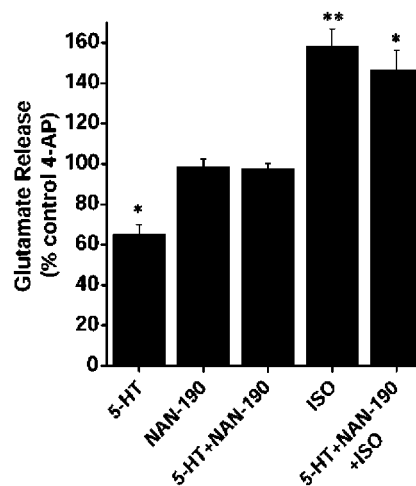
To confirm the extent to which the observed modulation of glutamate release by 5-HT-receptor activation was occurring via specific stimulation of 5-HT<sub>1A</sub> receptors, we looked at the effect of 5-HT in the presence of 5-HT<sub>1A</sub> receptor antagonist 1-(2-methoxyphenyl)-4-(4-phthalimidobutyl)piperazine (NAN-190) (Penington & Kelly, 1990). While NAN-190 (2  $\mu$ M) itself had no effect alone, it eliminated both the 5-HT (100  $\mu$ M)-mediated inhibition of glutamate release, and the 5-HT-mediated abrogation of ISO (50  $\mu$ M)-facilitation (Figure 5). The substantial extent of the latter effect implies that 5-HT<sub>1A</sub> receptors constitute a major proportion of the presynaptic 5-HT receptors being stimulated in our preparation.

The results hitherto suggest that there is antagonistic interaction between  $\beta$ -adrenoreceptors and 5-HT<sub>1A</sub> receptors with respect to the modulation of glutamate release from cerebrocortical synaptosomes. Interestingly, in common with this effect of presynaptic 5-HT receptors, we found a similar abrogatory effect on ISO-mediated potentiation of 4-AP-evoked glutamate release with the activation of inhibitory GABA<sub>B</sub> receptors localized in nerve terminals (Perkinton & Sihra, 1998; Wang & Sihra, unpublished observations). Next, to identify the locus in the second messenger cascade at



**Figure 4** Abrogation of ISO-mediated potentiation by 5-HT and the selective 5-HT<sub>1A</sub> agonist, 8-OH-DPAT. (a) Glutamate release was evoked by the addition of 1 mM 4-AP in (i) the absence (control) and in the presence of (ii) 50 μM ISO, (iii) 100 μM 5-HT or (iv) 100 μM 5-HT + 50 μM ISO, added 5 min before depolarization. (b) Quantitative comparison of the extent of glutamate release evoked by 1 mM 4-AP in the absence and presence of 50 μM ISO (added 5 min before depolarization) and absence and presence of 100 μM 5-HT or 10 μM 8-OH-DPAT (added 2 min before ISO). Results are means ± s.e.means of four independent experiments. \**P* < 0.05; \*\**P* < 0.01; different from control.

which the cross-talk between β-adrenoreceptors and 5-HT receptors takes place, we looked at the effect of 5-HT on the exogenous cAMP-mediated potentiation of glutamate release. If cross-talk occurs at locus downstream of cAMP production, 5-HT should eliminate the effect of cAMP analogue on 4-AP-evoked glutamate release. However, if it occurs more proximally, at the level of G<sub>i</sub> modulation of AC activity for instance, then the activation of PKA effected by the cAMP analogue should persist in causing the facilitation by 8-Br-cAMP observed in Figure 1. As before (Figure 1), these experiments employing cAMP analogues to stimulate PKA were performed in the presence of IBMX to both prevent the degradation of cAMP analogue, and obviate the inhibitory action of adenosine A<sub>1</sub> receptors. Figure 6 shows that the potentiation of control 4-AP-evoked release of 6.77 ± 1.77 nmol mg<sup>-1</sup> 5 min<sup>-1</sup> (Figure 6(i)) to 18.3 ± 3.04 nmol mg<sup>-1</sup> 5 min<sup>-1</sup> produced by 8-Br-cAMP + IBMX (Figure 6(ii)) was effectively blocked in synaptosomes pretreated with 5-HT to



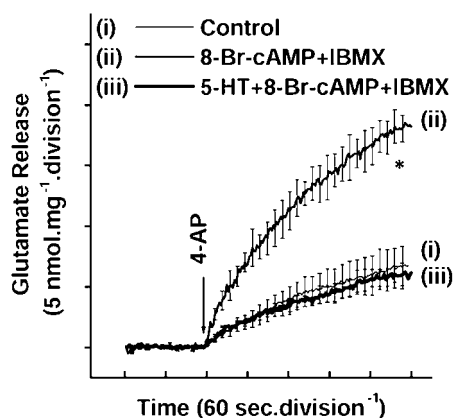
**Figure 5** Elimination of 5-HT receptor-mediated abrogation of β-adrenoreceptor-mediated facilitation of glutamate by the 5-HT<sub>1A</sub> antagonist, NAN-190. Modulation of 1 mM 4-AP-evoked glutamate release by 100 μM 5-HT, in the absence or presence of 2 μM NAN-190 (added 5 min before 5-HT). Lack of effect of 100 μM 5-HT on the 50 μM ISO-induced facilitation in the presence of 2 μM NAN-190. Results are presented as per cent of the control 4-AP-evoked release and are means ± s.e.means of three to five independent experiments. \**P* < 0.05; \*\**P* < 0.01; different from control.

produce release indistinguishable from control levels (5.95 ± 0.75 nmol mg<sup>-1</sup> 5 min<sup>-1</sup>, Figure 6(iii)). These results indicated that the interaction between the β-adrenoreceptor and 5-HT receptor likely occurs downstream of cAMP production and possibly at the level of PKA-dependent modulation of events, such as nerve terminal excitation, Ca<sup>2+</sup> influx, or the processes leading up to and possibly including exocytosis itself.

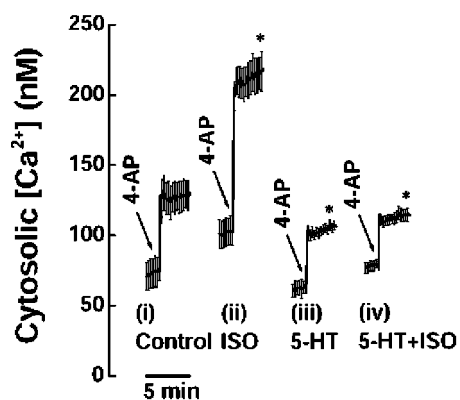
Looking directly at the involvement of the modulation of Ca<sup>2+</sup> influx in the cross-talk between β-adrenoreceptors and 5-HT receptors, we used Fura-2 to assess the effects of ISO and 5-HT on the 4-AP-evoked increase of [Ca<sup>2+</sup>]<sub>c</sub>. 4-AP (1 mM) caused a rise in [Ca<sup>2+</sup>]<sub>c</sub> to a plateau level of 128.3 ± 10.7 nM (Figure 7(i)). This 4-AP-evoked rise in [Ca<sup>2+</sup>]<sub>c</sub> was increased by 61.9 nM with ISO (50 μM) preincubation (Figure 7(ii)) and slightly decreased by 13.7 nM in the presence of 5-HT (100 μM) (Figure 7(iii)). Despite the modest inhibitory effect of 5-HT alone on Ca<sup>2+</sup> influx, the agonist markedly reduced the substantial potentiation produced by ISO (Figure 7(iv)). While ISO preincubation also caused a small but notable increase in the basal levels of [Ca<sup>2+</sup>]<sub>c</sub> prior to 4-AP-mediated depolarization, this effect was statistically not significant (Figure 7(ii)).

## Discussion

Our results demonstrate that 5-HT strongly suppresses the facilitatory effects of exogenous cAMP and the β-adrenoreceptor agonist, isoprenaline (ISO), on glutamate release from cerebrocortical nerve terminals. This suggests that there is antagonistic interaction or cross-talk, between presynaptic β-adrenoreceptor and 5-HT receptor signalling cascades. While previous electrophysiological studies in the basolateral amygdala had evinced presynaptic interaction indirectly by



**Figure 6** Antagonism of 8-Br-cAMP-mediated potentiation of glutamate release by 5-HT. Glutamate release was evoked by the addition of 1 mM 4-AP in (i) the absence (control) and in the presence of (ii) 100  $\mu$ M 8-Br-cAMP or (iii) 100  $\mu$ M 5-HT + 100  $\mu$ M 8-Br-cAMP, added 5 min before depolarization. In the experiments with 5-HT, the agonist was added 2 min before 8-Br-cAMP. Results are means  $\pm$  s.e.means of four to five independent experiments. Means and s.e.means were calculated at each time-point (2-s), but error bars are only shown every 10-s for clarity. \* $P < 0.05$ ; different from control.



**Figure 7** Facilitation of 4-AP-evoked  $\text{Ca}^{2+}$  influx by  $\beta$ -adrenoceptor activation is suppressed by 5-HT. Cytosolic free  $\text{Ca}^{2+}$  concentration (nM) was monitored using Fura-2. Synaptosomes were stimulated with 1 mM 4-AP in (i) the absence (control) and in the presence of (ii) 50  $\mu$ M ISO, (iii) 100  $\mu$ M 5-HT or (iv) 100  $\mu$ M 5-HT + 50  $\mu$ M ISO, added 5 min before stimulation. Results are means  $\pm$  s.e.means of five independent experiments. Means and s.e.means were calculated at each 3.75-s time point, but error bars are shown for every fourth reading for clarity. \* $P < 0.05$ ; different from control.

looking at changes in postsynaptic responses (Wang *et al.*, 1999), using the synaptosomal model, we provide direct evidence for a presynaptic locus and mechanism for the modulation of glutamatergic transmission by cross-regulation of these two GPCR signalling systems.

#### *$\beta$ -Adrenoceptor-mediated facilitation of glutamate release*

Electrophysiological studies have shown that elevation of cAMP and PKA stimulation can enhance excitatory transmission in the Schaeffer collateral-CA1 (Chavez-Noriega

& Stevens, 1992; Gereau & Conn, 1994; Huang & Kandel, 1994) and mossy fibre-CA3 (Weisskopf *et al.*, 1994) pathways of the hippocampus, as well as in the basolateral amygdala (Huang *et al.*, 1996; 1998). Here, the  $\beta$ -adrenoceptor agonist ISO was found to potentiate 4-AP-evoked glutamate release from cerebrocortical synaptosomes. Sensitivity of this effect to the PKA inhibitor, Rp-cAMPS, and the ability of the cAMP analogue, 8-Br-cAMP, to mimic the effect of  $\beta$ -adrenoceptor activation, implicates an AC/cAMP/PKA signalling cascade in the enhancement of glutamate release (Herrero & Sanchez-Prieto, 1996).

Posttranslational modification of proteins by phosphorylation may regulate neurotransmitter release at multiple loci in the stimulus-exocytosis cascade including: ion-channels modulating nerve terminal excitability (Frace & Hartzell, 1993; Roeper & Pongs, 1996; Dascal & Lotan, 1991; Li *et al.*, 1993), voltage-dependent  $\text{Ca}^{2+}$  channels (VDCCs) and, downstream of  $\text{Ca}^{2+}$  entry, components of the synaptic vesicle trafficking and exocytotic apparatus (Thompson *et al.*, 1993; Trudeau *et al.*, 1996; Sihra & Nichols, 1993). Given the significant effect of ISO on the 4-AP-evoked increase in  $[\text{Ca}^{2+}]_c$  observed here, it is tempting to postulate a PKA-mediated effect on either channels involved in determining synaptosomal excitability or VDCC subunits themselves, to upregulate  $\text{Ca}^{2+}$  influx/glutamate release.

In the absence of direct evidence of phosphorylation, a number of synaptosomal studies have suggested a delayed rectifier  $\text{K}^+$ -channel target for the modulation of nerve terminal excitability by protein phosphorylation (Sanchez-Prieto *et al.*, 1996). An effect of PKA at this locus has been suggested based on the enhancement of facilitation of 50  $\mu$ M 4-AP-mediated depolarization by forskolin (Herrero & Sanchez-Prieto, 1996). Notably however, in contrast to our clear demonstration of 100  $\mu$ M ISO-mediated facilitation of 1 mM 4-AP-evoked  $\text{Ca}^{2+}$  entry and glutamate release, no modulation was obtained with 1 mM 4-AP in this previous study. Although the reasons for this discrepancy are not immediately apparent, one obvious notable difference, apart from our use of a percoll-gradient purified synaptosome preparation (cf. Herrero & Sanchez-Prieto, 1996), was the significantly higher resting  $[\text{Ca}^{2+}]_c$  reported ( $\sim 160$  nM, Herrero & Sanchez-Prieto, 1996 versus  $\sim 70$  nM, current study). One consequence of raised  $[\text{Ca}^{2+}]_c$  would be the activation of nerve terminal  $\text{Ca}^{2+}$ -dependent protein kinases and phosphatases (Nichols *et al.*, 1990; Sihra *et al.*, 1995; Burley & Sihra, 2000). The inevitable alteration of the phosphorylation-state of key modulatory substrate proteins may subsequently affect the responsiveness of 4-AP-evoked release to modulation by PKA differentially, depending on the levels of voltage-dependent  $\text{Ca}^{2+}$ -entry (Herrero & Sanchez-Prieto, 1996).

Our observation, using membrane potential-sensitive fluorophors (Perkinton & Sihra, 1998), that 1 mM 4-AP-evoked depolarization of synaptosomes is minimally affected even after substantive stimulation of the cAMP/PKA pathway using the AC activator forskolin (Wang and Sihra, unpublished observations), argues against a major effect of PKA on synaptosomal excitability. Several previous studies have used the differential mechanisms of depolarization with 4AP and high- $[\text{K}^+]_{\text{external}}$  (high-KCl) to resolve effects on channels controlling synaptosomal excitability (4AP  $\neq$  high-KCl) as opposed to VDCCs (4AP  $\equiv$  high-KCl) (Barrie *et al.*,

1991; Coffey *et al.*, 1993; Sanchez-Prieto *et al.*, 1996 for review). However, the strong, clamped, tetrodotoxin-insensitive depolarization produced by KCl causes ion-channel inactivation and evidently adversely affects modulatory influences known to impinge on VDCCs as we have observed (Perkinton & Sihra, 1998; Davies & Sihra, unpublished observations). A lack of effect of PKA activation on KCl-mediated glutamate release, as has been reported (Herrero & Sanchez-Prieto, 1996), may not therefore be unequivocal evidence against a modulatory effect on VDCCs and for an upstream regulation of synaptosomal excitability. Importantly, in electrophysiological studies directly addressing the involvement of ion-channel activities during synaptic transmission, using patched neurons under voltage-clamp, delayed rectifier K<sup>+</sup>-currents were reported to be recalcitrant to modulation by PKA, while a clear modulation of Ca<sup>2+</sup> currents was demonstrable (Huang *et al.*, 1998). While this did not exclude the action of PKA at other channel targets determining synaptosomal excitability, in this type of study, PKA could be directly implicated in the modulation of P-type VDCCs.

Given that glutamate exocytosis is triggered by a localized increase in [Ca<sup>2+</sup>]<sub>c</sub> (Sihra *et al.*, 1992) via specific P/Q- and N-subtypes of VDCCs (Pocock & Nicholls, 1992; Turner *et al.*, 1993), direct regulation of Ca<sup>2+</sup> entry into the nerve terminals would be potent means to regulate synaptic transmission. Indeed, there is compelling evidence for the posttranslational modulation of VDCCs by direct phosphorylation of both  $\alpha_1$ - and  $\beta$ -subunits of VDCCs, and by a number of protein kinases, including PKA (Hell *et al.*, 1995; Chien & Hosey, 1998; Catterall, 2000). With both P/Q-type and N-type VDCCs, receptor-mediated increases in the PKA-dependent phosphorylation appear to facilitate channel activity (Gray & Johnston, 1987; Catterall, 2000). Our postulate, that a  $\beta$ -adrenoreceptor/AC/cAMP/PKA signaling cascade may be enhancing glutamate release by action at the VDCC level, is therefore consistent with these observations and others, explicitly showing ISO-mediated enhancement of P- and Q-type Ca<sup>2+</sup> currents recorded under voltage-clamp conditions (Huang *et al.*, 1996; 1998).

#### *5-HT Receptors mediate inhibition of glutamate release*

In contrast to  $\beta$ -adrenoreceptor stimulation, 5-HT receptor activation in nerve terminals suppressed glutamate release. There is a consensus that presynaptic inhibition by 5-HT is mediated by the 5-HT<sub>1</sub> receptor type and, depending on the system, specifically by 5-HT<sub>1A</sub> (Bobker & Williams, 1989; Cheng *et al.*, 1998; Lin *et al.*, 2001), 5-HT<sub>1B</sub> (Bobker & Williams, 1989; Tanaka & North, 1993; Singer *et al.*, 1996) and 5-HT<sub>1D</sub> (Travagli & Williams, 1996; Maura & Raiteri, 1996; Maura *et al.*, 1998) subtypes. In the current study, we found that the selective 5-HT<sub>1A</sub> receptor agonist, 8-OH-DPAT, mimicked the effect of 5-HT. On the other hand, we obtained no significant effect with the 5-HT<sub>1B</sub>/5-HT<sub>1D</sub> agonist sumatriptan (data not shown). Indeed, the reversal of inhibitory effect of 5HT alone, as well as its abrogation of the ISO-induced facilitation of release, by NAN-190, reinforces the involvement of presynaptic 5-HT<sub>1A</sub> receptors in the observed modulation of glutamate release. Interestingly, in contrast to these results with cerebrocortical synaptosomes, with cerebellar synaptosomes, a significant

inhibition of glutamate release by sumatriptan coupled with a lack of effect of 5-HT<sub>1A</sub> receptor activation, has implicated 5-HT<sub>1D</sub> receptors presynaptically, with 5-HT<sub>1A</sub> receptors being proposed to have post-synaptic function (Maura & Raiteri, 1996).

The reduction of 4-AP-evoked voltage-dependent Ca<sup>2+</sup> influx produced by 5-HT, points to the suppression of VDCCs as a potential mechanism underlying the inhibition of glutamate exocytosis by 5-HT<sub>1A</sub> receptor activation. Electrophysiological studies, in several neuronal systems, have demonstrated 5-HT-mediated inhibition of VDCCs, in general (Penington & Kelly, 1990; Rhee *et al.*, 1996; Cheng *et al.*, 1998), and of P/Q- and N-type VDCCs, specifically (Foehring, 1996; Bayliss *et al.*, 1997; Lin *et al.*, 2001). This suppression of Ca<sup>2+</sup> influx by 5-HT<sub>1A</sub> receptor activation is thought to occur through a direct, membrane-delimited, G<sub>i/o</sub>-coupled inhibition of VDCCs (Foehring, 1996; Cheng *et al.*, 1998; Lin *et al.*, 2001), and is akin to presynaptic inhibition produced by GPCRs like GABA<sub>B</sub> (Wu & Saggau, 1995; Perkinton & Sihra, 1998) and adenosine A<sub>1</sub> receptors (Barrie *et al.*, 1991; Wu & Saggau, 1994; Lu & Gean, 1999).

#### *Cross-talk of $\beta$ -adrenoreceptor and 5-HT signalling in nerve terminals*

In addition to the inhibitory effect of 5-HT receptor stimulation on 4-AP-evoked release of glutamate, 5-HT effectively blocked the facilitatory effect of PKA activation on glutamate release, whether the kinase was stimulated by endogenous cAMP produced by ISO-mediated  $\beta$ -adrenoreceptor activation of AC, or by exogenous cyclic nucleotide, 8-Br-cAMP. Given that co-application of ISO and 5-HT did not produce additive effects—the substantial ISO-mediated potentiation of glutamate release and Ca<sup>2+</sup> influx are completely abolished by 5-HT, despite the much more modest inhibitory effect of the latter alone—the interaction of  $\beta$ -adrenoreceptor and 5-HT receptor signalling pathways can be interpreted as the presynaptic coexistence and cross-talk of the two systems in a major population of cerebrocortical synaptosomes. This  $\beta$ -adrenoreceptor and 5-HT receptor cross-talk is consistent with a presynaptic origin for the previously reported inhibitory actions of 5-HT<sub>1A</sub> receptor activation on ISO-mediated enhancement of excitatory postsynaptic potentials recorded in amygdalar neurons (Wang *et al.*, 1999).

The antagonistic cross-talk between  $\beta$ -adrenoreceptors and 5-HT receptors described could in principle occur at one of several loci in GPCR signal transduction, viz. G-protein/AC coupling, cAMP metabolism, PKA activation, or alternatively, at the substrate level of presynaptic ion channels and/or release processes. At the level of AC, opposing homotypic and heterotypic interactions between stimulatory G<sub>s</sub>- and inhibitory G<sub>i</sub>-linked receptors have been reported (Pedarzi & Storm, 1996; Lopes *et al.*, 1999; Gerber & Gahwiler, 1994). Reciprocity with Ca<sup>2+</sup>/calmodulin-sensitive isoforms of AC (Mons & Cooper, 1995; Xia & Storm, 1997) may also occur by inhibitory receptors effecting membrane-delimited G<sub>i/o</sub>-mediated inhibition of VDCCs (Wu & Saggau, 1997). In the current experiments however, interaction at the level of AC appears unlikely because the 5-HT suppression of 4-AP-evoked glutamate release occurred even with AC activation circumvented using exogenous 8-Br-cAMP to activate PKA

directly.  $\beta$ -adrenoreceptors and 5-HT receptors therefore seem to cross-talk downstream of PKA activation and at the level of one or more of the protein kinase substrates.

Our observation that both ISO and 5-HT affect  $\text{Ca}^{2+}$ -entry, invokes the possibility that the two GPCR signalling cascades stimulated by these agonists may interact at the level of VDCCs to regulate glutamate release. Overtly, in concert with the 5-HT suppression of ISO-mediated facilitation of glutamate release, 5-HT strongly abrogated the facilitation of  $\text{Ca}^{2+}$ -influx by ISO, despite having a relatively modest inhibitory effect on  $\text{Ca}^{2+}$ -influx/release by itself. One possible implication of this is that,  $\text{G}_{\beta\gamma}$ -linked GPCRs causing inhibition of  $\text{Ca}^{2+}$  influx, converge with  $\text{G}_s$ -linked/AC GPCRs at the level of presynaptic VDCCs. In support of this notion, baclofen, a  $\text{GABA}_B$  receptor agonist known to inhibit presynaptic VDCCs (Wu & Saggau, 1995; Pfrieger *et al.*, 1994; Perkinson & Sihra, 1998), also suppressed ISO-mediated potentiation of glutamate release similarly to 5-HT (Wu & Saggau, 1997; Wang & Sihra, unpublished observations). However, the mechanism of the proposed abrogation of PKA-mediated facilitation of  $\text{Ca}^{2+}$  entry, by inhibitory

GPCRs negatively-coupled to VDCCs, remains to be elucidated.

Together with the well documented reciprocity of  $\text{G}_s$ - and  $\text{G}_{\beta\gamma}$ -coupled GPCRs at the level of AC, the current study suggests a presynaptic interaction of GPCR/ $\text{G}_s$ -protein/AC/PKA-mediated facilitation and GPCR/ $\text{G}_{\beta\gamma}$ -protein-mediated inhibition, possibly at the level of VDCCs, to affect  $\text{Ca}^{2+}$  entry and glutamate release. Physiologically, the coincidence and interplay between such opposing signalling cascades, by modulating nerve terminal activity, may play a crucial role in presynaptic plasticity. Pharmacologically, given that excitotoxic release of glutamate likely underlies the aetiology of a number of pathophysiological conditions (Meldrum & Garthwaite, 1990), the ability of 5-HT to moderate glutamate release suggests the potential clinical application of 5-HT<sub>1A</sub> receptors agonists as neuroprotective agents.

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