

Metabolomic Approaches to Defining the Role(s) of GABA_ρ Receptors in the Brain

Caroline Rae · Fatima A. Nasrallah · Vladimir J. Balcar ·
 Benjamin D. Rowlands · Graham A. R. Johnston ·
 Jane R. Hanrahan

Received: 6 December 2014 / Accepted: 26 December 2014 / Published online: 11 January 2015
 © Springer Science+Business Media New York 2015

Abstract The inhibitory neurotransmitter γ -aminobutyric acid (GABA) acts through various types of receptors in the central nervous system. GABA_ρ receptors, defined by their characteristic pharmacology and presence of ρ subunits in the channel structure, are poorly understood and their role in the cortex is ill-defined. Here, we used a targeted pharmacological, NMR-based functional metabolomic approach in Guinea pig brain cortical tissue slices to identify a distinct role for these receptors. We compared metabolic fingerprints generated by a range of ligands active at GABA_ρ and included these in a principal components analysis with a library of other metabolic fingerprints obtained using ligands active at GABA_A and GABA_B, with inhibitors of GABA uptake and with compounds acting to inhibit enzymes active in the GABAergic system. This enabled us to generate a metabolic “footprint” of the GABAergic system which revealed classes of metabolic activity associated with GABA_ρ which are distinct from other GABA receptors. Antagonised GABA_ρ

produce large metabolic effects at extrasynaptic sites suggesting they may be involved in tonic inhibition.

Keywords Functional metabolomics · ¹³C NMR spectroscopy · Principal components analysis · GABA(C)

Introduction

The actions of the major inhibitory neurotransmitter γ -aminobutyric acid (GABA) are mediated via several types of receptors. According to the classical concept these include the bicuculline sensitive ionotropic GABA_A receptors (Barnard et al. 1998), pentamers formed by several characteristic protein subunits (Collingridge et al. 2009) and the baclofen sensitive, metabotropic GABA_B receptors (Bowery et al. 2002). In addition, a receptor which is insensitive to both bicuculline and baclofen, and not affected by GABA_A modulators such as benzodiazepines and barbiturates, the GABA_ρ receptor (Drew et al. 1984; Johnston 1996), has been described.

The GABA_ρ receptor is a pentameric complex whose molecular components are ρ -subunits ($\rho 1$, $\rho 2$ and $\rho 3$). Originally believed only to coassemble with other ρ -subunits (Enz and Cutting 1998) there is now some evidence for “mixed” functional receptors which also exhibit pharmacologically distinct responses to those from homooligomeric GABA_ρ receptors (Enz and Cutting 1999). While convincing evidence exists only for the location of functional homomeric (ρ -subunits only) receptors in the retina (Enz and Cutting 1998) there are now strong indications of the presence of mixed receptors in the cerebellum (Harvey et al. 2006), hippocampus (Hartmann et al. 2004; Alakuijala et al. 2006) and brain stem (Milligan et al. 2004).

C. Rae (✉) · F. A. Nasrallah · B. D. Rowlands
 Neuroscience Research Australia, Barker St, Randwick,
 NSW 2031, Australia
 e-mail: c.rae@unsw.edu.au

C. Rae · B. D. Rowlands
 School of Medical Sciences UNSW, The University of New South
 Wales, Sydney, NSW 2052, Australia

V. J. Balcar
 Bosch Institute and School of Medical Sciences, The University of
 Sydney, Sydney, NSW 2006, Australia

G. A. R. Johnston
 Pharmacology, School of Medical Sciences, The University of
 Sydney, Sydney, NSW 2006, Australia

J. R. Hanrahan
 Faculty of Pharmacy, The University of Sydney, Sydney,
 NSW 2006, Australia

While the expression of $\rho 1$ subunits is very low in the cortex, there is evidence for expression of $\rho 2$ and $\rho 3$ receptor subunits in rat and human cortex (Enz et al. 1996; Boue-Grabot et al. 1998; Wegelius et al. 1998; Enz and Cutting 1999) but not in bovine parietal cortex (López-Chávez et al. 2005). It remains to be seen whether the mixed receptor activity reported to date arises from a truly heteromeric receptor or from GABAergic activity mediated via pseudohomomeric GABA $_{\rho}$ receptors.

Compared to most GABA $_A$ receptors, GABA $_{\rho}$ receptors show higher affinity for GABA. Additional characteristics include longer mean opening and closing times, smaller chloride conductance than GABA $_A$ receptors and slow (or a lack of) desensitization. Thus, GABA $_{\rho}$ receptor-mediated inhibition is thought to occur at lower concentrations of GABA and to last longer (Bormann and Feigenspan 1995). With these physiological characteristics in mind, it has been suggested that GABA $_{\rho}$ receptors are likely to be located extrasynaptically (Alakuijala et al. 2006).

We have developed a method for determining the metabolic effects of targeted pharmacological activity, using a Guinea pig cortical tissue slice model, $^{13}\text{C}/^1\text{H}$ NMR spectroscopy and multivariate statistics. The system has proven a robust indicator of subtle changes in metabolic activity brought about by modulating receptors (Rae et al. 2005, 2006, 2009; Nasrallah et al. 2007, 2009, 2010a) and other critical components of the synapse such as transporters (Rae et al. 2003; Bröer et al. 2007; Moussa et al. 2007). Here, we show distinct metabolic responses by Guinea pig cerebral cortical slices to ligands showing strong preference for GABA $_{\rho}$ receptors.

Materials and Methods

Materials

Guinea pigs (Dunkin-Hartley), weighing 400–800 g, were fed ad libitum on standard Guinea pig/rabbit pellets, with fresh cabbage leaves and lucerne hay roughage. Animals were maintained on a 12 h light/dark cycle. All experiments were conducted in accordance with the guidelines of the National Health and Medical Research Council of Australia and were approved by the institutional (UNSW) Animal Care Ethics Committee.

Sodium [$3\text{-}^{13}\text{C}$]pyruvate and sodium [^{13}C]formate were purchased from Cambridge Isotope Laboratories Inc (Andover, MA). CACA (*cis*-4-aminocrotonic acid) and resveratrol (3,4',5-trihydroxy-trans-stilbene) were purchased from Sigma Aldrich (St Louis, MO) and TPMPA (1,5,6-tetrahydropyridin-4-yl)methylphosphinic acid) were purchased from Tocris Cookson, (UK). (\pm)-*cis*-(3-Aminocyclopentyl) (butyl)phosphinic acid (*cis*-ACBPBA) was synthesised as published previously (Hanrahan et al.

2006), as was *cis*-2-(aminomethyl)-1-carboxycyclopropane (\pm)-CAMP (Duke et al. 1998) and *S*- and *R*-4-amino-1-cyclopent-1-en-1-yl(butyl)phosphinic acids, (*S*- and *R*-4-ACBPBA) (Kumar et al. 2008). All other reagents were of AR grade.

Modulation of GABA $_{\rho}$ Activity

Guinea pig cortical brain slices (350 μM in para-axial plane) were prepared as previously described (Rae and Balcar 2014). To determine the effect of modulation of GABA $_{\rho}$ receptors on metabolic activity, slices were incubated with 2 mM sodium [$3\text{-}^{13}\text{C}$]pyruvate (control) and also, in the case of ligand treatment groups, with one of two concentrations of the ligand. Pyruvate was used as the substrate of choice because it labels more compartments than glucose and hence provides more opportunity for discrimination (Rae et al. 2003, 2005, 2006; Bröer et al. 2007; Moussa et al. 2007; Nasrallah et al. 2007).

We used the GABA $_{\rho}$ partial agonist CACA (Allan et al. 1980; Kusama et al. 1993) at 20 and 100 μM . CACA has been demonstrated to show higher affinity for GABA $_{\rho}$ over other GABA $_A$ receptors than the alternative agonist *trans*-4-aminocrotonic acid (TACA); or alternatively to activate a bicuculline insensitive member of the large GABA $_A$ receptor family (Matthews et al. 1994) (Cl^- conductance $K_{1/2} \sim 50 \mu\text{M}$). CACA has also been reported to be inactive at GABA $_B$ (Kerr and Ong 1995) but, again, has been demonstrated to inhibit a GTP-dependent Ca^{2+} current at much lower concentrations ($<1 \mu\text{M}$) than that required to activate the Cl^- current, suggested to be a baclofen-insensitive member of the GABA $_B$ receptor family (Matthews et al. 1994).

The GABA $_{\rho}$ agonist (\pm)-CAMP (Duke et al. 1998) was used at 50 and 200 μM . (+)-CAMP has been reported to be the active diastereomer; $\rho 1$ ($K_d \sim 40 \mu\text{M}$) and $\rho 2$ ($K_d \sim 17 \mu\text{M}$) and both (+)- and (–)-CAMP are inactive at GABA transporters (Duke et al. 2000).

The GABA $_{\rho}$ antagonist TPMPA was used (at 10 and 100 μM). TPMPA has been reported to be selective for GABA $_{\rho}$ ($K_b = 2 \mu\text{M}$) over GABA $_A$ ($K_b \approx 320 \mu\text{M}$) and GABA $_B$ ($\text{EC}_{50} \approx 500 \mu\text{M}$) (Ragozzino et al. 1996).

The GABA $_{\rho}$ antagonist *cis*-ACBPBA (Table 1) was used at approximately equipotent concentrations to TPMPA (4 and 40 μM).

The GABA $_{\rho}$ antagonist SGS-742 (CGP36742; 3-aminopropyl(butyl)phosphinic acid) was used at 10 and 100 μM . SGS742 is an antagonist at GABA $_B$ but also has antagonist activity at GABA $_{\rho}$ and GABA $_A$ (Table 1). At 10 μM SGS742 should mostly antagonise GABA $_B$, while at 100 μM it should antagonise both GABA $_B$ and GABA $_{\rho}$, but have little activity at GABA $_A$ (Table 1).

The subtype-selective GABA $_{\rho}$ antagonists *S*-ACBPBA (5 μM) and *R*-ACBPBA (3 μM) were used at concentrations equipotent at $\rho 2$ (Table 1). This meant that *R*-ACBPBA was

Table 1 Kinetic properties of ligands active at GABA_A receptors

	GABA _A	GABA _ρ	GABA _B
GABA	$\alpha_1\beta_2\gamma$ EC ₅₀ =21.1 μ M(Kumar et al. 2008) $\alpha_4\beta_3\delta$ EC ₅₀ =2.7 μ M(Storustovu and Ebert 2006) $\alpha_6\beta_3\delta$ EC ₅₀ =0.49 μ M(Storustovu and Ebert 2006)	ρ_1 EC ₅₀ =2.5 μ M(Kusama et al. 1993) ρ_2 EC ₅₀ =2.5 μ M(Kusama et al. 1993) ρ_3 EC ₅₀ =4.0 μ M(Kusama et al. 1993)	1.7 μ M(Kumar et al. 2008)
Muscimol	Agonist $\alpha_1\beta_2\gamma$ EC ₅₀ =7.1 μ M(Ebert et al. 1997) $\alpha_4\beta_3\delta$ EC ₅₀ =1.1 μ M, I _{max} =144 % (Storustovu and Ebert 2006) $\alpha_6\beta_3\delta$ EC ₅₀ =0.16 μ M, I _{max} =183 % (Storustovu and Ebert 2006)	Partial agonist ρ_1 EC ₅₀ =3.5 μ M, I _{max} =83 % (Kusama et al. 1993) ρ_2 EC ₅₀ =1.4 μ M, I _{max} =63 % (Kusama et al. 1993) ρ_3 EC ₅₀ =1.9 μ M(Vien et al. 2002)	Inactive
Isoguvacine	Agonist EC ₅₀ =310 μ M(Murata et al. 1996)	Partial agonist EC ₅₀ =99 μ M, I _{max} =25 % (Woodward et al. 1993)	Inactive
THIP	Agonist $\alpha_1\beta_2\gamma$ EC ₅₀ =355 μ M(Ebert et al. 1997) $\alpha_4\beta_3\delta$ EC ₅₀ =51.8 μ M, I _{max} =215 % (Storustovu and Ebert 2006) $\alpha_6\beta_3\delta$ EC ₅₀ =8.2 μ M, I _{max} =309 % (Storustovu and Ebert 2006)	Antagonist ρ_1 K _B =30.3 μ M(Carland et al. 2004) ρ_3 IC ₅₀ =10.2 μ M(Vien et al. 2002)	Inactive
CACA	Inactive<5000 μ M	Partial agonist ρ_1 EC ₅₀ =74 μ M, I _{max} =78 % (Duke et al. 2000) ρ_2 EC ₅₀ =70 μ M, I _{max} =82 % (Duke et al. 2000) ρ_3 EC ₅₀ =139 μ M, I _{max} =57 % (Vien et al. 2002)	Inactive
TACA	Agonist EC ₅₀ =133 μ M(Ragozzino et al. 1996)	Agonist ρ_1 EC ₅₀ =0.44 μ M(Chebib et al. 1997) ρ_2 EC ₅₀ =0.3 μ M(Kusama et al. 1993) ρ_3 EC ₅₀ =3.8 μ M(Vien et al. 2002)	Inactive
(+)-CAMP	V weak antagonist(Duke et al. 2000)	Agonist ρ_1 EC ₅₀ =40 μ M, I _{max} =104 % (Duke et al. 2000) ρ_2 EC ₅₀ =17 μ M, I _{max} =108 % (Duke et al. 2000)	Inactive
(-)-CAMP	V weak antagonist(Duke et al. 2000)	V weak agonist ρ_1 IC ₅₀ =900 μ M(Duke et al. 2000) ρ_2 IC ₅₀ =400 μ M(Duke et al. 2000)	Inactive
(±)-CAMP		Partial agonist ρ_3 EC ₅₀ =4.8 μ M, I _{max} =57 % (Vien et al. 2002)	Inactive
(+)-TAMP	Partial agonist EC ₅₀ =484 μ M, I _{max} =51 % (Duke et al. 2000)	Partial agonist ρ_1 EC ₅₀ =60 μ M, I _{max} =39 % (Duke et al. 2000) ρ_2 EC ₅₀ =30 μ M, I _{max} =61 % (Duke et al. 2000)	Inactive
(-)-TAMP	Partial agonist EC ₅₀ =50–60 μ M, I _{max} =51 % (Duke et al. 2000)	Partial agonist ρ_1 EC ₅₀ =9 μ M, I _{max} =39 % (Duke et al. 2000) ρ_2 EC ₅₀ =3 μ M, I _{max} =55 % (Duke et al. 2000)	Inactive
(±)-TAMP		Antagonist ρ_3 K _B =4.8 μ M(Vien et al. 2002)	
*Picrotoxin	Antagonist \wedge IC ₅₀ =5 μ M(Xu et al. 1995)	Antagonist ρ_1 \wedge IC ₅₀ =0.8 μ M(Carland et al. 2008)	Inactive
TPMPA	V weak antagonist K _B =320 μ M(Murata et al. 1996)	Antagonist ρ_1 K _B =2.1 μ M(Murata et al. 1996) ρ_2 K _B =15.6 μ M(Chebib et al. 1998a) ρ_3 K _B =10.2 μ M(Vien et al. 2002)	V weak agonist EC ₅₀ ~500 μ M(Murata et al. 1996)
(±)-cis-ACBPBA	V weak antagonist 1 mM inhibits GABA EC ₅₀ by 27 %	Antagonist (Chebib et al. 2009b) ρ_1 K _B =5.06 μ M ρ_2 K _B =11.08 μ M	Weak antagonist (Chebib et al. 2009b) 1 mM inhibits GABA EC ₅₀ by 33 %

Table 1 (continued)

	GABA _A	GABA _ρ	GABA _B
(±)-trans-ACPBPA	V weak antagonist 1 mM inhibits GABA EC ₅₀ by 35 %	Antagonist (Chebib et al. 2009b) ρ ₁ K _B =72.58 μM ρ ₂ K _B =189.7 μM	Weak antagonist(Chebib et al. 2009b) 1 mM inhibits GABA EC ₅₀ by 28 %
(R)-4-ACPBPA	Inactive at 600 μM(Kumar et al. 2008)	Antagonist ρ ₁ K _B =59.3 μM(Kumar et al. 2008) ρ ₂ K _B =6.22 μM(Hanrahan and Chebib)	Inactive<300 μM(Kumar et al. 2008)
(S)-4-ACPBPA	Inactive<600 μM(Kumar et al. 2008)	Antagonist ρ ₁ K _B =4.97 μM(Kumar et al. 2008) ρ ₂ K _B =11.24 μM(Hanrahan and Chebib)	Inactive<300 μM(Kumar et al. 2008)
Baclofen	Inactive	Inactive	Agonist EC ₅₀ =0.8 μM(Bolser et al. 1995)
Guanidinoacetate	Partial agonist EC ₅₀ =167±40 μM (Neu et al. 2002)	Antagonist ρ ₁ [#] IC ₅₀ =5.4 μM(Chebib et al. 2009a)	Inactive (Neu et al. 2002)
SGS742	Antagonist IC ₅₀ =508 μM(Chebib et al. 1997)	Antagonist ρ ₁ [#] IC ₅₀ =62 μM(Chebib et al. 1997)	Antagonist IC ₅₀ =38 μM(Chebib et al. 1997)
SCH50911	Inactive<100 μM	Inactive≤100 μM	Antagonist(Bolser et al. 1995) IC ₅₀ =3.5 μM
Resveratrol		Non-competitive inhibitor (Lee et al. 2013) ρ ₁ IC ₅₀ =28.9±2.8 μM	

*Also an inhibitor at other cys loop receptors e.g., glycine, 5HT₃ receptors

[#] At GABA EC₅₀

[^]Depends on α subunit

active at ρ₂ but would have had little activity at ρ₁, while S-ACPBPA would be expected to be active at both subtypes.

Resveratrol, reported to be a non-competitive antagonist at GABA_ρ with an IC₅₀ of 28.9±2.8 μM (Lee et al. 2013), was used at 48 μM.

Slices were incubated for 1 h with 2 mM [3-¹³C]pyruvate (control) and the different concentrations of each of the ligands.

Preparation of Samples and NMR Analysis

On completion of the incubation period, slices were removed from the incubation buffer by rapid filtration and double-extracted in methanol/chloroform. Extracts were lyophilized, and the pellet retained for protein estimation by the Lowry technique. Lyophilized supernatants were stored at -20 °C until required for NMR analysis. Samples were resuspended in 0.65 mL D₂O containing 2 mM sodium [¹³C]formate as an internal intensity and chemical shift reference (¹³C δ 171.8). Fully relaxed ¹H and ¹H[¹³C-decoupled] spectra (total cycle of 30 s, comprising 24 s relaxation delay, 4 s water suppression and ~2 s acquisition time), WURST-40 with a 112-step phase cycle, decoupling during acquisition) were obtained at

600.13 MHz on a Bruker DRX-600 spectrometer with a 5 mm dual ¹H/¹³C probe, followed by ¹³C [¹H-decoupled] spectra (typically 3000-5000 transients, cycle of 4 s comprising 2 s relaxation delay and ~2 s acquisition time, continuous WALTZ-16 decoupling, 131072 data points).

Resveratrol samples and control spectra were acquired on a Bruker AVANCE III HD 600 spectrometer fitted with a cryoprobe (TCI) and refrigerated sample changer. ¹H{¹³C-decoupled} spectra were acquired using bilev composite pulse decoupling across an effective bandwidth of 48,000 Hz during the acquisition time, on a 30s duty cycle, while ¹³C{¹H-decoupled} spectra were acquired on a 4 s duty cycle using continuous WALTZ-65 decoupling.

Assignments were made as described previously (Rae et al. 2000).

¹³C [¹H-Decoupled] spectra were transformed using 3 Hz exponential line-broadening and peak areas were determined by integration using standard Bruker software (TOPSPIN, Versions 1.3, or 3.1) following baseline correction. Peak areas were adjusted for nuclear Overhauser effect, saturation and natural abundance effects and quantified by reference to the area of the internal standard resonance of [¹³C]formate. Glu C3 was not quantified due to possible resonance overlap with

residual pyruvate signal. Metabolite pool sizes (lactate, alanine, GABA, glutamate, glutamine and aspartate) were determined by integration of resonances in fully relaxed 600 MHz $^1\text{H}[^{13}\text{C}\text{-decoupled}]$ spectra using $[^{13}\text{C}]\text{formate}$ as the internal intensity reference.

Experimental data ($N=4$) are given as means (standard deviation). Statistical analysis was done using ANOVA for comparing ligand-treated metabolism at each receptor with control ($N=4$, followed, only where statistical significance was indicated by Scheffe F-test, by a nonparametric test (Mann–Whitney U test) (Statview Student)). Significance was assumed at $\alpha=0.05$. Data are shown graphically as change relative to control (mean) in order more clearly to show the metabolic “pattern” generated by the ligand relative to control.

Pattern Recognition of the Data

Multivariate pattern recognition and data reduction tools are capable of taking into account several predictive variables simultaneously. They are especially useful for analysis of the type of metabolic data presented here as they can objectively distil the major response variables to a few controlled factors. Differences and similarities in the data are then easier to visualise and interpret. Principal components analysis (PCA) is an unsupervised projection method designed objectively to identify the variance in the dataset. The approach classifies the data into groups based solely on the variables that change the most, with no information about class membership being used. The robustness of the resultant model is assessed by a goodness of fit algorithm, with $R^2>0.60$ representing a model which accounts for the majority of variance in the dataset (Eriksson et al. 1999). In this work $R^2=0.7\%$.

In our experiments the major variance in the data is accounted for by the difference between the ligand and control experiments (for examples see (Rae et al. 2005; Rae et al. 2006; Moussa et al. 2007)), which is less interesting. Here, therefore, we followed an alternative approach (Nasrallah et al. 2007) where the data for each variable are converted to ratio relative to their control mean ($N=4$) and these data are then used for multivariate statistical analysis. This allows us to disregard the major variance in the data, which is described by the difference between control and ligand treated data. While this difference may be important, and is analysed and described in our group-wise comparisons (see Results), it is of lesser interest to us than how the datasets vary from one another. It is this variation in the data that we wish to uncover in these multivariate analyses.

We used data from this work, plus data from our previously published work investigating other GABA receptors (Nasrallah et al. 2007, 2009, 2010a, b; Rae et al. 2014) in order to assess the relative metabolic effects of GABA_p receptors compared to the rest of the GABAergic system.

Briefly, the data were imported into SIMCA P+ (Umetrics, Umeå, Sweden) and the data reduction tool PCA used following scaling of the data using unit variance scaling. This is where the data are centred around the mean, then divided by $1/s_\sigma$ where s_σ is the standard deviation of that variable. This ensures that each variable contributes equally to the model. The data were then subjected to PCA, which reduces the variances in the data to a series of latent variables known as principal components (PC's). To visualise the contributors to each of these PCs the corresponding loadings plots can then be examined.

Results

Metabolic “Fingerprints”

Metabolic fingerprints generated by agonists at GABA_p receptors are shown in Fig. 1. The fingerprints generated by 20 and 100 μM CACA are similar to that from 200 μM (\pm)-CAMP, while that generated by 50 μM (\pm)-CAMP is distinct with mildly decreased net incorporation of label into Krebs cycle intermediates (Glu C2, C4, Asp C2, C3) and no significant incorporation of label into Gln C4. This difference is shown more clearly in the plot of the first two principal components of the PCA model (Fig. 2, discussed below), which shows 20 and 100 μM CACA clustering in the right hand lower quadrant of the Hotelling circle, while 50 μM (\pm)-CAMP, which could be expected mostly to act on ρ_2 receptors at this concentration (Table 1), clusters in the top left hand quadrant.

Metabolic fingerprints generated by antagonists at GABA_p are shown in Fig. 3. Similar metabolic fingerprints were generated by 10 μM TPMPA, 4 μM *cis*-ACPBPA and 100 μM SGS742 which typically involved significantly increased incorporation of label into all Krebs cycle intermediates, and a very large incorporation of label into Gln C4. The “typical” pattern generated by these antagonists is shown in panel B of Fig. 2.

A large incorporation of label into Gln C4 was also demonstrated with the ρ_2 -specific antagonist *R*-ACPBPA but the relative increase in Krebs cycle labelling was much reduced in comparison with 10 μM TPMPA, 4 μM *cis*-ACPBPA and 100 μM SGS742. *R*-ACPBPA produced a markedly different metabolic response to *S*-ACPBPA (Fig. 3). *R*-ACPBPA clustered proximate to the “typical” antagonists suggesting that much of the metabolic response elicited by the “typical” antagonists was at ρ_2 -containing receptors.

SGS742 at 10 μM produced a metabolic fingerprint similar to that of the GABA_B antagonists 5 μM SCH50911 and 50 μM CGP 35348 (Fig. 4), clustering proximate to these ligands in the PCA model.

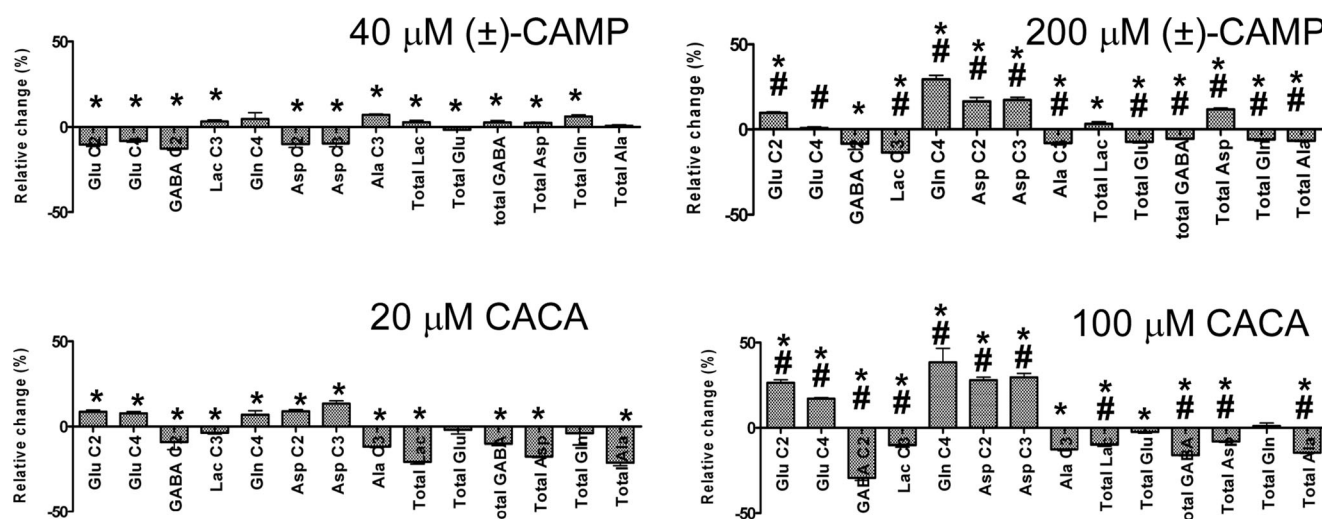


Fig. 1 Relative effect of GABA_p agonists on net flux of ¹³C and on total metabolite pool sizes in brain cortical tissue slices incubated 1 h with sodium [3-¹³C]pyruvate. Data are shown as relative to the control mean, with control metabolism centred to zero. Error bars represent

standard deviations. Statistically significant changes (calculated on the raw data not the relative change in flux or pool size) are indicated by * ($P < 0.05$, different to control) or # ($P < 0.05$, different to the other concentration of ligand used)

Resveratrol (48 μM) produced a metabolic fingerprint quite distinct from other GABA_p antagonists (Fig. 3)

consistent with resveratrol having other actions besides its reported activity at GABA_p.

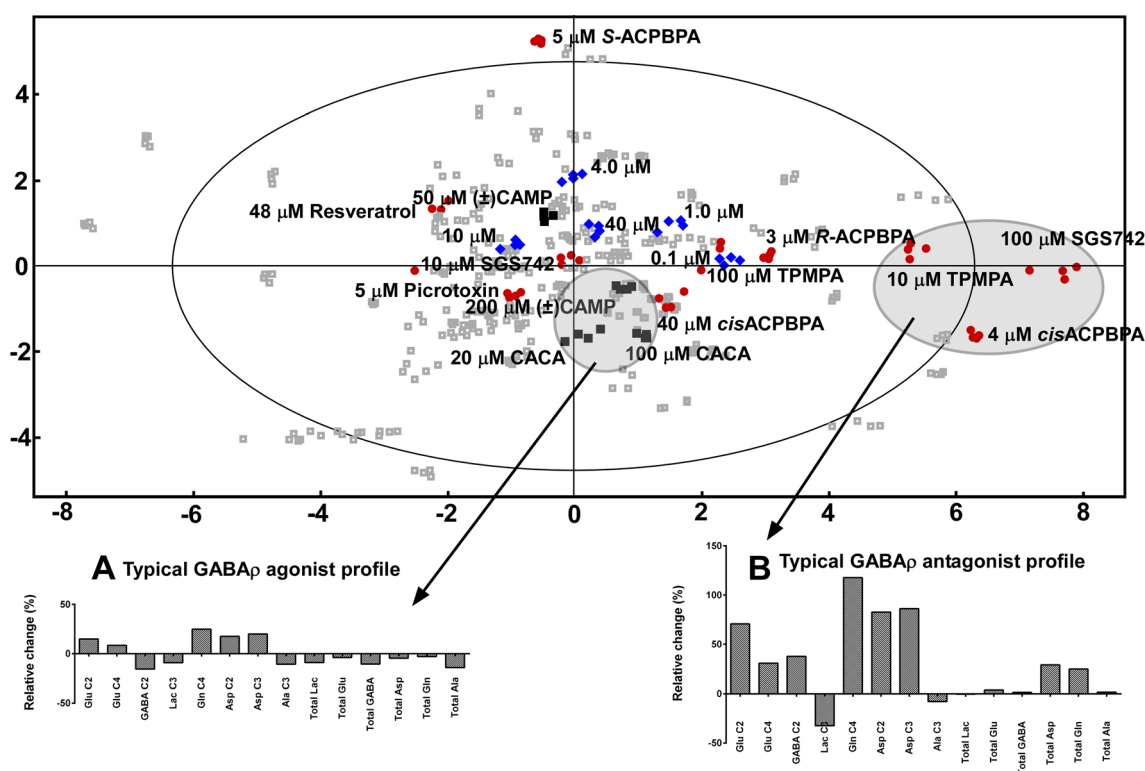


Fig. 2 PCA of labelling and total metabolite concentrations for ligands active in the GABAergic system. The figure shows the first two principal components (PCs) of the analysis. This plot represents the metabolic “footprint” of the GABAergic system and demonstrates separation of metabolic profiles according to the amount of Krebs cycle activity, which increases with positive loading in PC1. The large outer ellipse represents the 95 % confidence interval (Hotellings score). The metabolic profile

most typical of agonist activity at GABA_p is shown in insert A and the area corresponding to GABA_p agonist activity indicated by the circled shading. Similarly, the metabolic profile typical of antagonist activity at GABA_p is indicated in the shaded ellipse on the right hand side of the Hotellings circle in the plot and the typical metabolic fingerprint is shown in insert B

Multivariate Statistical Analysis of GABA ρ Ligands

Data from experiments using a range of ligands active at GABA $_A$ (Rae et al. 2008), GABA $_B$ (Nasrallah et al. 2007), GABA transporters (Nasrallah et al. 2010a) as well as from experiments exploring the effects of drugs active in the GABAergic system such as γ -hydroxybutyrate (Nasrallah et al. 2010b), vigabatrin (Nasrallah et al. 2011) and ethanol (Rae et al. 2014) were imported along with data from this work, and used to construct a principal components model. The model resulted in three principal components accounting for 82 % of the variance in the data (47, 27 and 8 %, respectively) with a cross-validation Q2 of 0.7. The first two components of this model are shown in Fig. 2.

Multivariate Statistical Analysis of GABA ρ and GABA $_A$ Ligands

To identify the defining metabolic markers of GABA $_A$ vs GABA ρ receptors, a multivariate model was constructed comprising ligands with agonist activity at these receptors, which yielded four principal components accounting for 39, 28, 13 and 9 % of the variance in the data, respectively ($R^2=89$ %, $Q^2=75$ %). The first two components of this model are plotted in Fig. 5a. There was overlap between (\pm)-CAMP (200 μ M) and isoguvacine (100 μ M) which cluster together. Similarly, the lower concentrations of these two drugs (50 μ M (\pm)-CAMP and 10 μ M isoguvacine) are located close together, although they are separated along PC1. The benzodiazepine selective for $\alpha 5$ -containing GABA $_A$ receptors LY655,708 (0.1 nM) is also located near to 50 μ M (\pm)-CAMP.

The GABA ρ agonist CACA does not cluster near to any other ligand (Fig. 5a). PC1 is loaded by increases in net flux into Glu C2 and C4, Asp C2 and C3 and GABA C2 and Gln C4 plus increases in the total metabolite pools of Asp, Gln and Glu. PC2 is loaded by increases in net flux into Ala C3 and lactate C3 and increases in the pool size of lactate, Glu, GABA, Asp and Ala. There are minor contributions from decreases in net flux into Glu C4 and Asp C2 and C3.

To see whether a separation between the two classes of metabolic profiles could be effected by a supervised approach, partial least squares discriminant analysis (PLS-DA) was used which introduced the class membership of each observation as a dummy variable. A model with three major principal components was generated accounting for 62 % of the variance (R^2 ; $Q^2=56$ %); the first two components of which (39 and 20 % of the variance, respectively) are shown in Fig. 5b. This model shows clear separation of GABA ρ and GABA $_A$ ligands from one another along component 1.

Component 1 is dominated by decreased net flux into GABA C2 and increased net flux into Gln C4, with contributions from decreased total GABA and total alanine. PC2 is loaded by decreased net flux into GABA C2, increased net

flux into Gln C4 and Ala C3 with increased total lactate. The contributions of the loadings to the plot are shown in Fig. 5c. The metabolites which most predict whether a dataset is from a GABA $_A$ or a GABA ρ ligand are net flux into GABA C2 and Gln C4, with increased net flux into Gln C4 and decreased net flux into GABA C2 most predictive of GABA ρ , and decreased net flux into Gln C4 and increased net flux into GABA C2 most predictive of membership of the GABA $_A$ class.

Discussion

The present study clearly demonstrates significant changes in metabolic activity induced by modulation of GABA ρ receptors in the cerebral cortex. The agonist (\pm)-CAMP is specific for GABA ρ , with no reported activity at GABA transporters and K_d s of 65 and 26 μ M at $\rho 1$ and $\rho 2$ subunits, respectively (Duke et al. 2000) indicating that at the concentrations used here (50 and 200 μ M) little subunit specificity would have been demonstrated. The agonist CACA shows little selectivity between $\rho 1$ and $\rho 2$ subunits with similar K_d reported at both (Duke et al. 2000) although recently it has been reported to be more potent at homomeric $\rho 2$ receptors (Alakuijala et al. 2005). It has been reported to be a weak inhibitor of GABA uptake ($K_i=750$ μ M) (Chebib and Johnston 1997) indicating that at the concentrations used in this study, it is not likely to be causing any significant inhibition of GABA uptake. CACA has been reported to be active at $\alpha 6$ -containing GABA $_A$ receptors (Wall 2001). The expression of this subunit in the mouse brain is confined to the cerebellum (Kato 1990), as it is in the rat brain (Wisden et al. 1992) so by analogy it may not be expressed in the Guinea pig cortex either.

This suggests that the metabolic profile typical of activity at GABA ρ (equal activity at $\rho 1$ and $\rho 2$) is described by the metabolic fingerprint shown in Fig. 2. There is a mild increase in net flux into Krebs cycle intermediates and Gln C4, coupled with decreased incorporation into GABA C2 and the glycolytic byproducts Lactate C3 and Ala C3. More specific activity at $\rho 2$ would be reflected by the metabolic profile generated by 50 μ M (\pm)-CAMP (Fig. 1). While quite different, it also shows increased net flux into Gln C4.

TPMPA has been reported to be an antagonist at GABA ρ , showing higher affinity for GABA ρ than GABA $_A$ (>100 times) and very weak agonist effects at GABA $_B$ (Ragozzino et al. 1996). It is eight-fold more potent at $\rho 1$ receptors than $\rho 2$ ($K_b=2.0$ vs 15.6 μ M, (Chebib et al. 1998b). *Cis*-ACBPBA is a weak agonist at GABA $_B$ and a weak antagonist at GABA $_A$ (Chebib et al. 2009b) and a competitive antagonist at GABA ρ ($\rho 1$, $K_B=5.06$ μ M).

SGS 742, originally promoted for its antagonism at GABA $_B$, is plainly a potent GABA ρ antagonist with

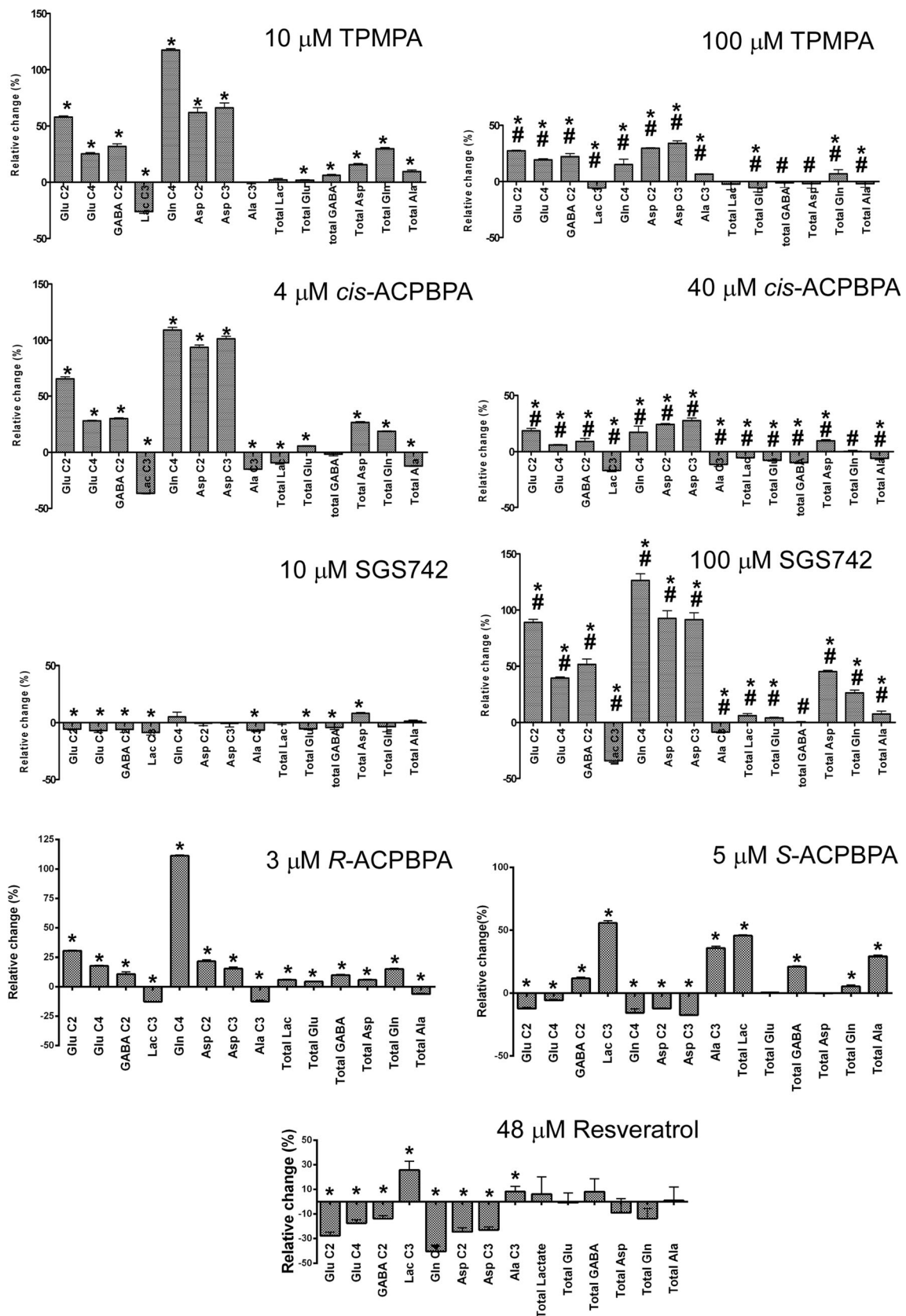


Fig. 3 Relative effect of GABA_B antagonists on net flux of ¹³C and on total metabolite pool sizes in brain cortical tissue slices incubated 1 h with sodium [^{3-¹³C}]pyruvate. Data are shown as relative to the control mean, with control metabolism centred to zero. Error bars represent standard deviations. Statistically significant changes (calculated on the raw data not the relative change in flux or pool size) are indicated by * ($P < 0.05$, different to control) or # ($P < 0.05$, different to the other concentration of ligand used)

significant metabolic effects as a consequence (Figs. 2 and 3). It has been undergoing clinical trials for a range of disorders (Froestl et al. 2004), although the mechanism by which it induces its outcomes has not yet been clarified.

The results indicate that inhibition of GABA_B activity by receptor antagonism results in greatly increased metabolic activity (Figs. 1 and 3) indicating that GABA_B likely plays a significant role in maintaining inhibitory tone in brain activity.

Significantly, comparison with the effects of GABA itself indicate that the responses to activation of GABA_B in the present study are similar to those evoked by exogenous GABA at concentrations close to the EC₅₀ for GABA reported for GABA_B receptors (ρ2 subunit, EC₅₀ 2.5 μM; (Kusama et al. 1993)).

Perhaps more significantly, the present results indicate that the responses engendered by modulation of GABA_B are very different to those produced by modulation of GABA_A. Although Fig. 5a shows clustering of high concentrations of isoguvacine (100 μM) with the GABA_B agonist (±)-CAMP this may well be due to activity of isoguvacine at GABA_B receptors where it has been reported to be a weak partial agonist ($K_d = 137$ μM at human ρ1, 110 μM at human ρ2, (Chebib et al. 1998b)). The metabolic effects of modulation of GABA_B and GABA_A receptors can easily and clearly be separated from one another, as shown in Fig. 5b. The fact that membership of either the GABA_A or GABA_B classes are

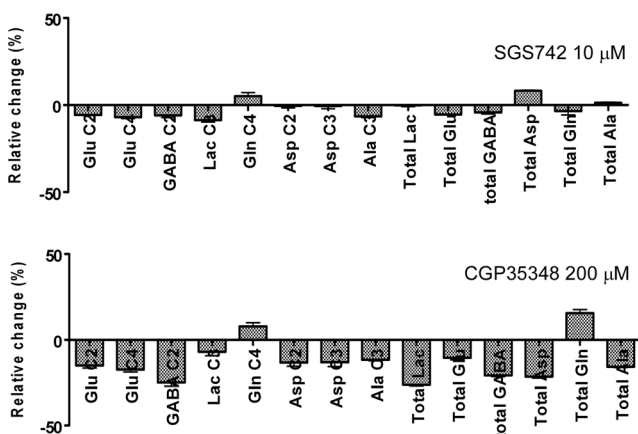


Fig. 4 Comparison of metabolic profiles of 10 μM SGS742 and 200 μM CGP 35348, a GABA_B antagonist. Data are shown as relative to the control mean, with control metabolism centred to zero. Error bars represent standard deviations. Statistically significant changes (calculated on the raw data not the relative change in flux or pool size) are indicated by * ($P < 0.05$, different to control)

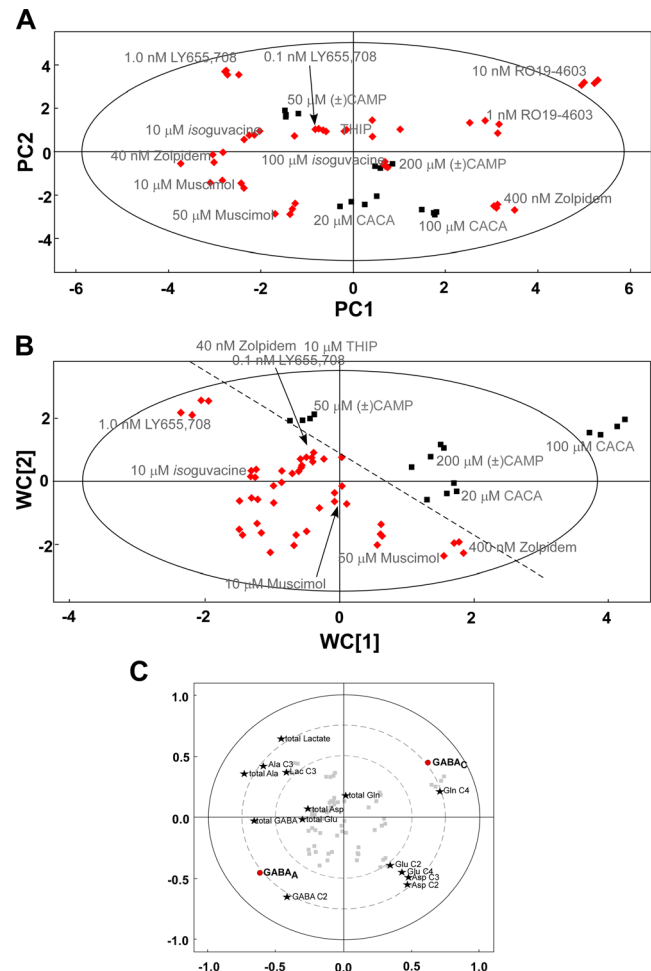


Fig. 5 Multivariate statistical analysis of labelling and total metabolite concentration data for GABA_B agonists and selected GABA_A receptor ligands. **a.** PCA including labelling and metabolite pool data from experiments using CACA and (±)-CAMP, the GABA_B receptor agonists muscimol, THIP and isoguvacine and the GABA_A allosteric modulators Zolpidem, LY655,708 and RO19-4603. All data from GABA_A receptor ligands is published elsewhere (Nasrallah et al. 2009). The figure shows the first two PCs of the analysis. **b.** PLS-DA of the same data. The figure shows the first two components of the analysis. The dashed line is included to mark the boundary between GABA_A and GABA_B data. The large outer ellipse represents the 95 % confidence interval (Hotellings score). **c.** A scores and loadings bi-plot from the PLS-DA model shown in **b.** The data from the model are shown in grey squares, the variables with black stars and the class membership in red (grey) circles. The data show the variables which most predict class membership are net flux into Glu C4 and GABA C2

associated with distinct and opposite metabolic outcomes (increased net flux into Gln C4 and decreased net flux into GABA C2 in the case of GABA_B, and decreased net flux into Gln C4 and increased net flux into GABA C2) suggests the two receptor classes are acting in opposite but complementary ways. Increased net flux into Gln C4 is associated with increased glutamate/glutamine cycling in tissue slices (Rae et al. 2003, 2006) while increased net flux into GABA C2 is associated with increased inhibition of metabolism (Nasrallah

et al. 2007). The location of the receptors may also play an important role, with GABA_ρ located extrasynaptically as befits high affinity GABA receptors; furthermore, it may be that as a consequence of this that GABA_ρ preferentially engage different metabolic pools to those engaged by GABA_A. The two receptor types also show markedly different effects on the total pool sizes of various metabolites (Fig. 5c, suggesting that they in fact do engage quite different metabolic compartments).

This finding of complementary activity in GABA_A and GABA_ρ is consistent with electrophysiological studies showing opposite effects on excitability in the superior colliculus (Pasternack et al. 1999) and the electroretinogram recorded from rat retina (Kapousta-Bruneau 2000) together with behavioural data showing opposite effects on memory by drugs active at these receptors (Gibbs and Johnston 2005).

In summary, we have shown significant and distinct patterns of metabolic activity arising from modulation of GABA_ρ in the Guinea pig cortical tissue slice. The finding of opposing metabolic outcomes of GABA_ρ and GABA_A would be consistent with a proposal that GABA_ρ represent not just a pharmacologically but, also, physiologically (with opposing effects on brain metabolic function) distinct class of receptors. The opposite and complementary effects associated with each receptor type may also imply that GABA_ρ are acting to modulate the activity of GABA_A receptors, and vice versa. Proper balance between the activation of GABA_A and GABA_ρ may be of critical importance for the normal GABAergic inhibition in the mammalian cerebral cortex.

Acknowledgments The authors are grateful to Dr Jim Hook, Dr Adele Amore and Dr Don Thomas of the UNSW Mark Wainwright Analytical Centre for expert technical assistance.

Funding This work was supported by UNSW, NewSouth Global and the National Health and Medical Research Council of Australia (#568767 & 630516).

Conflict of Interest CDR has received a speaker honorarium from Philips Healthcare. BDR, GARJ, VJB, FAN and JRH have no conflict of interest to declare.

References

- Alakuijala A, TalviOja K, Pasternack A, Pasternack M (2005) Functional characterization of rat $\rho 2$ subunits expressed in HEK 293 cells. *Eur J Neurosci* 21:692–700
- Alakuijala A, Alakuijala J, Pasternack M (2006) Evidence for a functional role of GABA_C receptors in the rat mature hippocampus. *Eur J Neurosci* 23:514–520
- Allan RD, Curtis DR, Headley PM, Johnston GAR, Lodge D, Twitchin B (1980) The synthesis and activity of *cis*- and *trans*-2-(aminomethyl) cyclopropanecarboxylic acid as conformationally restricted analogues of GABA. *J Neurochem* 34:652–654
- Barnard EA, Skolnick P, Olsen RW, Möhler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ (1998) International union of pharmacology. XV. Subtypes of γ aminobutyric acidA receptors: classification on the basis of subunit structure and receptor function. *Pharmacol Rev* 50:291–313
- Bolser DC, Blythin DJ, Chapman RW, Egan RW, Hey JA, Rizzo C, Kuo SC, Kreutner W (1995) The pharmacology of SCH-50911 - a novel, orally-active GABA-B receptor antagonist. *J Pharmacol Exp Ther* 274:1393–1398
- Bormann J, Feigenspan A (1995) GABAC receptors. *Trends Neurosci* 18:515–519
- Boue-Grabot E, Roudbaraki M, Bascles L, Tramu G, Bloch B, Garret M (1998) Expression of GABA receptor ρ subunits in rat brain. *J Neurochem* 70:899–907
- Bowery NG, Bettler B, Froestl W, Gallagher JP, Marshall F, Raiteri M, Bonner TI, Enna SJ (2002) International union of pharmacology. XXXIII. Mammalian γ -aminobutyric acid_B receptors: structure and function. *Pharmacol Rev* 54:247–264
- Bröer S, Bröer A, Hansen JT, Bubb WA, Balcar VJ, Nasrallah FA, Garner B, Rae C (2007) Alanine metabolism, transport and cycling in the brain. *J Neurochem* 102:1758–1770
- Carland JE, Moore AM, Hanrahan JR, Mewett KN, Duke RK, Johnston GAR, Chebib M (2004) Mutations of the 2' proline in the M2 domain of the human GABA(C) rho 1 subunit alter agonist responses. *Neuropharmacol* 46:770–781
- Carland JE, Johnston GAR, Chebib M (2008) Relative impact of residues at the intracellular and extracellular ends of the human GABA(C) rho 1 receptor M2 domain on picrotoxinin activity. *Eur J Pharmacol* 580:27–35
- Chebib M, Johnston GAR (1997) Stimulation of [³H]GABA and β -[³H]alanine release from rat brain slices by *cis*-4-aminocrotonic acid. *J Neurochem* 68:786–794
- Chebib M, Vandenberg RJ, Froestl W, Johnston GAR (1997) Unsaturated phosphinic analogues of γ -aminobutyric acid as GABA_C receptor antagonists. *Eur J Pharmacol* 329:223–229
- Chebib M, Mewett KN, Johnston GAR (1998a) GABAC receptor antagonists differentiate between human $\rho 1$ and $\rho 2$ receptors expressed in *Xenopus* oocytes. *Eur J Pharmacol* 357:227–234
- Chebib M, Mewett KN, Johnston GAR (1998b) GABAC receptor antagonists differentiate between human [rho]1 and [rho]2 receptors expressed in *Xenopus* oocytes. *Eur J Pharmacol* 357:227–234
- Chebib M, Gavande N, Wong KY, Park A, Premoli I, Mewett KN, Allan RD, Duke RK, Johnston GAR, Hanrahan JR (2009a) Guanidino acids act as rho 1 GABA(C) receptor antagonists. *Neurochem Res* 34:1704–1711
- Chebib M, Hinton T, Schmid KL, Brinkworth D, Qian H, Matos S, Kim H, Abdel-Halim H, Kumar RJ, Johnston GAR, Hanrahan JR (2009b) Novel, potent and selective GABAC antagonists inhibit myopia development and improve memory. *J Pharmacol Exp Therap* 328: 448–457
- Collingridge GL, Olsen RW, Peters J, Spedding M (2009) A nomenclature for ligand-gated ion channels. *Neuropharmacol* 56:2–5
- Drew CA, Johnston GAR, Weatherby RP (1984) Bicuculline-insensitive GABA receptors: studies on the binding of (–)-baclofen to rat cerebellar membranes. *Neurosci Lett* 52:317–321
- Duke RK, Allan RD, Chebib M, Greenwood JR, Johnston GAR (1998) Resolution and conformational analysis of diastereomeric esters of *cis* and *trans*-2-(aminomethyl)-1-carboxycyclopropanes. *Tetrahedron Asymmetry* 9:2533–2548
- Duke RK, Chebib M, Balcar VJ, Allan RD, Mewett KN, Johnston GAR (2000) (+) and (–)-*cis*-2-Aminomethylcyclopropanecarboxylic acids show opposite pharmacology at recombinant $\rho 1$ and $\rho 2$ GABA_C receptors. *J Neurochem* 75:2602–2610
- Ebert B, Thompson SA, Saounatou K, McKernan R, Krogsgaard-Larsen P, Wafford KA (1997) Differences in agonist/antagonist binding affinity and receptor transduction using recombinant human gamma-aminobutyric acid type A receptors. *Mol Pharmacol* 52: 1150–1156

- Enz R, Cutting GR (1998) Molecular composition of GABAC receptors. *Vis Res* 38:1431–1441
- Enz R, Cutting GR (1999) GABAC receptor ρ subunits are heterogeneously expressed in the human CNS and form homo- and heterooligomers with distinct physical properties. *Eur J Neurosci* 11:41–50
- Enz R, Brandstätter JH, Hartveit E, Wässle H, Bormann J (1996) Expression of GABA receptor $\rho 1$ and $\rho 2$ subunits in the retina and brain of the rat. *Eur J Neurosci* 7:1495–1501
- Eriksson L, Johansson E, Kettaneh-Wold N, Wold S (1999) Introduction to multi- and megavariable data analysis using projection methods (PCA and PLS). In: Umeå, Sweden: Umetrics
- Froestl W, Gallagher M, Jenkins H, Madrid A, Melcher T, Teichman S, Mondadori CG, Pearlman R (2004) SGS742: the first GABA_B receptor antagonist in clinical trials. *Biochem Pharmacol* 68:1479–1487
- Gibbs ME, Johnston GA (2005) Opposing roles for GABAA and GABAC receptors in short-term memory formation in young chicks. *Neurosci* 131:567–576
- Hanrahan JR, Mewett KN, Chebib M, Matos S, Eliopoulos CT, Crean C, Kumar RJ, Burden P, Johnston GAR (2006) Diastereoselective synthesis of (+/-)-(3-aminocyclopentane)alkylphosphinic acids, conformationally restricted analogues of GABA. *Org Biomol Chem* 4:2642–2649
- Hartmann K, Stief F, Draguhn A, Frahm C (2004) Ionotropic GABA receptors with mixed pharmacological properties of GABA_A and GABA_C receptors. *Eur J Pharmacol* 497:139–146
- Harvey VL, Duguid IC, Cornelius K, Stephens GJ (2006) Evidence that GABA ρ subunits contribute to functional ionotropic GABA receptors in mouse cerebellar Purkinje cells. *J Physiol* 577:127–139
- Johnston GAR (1996) GABAC receptors: relatively simple transmitter-gated ion channels? *Trends Pharmacol Sci* 17:319–323
- Kapousta-Bruneau NV (2000) Opposite effects of GABA_A and GABA_C receptor antagonists on the B-wave of ERG recorded from the isolated rat retina. *Vis Res* 40:1653–1665
- Kato K (1990) Novel GABAA receptor alpha subunit is expressed only in cerebellar granule cells. *J Mol Biol* 214:619–624
- Kerr DIB, Ong J (1995) GABA_B receptors. *Pharmacol Ther* 67:187–246
- Kumar RJ, Chebib M, Hibbs DE, Kim HL, Johnston GAR, Salam NK, Hanrahan JR (2008) Novel gamma-aminobutyric acid rho(1) receptor antagonists; synthesis, pharmacological activity and structure-activity relationships. *J Med Chem* 51:3825–3840
- Kusama T, Wang TL, Guggino WB, Cutting GR, Uhl GR (1993) GABA rho-2-receptor pharmacological profile - GABA recognition site similarities to rho-1. *Eur J Pharmacol* 245:83–84
- Lee BH, Choi SH, Hwang SH, Kim HJ, Lee JH, Nah SY (2013) Resveratrol inhibits GABAC rho receptor-mediated ion currents expressed in xenopus oocytes. *Korean J Physiol Pharmacol : Off J Korean Physiol Soc Korean Soc Pharmacol* 17:175–180
- López-Chávez A, Miledi R, Martínez-Torres A (2005) Cloning and functional expression of the bovine GABA_C $\rho 2$ subunit. Molecular evidence of a widespread distribution in the CNS. *Neurosci Res* 53:421–427
- Matthews G, Ayoub GS, Heidelberger R (1994) Presynaptic inhibition by GABA is mediated via two distinct GABA receptors with novel pharmacology. *J Neurosci* 14:1079–1090
- Milligan CJ, Buckley NJ, Garret M, Deuchars J, Deuchars SA (2004) Evidence for inhibition mediated by coassembly of GABA_A and GABA_C receptor subunits in native central neurons. *J Neurosci* 24:7241–7250
- Moussa CE-H, Rae C, Bubb WA, Griffin JL, Deters NA, Balcar VJ (2007) Inhibitors of glutamate transport modulate distinct patterns in brain metabolism. *J Neurosci Res* 85:342–350
- Murata Y, Woodward RM, Miledi R, Overman LE (1996) The first selective antagonist for a GABA(C) receptor. *Bioorg Med Chem Lett* 6:2073–2076
- Nasrallah F, Griffin JL, Balcar VJ, Rae C (2007) Understanding your inhibitions. Modulation of brain cortical metabolism by GABA-B receptors. *J Cereb Blood Flow Metab* 27:1510–1520
- Nasrallah F, Griffin JL, Balcar VJ, Rae C (2009) Understanding your inhibitions. Effects of GABA and GABAA receptors on brain cortical metabolism. *J Neurochem* 108:57–71
- Nasrallah FA, Balcar VJ, Rae C (2010a) A metabolomic study of inhibition of GABA uptake in the cerebral cortex. *Metabolomics* 6:67–77
- Nasrallah FA, Maher AD, Hanrahan JR, Balcar VJ, Rae CD (2010b) γ -Hydroxybutyrate and the GABAergic footprint. A metabolomic approach to unpicking the actions of GHB. *J Neurochem* 115:58–67
- Nasrallah FA, Balcar VJ, Rae CD (2011) Activity dependent GABA release controls brain cortical tissue slice metabolism. *J Neurosci Res* 89:1935–1945
- Neu A, Neuheff H, Trube G, Fehr S, Ullrich K, Roeper J, Isbrandt D (2002) Activation of GABAA receptors by guanidinoacetate: a novel pathophysiological mechanism. *Neurobiol Dis* 11:298–307
- Pasternack M, Boller M, Pau B, Schmidt M (1999) GABA_A and GABA_C receptors have contrasting effects on excitability in superior colliculus. *J Neurophysiol* 82:2020–2023
- Rae C, Balcar V (2014) A chip off the old block: the brain slice as a model for metabolic studies of brain compartmentation and neuropharmacology. In: Hirrlinger J, Waagepetersen HS (eds) Brain energy metabolism. Springer, New York, pp 217–241
- Rae C, Lawrance ML, Dias LS, Provis T, Bubb WA, Balcar VJ (2000) Strategies for studies of potentially neurotoxic mechanisms involving deficient transport of L-glutamate: antisense knockout in rat brain in vivo and changes in the neurotransmitter metabolism following inhibition of glutamate transport in guinea pigs brain slices. *Brain Res Bull* 53:373–381
- Rae C, Hare N, Bubb WA, McEwan SR, Bröer A, McQuillan JA, Balcar VJ, Conigrave AD, Bröer S (2003) Inhibition of glutamine transport depletes glutamate and GABA neurotransmitter pools: further evidence for metabolic compartmentation. *J Neurochem* 85:503–514
- Rae C, Moussa CE-H, Griffin JL, Bubb WA, Wallis T, Balcar VJ (2005) Group I and II metabotropic glutamate receptors alter brain cortical metabolic and glutamate/glutamine cycle activity: a ¹³C NMR spectroscopy and metabolomic study. *J Neurochem* 92:405–416
- Rae C, Moussa CE-H, Griffin JL, Parekh SB, Bubb WA, Hunt NH, Balcar VJ (2006) A metabolomic approach to ionotropic glutamate receptor subtype function: a nuclear magnetic resonance in vitro investigation. *J Cereb Blood Flow Metab* 26:1005–1017
- Rae C, Nasrallah FA, Griffin JL, Balcar VJ (2008) Understanding your inhibitions: neuropharmacological perturbations of GABAergic systems, metabolic outcomes and network correlations. *Neuroimage* 41:57
- Rae C, Nasrallah FA, Griffin JL, Balcar VJ (2009) Now I know my ABC. A systems neurochemistry and functional metabolomic approach to understanding the GABAergic system. *J Neurochem* 109(Suppl 1): 109–116
- Rae CD, Davidson JE, Maher AD, Rowlands BD, Kashem MA, Nasrallah FA, Rallipalli SK, Cook JM, Balcar VJ (2014) Ethanol, not detectably metabolized in brain, significantly reduces brain metabolism, probably via action at specific GABA(A) receptors and has measurable metabolic effects at very low concentrations. *J Neurochem* 129:304–314
- Ragozzino D, Woodward RM, Murata Y, Eusebi F, Overman LE, Miledi R (1996) Design and in vitro pharmacology of a selective γ -aminobutyric acid_C receptor antagonist. *Mol Pharmacol* 50:1024–1030
- Storustov SI, Ebert B (2006) Pharmacological characterization of agonists at delta-containing GABA(A) receptors: functional selectivity for extrasynaptic receptors is dependent on the absence of gamma(2). *J Pharmacol Exp Ther* 316:1351–1359

- Vien J, Duke RK, Mewett KN, Johnston GA, Shingai R, Chebib M (2002) Trans-4-Amino-2-methylbut-2-enoic acid (2-MeTACA) and (+/–)-trans-2-aminomethylcyclopropanecarboxylic acid ((+/-)-TAMP) can differentiate rat rho3 from human rho1 and rho2 recombinant GABA(C) receptors. *Br J Pharmacol* 134:883–890
- Wall MJ (2001) Cis-4-amino-crotonic acid activates [alpha]6 subunit-containing GABAA but not GABAC receptors in granule cells of adult rat cerebellar slices. *Neurosci Lett* 316:37–40
- Wegelius K, Pasternack M, Hiltunen JO, Rivera C, Kaila K, Saarma M, Reeben M (1998) Distribution of GABA receptor ρ subunit transcripts in the rat brain. *Eur J Neurosci* 10:350–357
- Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992) The distribution of 13 GABA-A receptor subunit messenger-RNAs in the rat brain. 1. Telencephalon, Diencephalon, Mesencephalon. *J Neurosci* 12: 1040–1062
- Woodward RM, Polenzani L, Miledi R (1993) Characterization of bicuculline/baclofen-insensitive (rho-like) gamma-aminobutyric acid receptors expressed in *Xenopus* oocytes. II. Pharmacology of gamma-aminobutyric acidA and gamma-aminobutyric acidB receptor agonists and antagonists. *Mol Pharmacol* 43:609–625
- Xu M, Covey DF, Akabas MH (1995) Interaction of picrotoxin with GABA(A) receptor channel-lining residues probed in cysteine mutants. *Biophys J* 69:1858–1867