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Acute increases in synaptic GABA detectable in the living human brain: a [^{11}C]Ro15-4513 PET study

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Abstract

The inhibitory γ-aminobutyric acid (GABA) neurotransmitter system is associated with the regulation of normal cognitive functions and dysregulation has been reported in a number of neuropsychiatric disorders including anxiety disorders, schizophrenia and addictions. Investigating the role of GABA in both health and disease has been constrained by difficulties in measuring acute changes in synaptic GABA using neurochemical imaging. The aim of this study was to investigate whether acute increases in synaptic GABA are detectable in the living human brain using the inverse agonist GABA-benzodiazepine receptor (GABA-BZR) positron emission tomography (PET) tracer, [^{11}C]Ro15-4513. We examined the effect of 15mg oral tiagabine, which increases synaptic GABA by inhibiting the GAT1 GABA uptake transporter, on [^{11}C]Ro15-4513 binding in 12 male participants using a paired, double blind, placebo-controlled protocol. Spectral analysis was used to examine synaptic α1 and extrasynaptic α5 GABA-BZR subtype availability in brain regions with high levels of [^{11}C]Ro15-4513 binding. We also examined the test-retest reliability of α1 and a5-specific [^{11}C]Ro15-4513 binding in a separate cohort of 4 participants using the same spectral analysis protocol. Tiagabine administration produced significant reductions in hippocampal, parahippocampal, amygdala and anterior cingulate synaptic α1 [^{11}C]Ro15-4513 binding, and a trend significance reduction in the nucleus accumbens. These reductions were greater than test-retest reliability, indicating that they are not the result of chance observations. Our results suggest that acute increases in endogenous synaptic GABA are detectable in the living human brain using [^{11}C]Ro15-4513 PET. These findings have potentially major implications for the investigation of GABA function in brain disorders and in the development of new treatments targeting this neurotransmitter system.

Keywords: GABA, tiagabine, [^{11}C]Ro15-4513, PET, synaptic

Introduction

Gamma-aminobutyric acid (GABA) is the most important and ubiquitous inhibitory neurotransmitter in the brain. The GABA system is associated with a number of important cognitive functions including memory (Michels et al., 2012), attention (Smolnik et al., 1998) and associative learning (Makkar et al., 2010). Dysregulation within the GABA system has been reported in many neuropsychiatric disorders, such as anxiety and panic disorders (Malizia et al., 1998), epilepsy (Baulac et al., 2001; Loup et al., 2000), schizophrenia (Ahn et al., 2011; Hoftman et al., 2013), and addiction (Lingford-Hughes et al., 2012). The ability to image synaptic GABA levels in the living human brain is therefore critical for our understanding of the role of the GABA system in both health and disease.

Currently, human GABA *in vivo* neuroimaging research predominantly uses positron emission tomography (PET) to quantify GABA-benzodiazepine receptor (GABA-BZR) availability and magnetic resonance spectroscopy (MRS) to index overall GABA levels. However, GABA MRS has a number of limitations such as difficulties isolating GABA in the spectrum, the need to sample very large voxels, and difficulties in interpreting how much of the MRS signal originates from the neurotransmitter or synaptic pool compared with the larger metabolic pool. Thus, although MRS studies report differences in GABA levels in clinical populations, such as in unipolar depression (Sanacora et al., 1999) or after anticonvulsant treatment in epilepsy (Doelken et al., 2010), it is impossible to say whether these differences are neurotransmitter or metabolic in origin. In addition, the sensitivity of GABA MRS to measure expected acute changes in synaptic GABA levels after a pharmacological

challenge is inconsistent (Henry et al., 2010; Kupers et al., 2009; Valentine et al., 2011). In contrast, PET offers a unique opportunity to assess dynamic changes in synaptic GABA levels.

Two PET ligands, [^{11}C]flumazenil and [^{11}C]Ro15-4513, are mainly used for *in vivo* neurochemical imaging of the human GABA system and both bind to the benzodiazepine site on the GABA-A receptor. GABA-benzodiazepine receptors (GABA-BZR's) contain two subunits from one of six α subunits (α1-α6); GABA-BZR's containing α1-α3 subunits are located intra-synaptically and those containing α4-α6 are predominantly or exclusively located extra-synaptically (Brunig et al., 2002; Kasugai et al., 2010). Measurement of synaptic GABA levels using PET is therefore theoretically dependent on the ability to isolate a specific synaptic α1-α3 signal. [^{11}C]Flumazenil, the more commonly used radioligand, binds with similar affinity to α1-α3 and α5 subtypes and also with somewhat lower affinity to α4 and α6 subtypes (Hadingham et al., 1993). It is not therefore currently possible to isolate a specific synaptic GABA-BZR PET signal using [^{11}C]flumazenil. Although [^{11}C]Ro15-4513 is more selective for the α5 subtype, there is also measurable *in vivo* binding at the α1 subtype (Hadingham et al., 1993). We have recently shown that a specific α1 subtype signal can be quantified in [^{11}C]Ro15-4513 PET studies, by partitioning fast and slow ligand kinetics using spectral analysis (Myers et al., 2012).

The pharmacological properties of Ro15-4513 also offer further advantages over flumazenil. Ro15-4513 is an inverse GABA-BZR agonist whereas flumazenil is generally considered to be an antagonist. The affinity of benzodiazepine full agonists or partial agonists, but not antagonists, at the GABA-BZR can alter as a result of

changes in synaptic GABA levels – the so called GABA shift (Braestrup et al., 1982; Tallman et al., 1978). Consistent with the GABA shift is evidence from autoradiographic studies of the primate and rat brain demonstrating that [^{11}C]Ro15-4513 and [^{3}H]Ro15-4513 binding respectively is markedly reduced by increased GABA levels while [^{11}C]flumazenil binding is little affected (Onoe et al., 1996; Tyacke et al., 2009). However, whilst flumazenil shows no GABA shift in vitro (Braestrup et al., 1982), in some circumstances in vivo it appears to behave as a partial agonist (Higgitt et al., 1986; Miller et al., 1988) with a positive shift. This may explain the increased binding of [^{11}C]flumazenil in recent human PET studies (Frankle et al., 2012; Frankle et al., 2009).

The aim of this present study was to determine whether a reduction in synaptic α1-subtype GABA-BZR affinity produced by increases in synaptic GABA levels could be detected in the living human brain. We used tiagabine, which increases synaptic GABA levels by selectively inhibiting the GABA transporter 1 (GAT1) in experimental animal studies (Fink-Jensen et al., 1992; Richards and Bowery, 1996; Sybirska et al., 1993) and in a human case report (During et al., 1992). Tiagabine also produces physiological changes consistent with increased GABA concentrations in recent MEG studies (Muthukumaraswamy et al., 2013; Muthukumaraswamy et al., 2012). Our hypothesis was that tiagabine administration would increase synaptic GABA levels and hence reduce the affinity and binding of [^{11}C]Ro15-4513 in brain regions with the highest levels of [^{11}C]Ro15-4513 binding where the identification and quantification of α1 and α5 binding is most robust and reliable. To examine the extent to which these findings differed from chance-level observations, we also sought to compare the magnitude of change detected after a tiagabine challenge to

the test–retest reliability of α1 and α5-subtype [^{11}C]Ro15-4513 binding in identical regions of interest in a separate cohort of participants.

Material and methods

Participants

Twelve healthy male participants (mean age 50.1 years ± SD: 7.1; 6 non-cigarette smokers, 5 ex-cigarette smokers, 1 participant with unknown smoking status) completed the tiagabine challenge study, and four healthy male participants (mean age 41.5 years ±SD: 4.4; 3 non-smokers, 1 participant with unknown smoking status) completed the test-retest reliability study. Exclusion criteria for all participants included current or previous significant mental health disorders, alcohol or recreational drug dependency as defined by DSM-IV, serious physical illness, past neurological disorders, and previous use of psychotropic medications. Potential participants underwent urine drug screening to exclude recent recreational drug use prior to their enrolment in the study. For the tiagabine challenge study participants' baseline anxiety and depression symptoms were assessed using the Beck Depression Inventory (BDI) (Beck et al., 1961) and the Spielberger Trait Anxiety Inventory (STAI) (Spielberger et al., 1970). Verbal and visuospatial memory was assessed using the logical memory subtest of the Wechsler Memory Scale (Wechsler, 1981) and the Rey-Osterrieth Complex Figure Test (Shin et al., 2006), respectively. All participants provided written informed consent to take part in the study which was approved both by the Hammersmith Research Ethics Committee and the Administration of Radioactive Substances Advisory Committee, UK.

Tiagabine challenge study participants underwent two [^{11}C]Ro15-4513 PET scans using a double-blind placebo-controlled protocol with at least one week between scans. These participants received an oral administration of either 15 mg tiagabine

or placebo ninety minutes before each scan so that peak plasma concentrations were highest while the participant was in the scanner. The effects of tiagabine administration on sedation, agitation, disorientation and 'alcohol like drug effect' were rated by investigators using ten point Likert scales. Test-retest participants completed two [^{11}C]Ro15-4513 PET scans.

[^{11}C]Ro15-4513 PET imaging

All PET scans were acquired using an ECAT HR+ 962 scanner (CTI/Siemens) with an axial field of view of 15.5cm. A 10 minute transmission scan was performed prior to each emission scan to measure tissue attenuation in two dimensional mode. Each participant received a fast intravenous bolus injection of 479.6MBq ±25.6 [^{11}C]Ro15 4513 through an intravenous cannula sited in the dominant antecubital fossa vein. Twenty four dynamic frames (1x30, 4x15, 4x60, 2x150, 10x300, 3x600 s) of data were acquired in 3D mode over 90 min and produced images containing 63 contiguous slices. Arterial blood sampling was used to produce a metabolite-corrected plasma input function as described previously (Lingford-Hughes et al., 2002).

MR Imaging

All participants underwent a structural T1 MRI scan for co-registration purposes. MRI scans were acquired using a 1.5T scanner for tiagabine challenge participants (1.5 Eclipse system, Marconi Medical Systems, Cleveland, OH, USA; TR=30 ms, TE=3ms, flip angle=30º, NSA=1, voxel dimensions 0.98x1.6x1.6mm^3) and a 3T scanner for test-retest participants (3T Intera Philips Medical Systems; TR=9.6ms, TE=4.6ms, flip angle=8 º, NSA=1, voxel dimensions 0.94x0.94x1.2mm^3).

[^{11}C]Ro15-4513 PET image analysis

All dynamic scans were corrected for head movement using frame by frame (FBF) realignment (Montgomery et al., 2006). This procedure was applied to all frames to generate a FBF corrected dynamic image, which was then analysed using an automated region of interest (ROI) analysis. FBF corrected reconstructed [^{11}C]Ro15-4513 images were analysed using Analyze AVW version 9.0 (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN), Matlab 6, 6.5 and SPM5 (available via http://www.fil.ion.ucl.ac.uk/spm/). In line with our hypothesis that tiagabine administration would reduce synaptic GABA-BZR α1-subtype binding, we chose brain regions with high levels of [^{11}C]Ro15-4513 binding (the nucleus accumbens, hippocampus, parahippocampal gyrus, amygdala and anterior cingulate gyrus) where change in synaptic GABA-BZR α1-subtype binding would be most evident. These regions of interest (ROIs) are also of major interest in a number of neuropsychiatric disorders. ROIs were placed on the [^{11}C]Ro15-4513 images using a maximum probability map based on 30 participants, which defined 83 regions (Gousias et al., 2008; Hammers et al., 2003). Each structural MRI image was rigid-body co-registered to the weighted summed counts image. The co-registered MRI was normalised to stereotaxic space using bias-corrected segmentation in SPM5 (Ashburner and Friston, 2005). This normalisation was reversed to warp the maximum probability map to the co-registered structural MRI and the PET dynamic image. Goodness-of-fit was checked visually prior to regional sampling using object maps in the ROI tool in Analyze 9.0. Sampling regions of interest in each dynamic frame enabled the collection of time-activity curves (TACs) for each ROI.

[^{11}C]Ro15-4513 specific volume of distribution (described here as V_S) values were generated using spectral analysis. Spectral analysis is the only analysis method currently available that allows isolation of specific GABA-BZR α1 and α5 subtype receptor signals from [^{11}C]Ro15-4513 PET images. We did not use compartmental modelling as this does not allow for the identification of specific α1 and α5 subtype binding and no appropriate reference region for each subtype exists (Myers et al., 2012). Spectral analysis (Cunningham and Jones, 1993; Turkheimer et al., 1994) is a basis function technique, used to convolve the plasma input function with first order poly-exponentials, to fit regional TAC data, assuming system linearity and time-invariance. The "spectrum" of dissociation rates, each represented by one or several closely related peaks, that effect the shape of the TAC can be divided into ranges that represent the different compartments of [^{11}C]Ro15-4513 binding (Myers et al., 2012). The range of decay values was 0.0030- 0.040 s^{-1} for the α1 subtype binding site and 0.00063-0.003 s^{-1} for the α5 subtype binding site. α1 and α5 subtype [^{11}C]Ro15-4513 distribution volumes were calculated by summing the peak heights within the prescribed band (Myers et al., 2012). Spectral analysis was also used to calculate [^{11}C]Ro15-4513 plasma clearance (K_1) by summing the peak height across the total range of the spectrum, not including the fast peaks representing blood volume.

Statistical Analysis

Scan data and demographics were assessed using two-tailed t-tests. For the tiagabine challenge dataset, decreases in α1-specific V_S values in *a prori* ROIs were assessed using a one-tailed paired t-test, given our hypothesis that a decrease in α1-specific [^{11}C]Ro15-4513 binding would occur in these regions after tiagabine administration. Changes in α5-specific V_S values in these areas after tiagabine

administration were assessed using two-tailed paired t-tests. Change in overall brain α1- and α5-specific [^{11}C]Ro15-4513 binding after tiagabine administration was assessed using a two-way repeated measures ANOVA.

For the test-retest dataset, α1- and α5-specific [^{11}C]Ro15-4513 reliability was calculated using an intra-class correlation coefficient (ICC) based on a two way mixed effect model (Egerton et al., 2010; Shrout and Fleiss, 1979) where ICC values approaching +1 indicate that variance is largely due to between rather than within-subject variation (Egerton et al., 2010). Reproducibility was calculated as the percentage test-retest difference (%VAR) (Egerton et al., 2010): ((retest value-test value)/0.5(test value+retest value)) x 100. All statistical analyses were performed using SPSS 20.0 (SPSS, Chicago, Illinois, USA) and all values are expressed as mean (SD). Power calculations were performed using GPower 3.0 (Faul et al., 2007).

Results

Tiagabine behavioural effects

The mean tiagabine dose administered to tiagabine challenge participants was 0.18±0.02 mg/kg. Tiagabine was generally well tolerated, although one participant became symptomatically hypotensive for around an hour at the end of an imaging session. Tiagabine administration produced increases in ‘alcohol like drug effect’ (mean±SD placebo and tiagabine scores: 1.2±0.9, 3.9±1.6; p=0.002), disorientation (mean±SD placebo and tiagabine scores: 1.2±0.6, 2.3±0.9; p=0.009), and sedation (mean±SD placebo and tiagabine scores: 1.6±1.4, 2.6±1.6; p=0.09), but had no effect on agitation (mean±SD placebo and tiagabine scores: 1.2±0.6, 1.2±0.6; p=1.0). These effects are similar to those from our previous studies where the main behavioural effects of 15mg tiagabine were described as ‘feeling of intoxication', ‘dizziness', and ‘drug effect is like alcohol' (Muthukumaraswamy et al., 2013; Muthukumaraswamy et al., 2012).

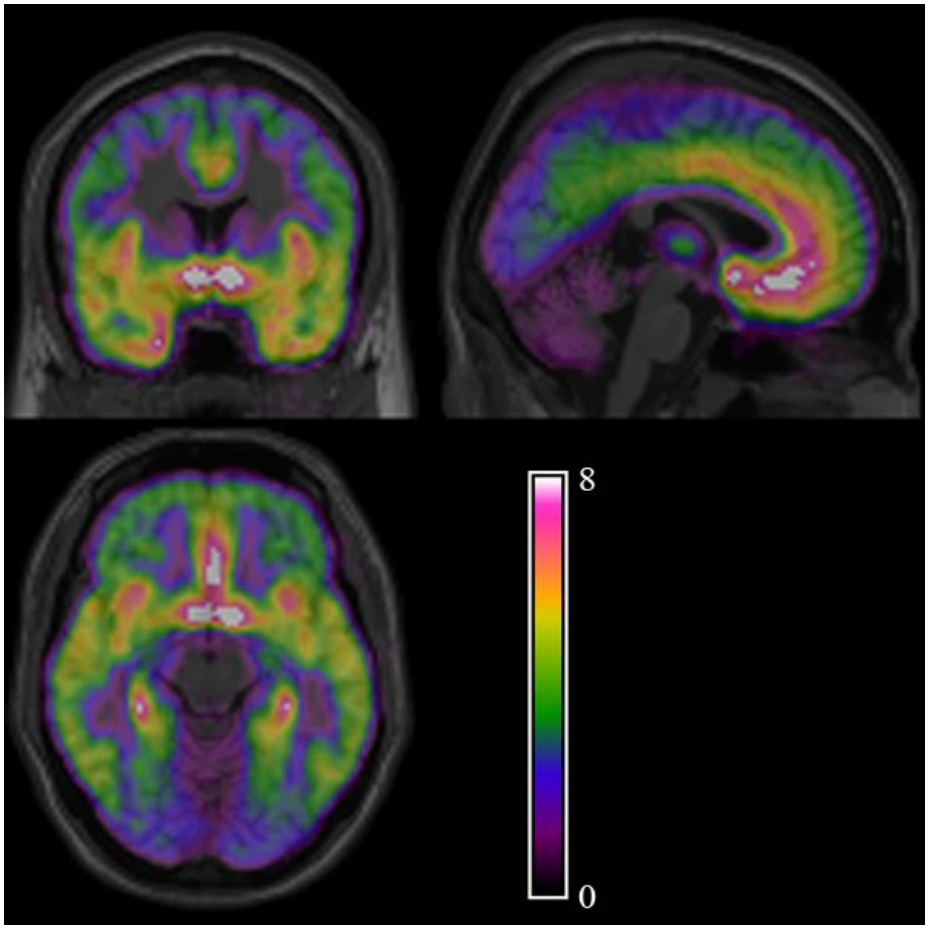


Figure 1: Representative example of a [^{11}C]Ro15-4513 PET image from which α1- and α5-subtype specific GABA-BZR receptor binding is derived (average parametric total VT map warped into MNI space from 5 healthy participants). Coronal view is displayed upper left, sagittal view is displayed upper right, axial view is displayed lower left and the colour bar, displayed lower right, indicates extent of total VT binding.

[^{11}C]Ro15-4513 PET imaging

A representative example of a [^{11}C]Ro15-4513 PET image, from which α1- and α5-subtype specific GABA-BZR receptor binding is derived, is shown in Figure 1. The mean duration between tiagabine and placebo scans was 47.9±35.3 days, and the

mean duration between test-retest scans was 16.2±5.1 days. Duration between scans was not significantly different between the tiagabine and test-retest cohorts (p=0.12). Whole brain K_1 values or K_1 values from any of the ROI's studied were not significantly different across treatment conditions for the tiagabine challenge study (all *p* values> 0.05). [^{11}C]Ro15-4513 dose injected or Ro15-4513 mass injected also was not significantly different across treatment conditions (all *p* values> 0.05).

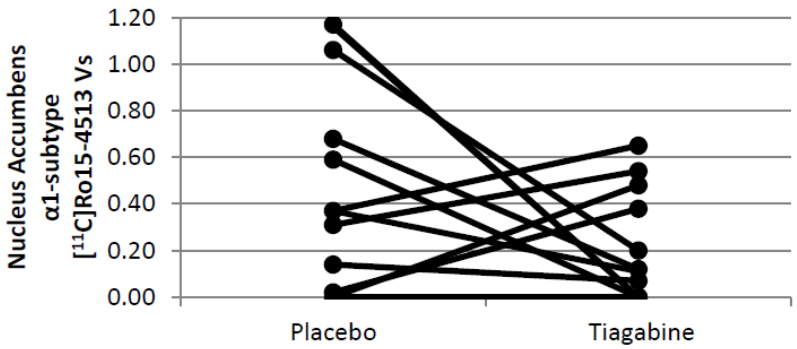
	α1-subtype V_S					α5-subtype V_S				
Region	Placebo	Tiagabine	% decrease	P value	Effect size	Placebo	Tiagabine	% increase	P value	Effect size
Nucleus Accumbens	0.49 ±0.44	0.21 ±0.24	54 ±74	0.06	0.79	12.2 ±3.1	12.8 ±4.0	12 ±42	0.68	0.17
Hippocampus	0.89 ±0.50	0.49 ±0.66	39 ±81	0.02	0.68	7.2 ±0.9	8.8 ±2.6	25 ±40	0.06	0.82
Parahippocampus	0.73 ±0.54	0.30 ±0.48	29 ±121	0.05	0.84	6.7 ±1.1	7.3 ±1.5	14 ±23	0.18	0.46
Amygdala	1.08 ±0.86	0.48 ±0.57	61 ±42	0.01	0.82	7.7 ±1.3	8.3 ±3.0	11 ±41	0.52	0.26
Anterior Cingulate	1.41 ±0.89	0.60 ±0.87	51 ±82	0.02	0.92	6.2 ±1.2	7.3 ±1.6	23 ±34	0.05	0.78

Table 1: Changes in mean ±SD α1- and α5-subtype [^{11}C]Ro15-4513 binding (V_S) after tiagabine administration

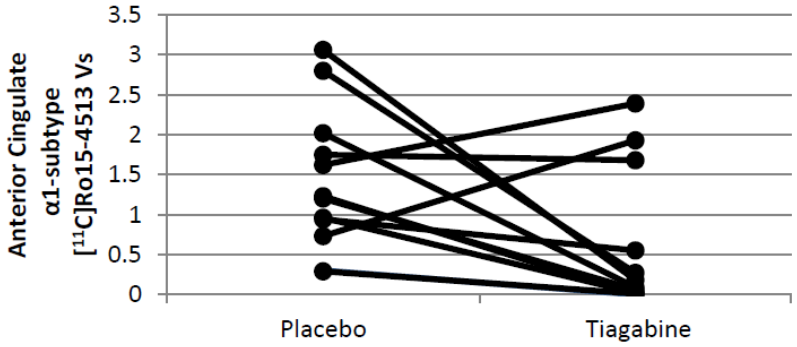
α1-subtype tiagabine challenge results

Tiagabine administration was associated with reductions in synaptic α1-subtype [^{11}C]Ro15-4513 binding in all *a priori* ROI regions studied (see Table 1 and Figure 2). Mean α1-subtype [^{11}C]Ro15-4513 V_S was significantly reduced by 61% in the amygdala, 51% in the anterior cingulate gyrus, 39% in the hippocampus and 29% in the parahippocampal gyrus (all *p* values<0.05). α1-subtype V_S was reduced by 54% at a trend significance level in the nucleus accumbens (*p*=0.06). Further exploratory *post hoc* analysis found that tiagabine administration was associated with a 12% reduction in whole brain α1-subtype [^{11}C]Ro15-4513 binding (mean±SD whole brain placebo and tiagabine α1-subtype V_S: 1.67±0.39, 1.44±0.56; p<0.0001). Figure 3 shows overall [^{11}C]Ro15-4513 spectra, divided into α1 and α5 frequency bands, during the placebo and tiagabine condition.

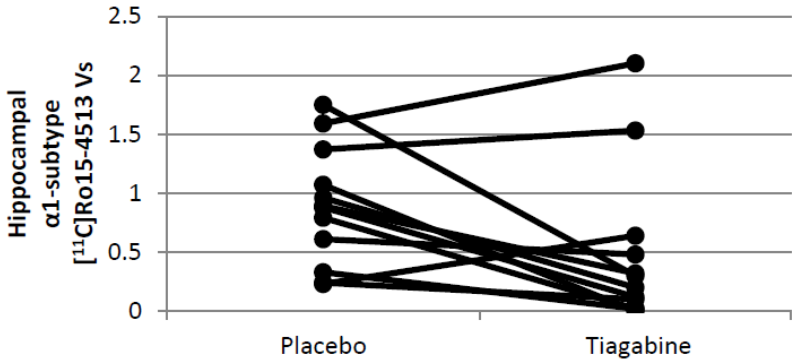
A.


Nucleus Accumbens
α1-subtype
[11C]Ro15-4513 Vs
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Placebo
Tiagabine

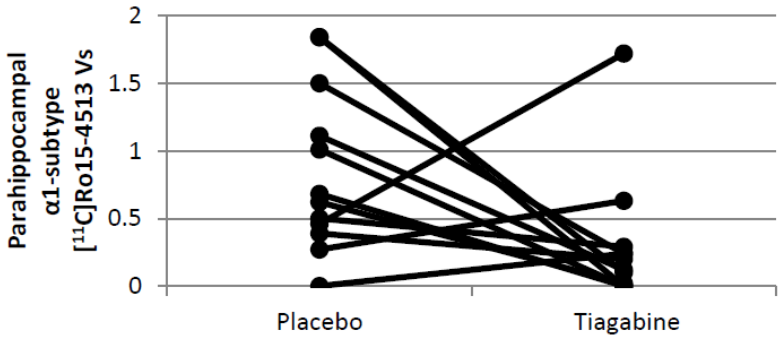
B.


Anterior Cingulate
α1-subtype
[11C]Ro15-4513 Vs
3.5
3
2.5
2
1.5
1
0.5
0
Placebo
Tiagabine

C.


Hippocampal
α1-subtype
[11C]Ro15-4513 Vs
2.5
2
1.5
1
0.5
0
Placebo
Tiagabine

D.


Parahippocampal
α1-subtype
[11C]Ro15-4513 Vs
2
1.5
1
0.5
0
Placebo
Tiagabine

E.

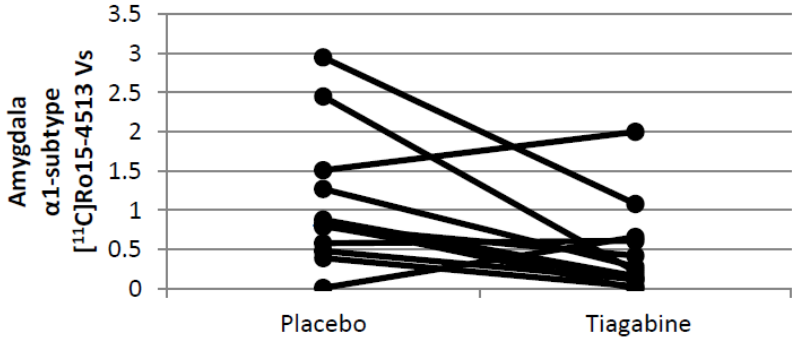

Amygdala
α1-subtype
[11C]Ro15-4513 Vs
3.5
3
2.5
2
1.5
1
0.5
0
Placebo
Tiagabine

Figure 2: Change in α1-subtype [^{11}C]Ro15-4513 binding after tiagabine administration in the nucleus accumbens (A), anterior cingulate gyrus (B), hippocampus (C), parahippocampal gyrus (D), and amygdala (E).

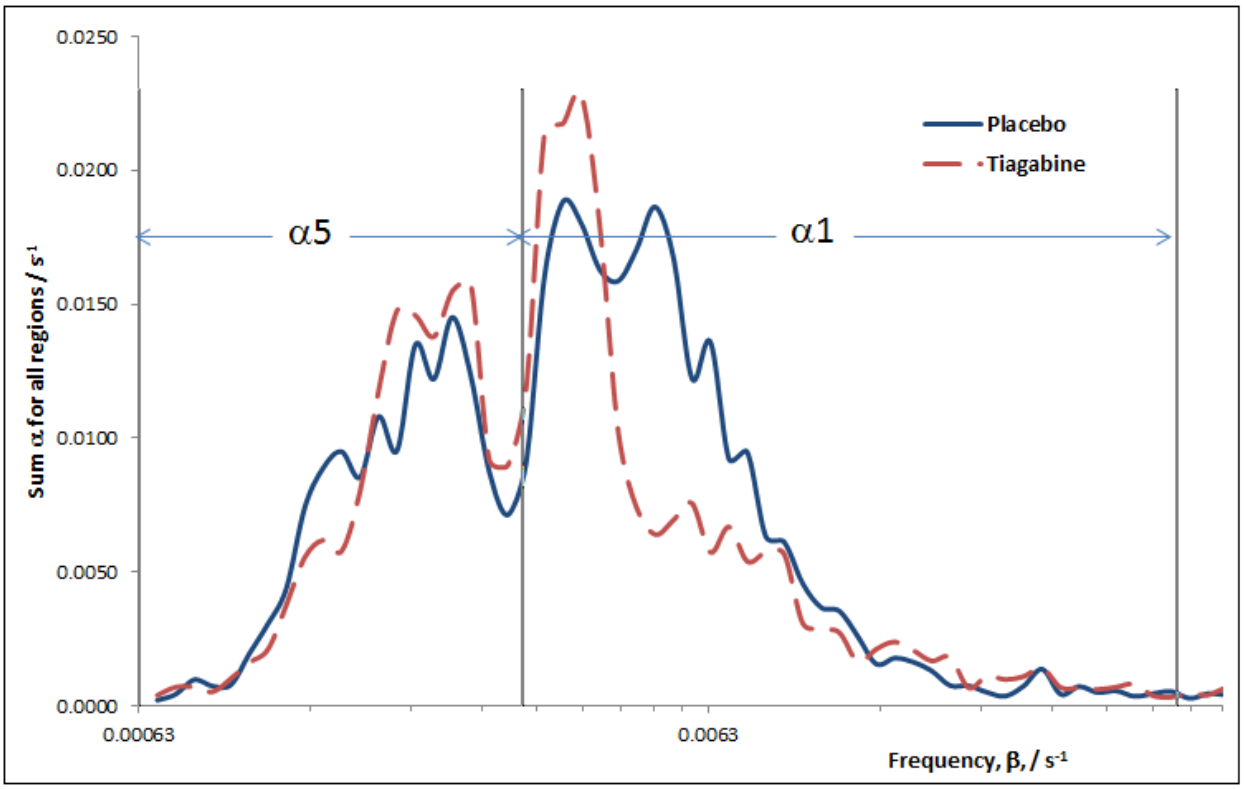
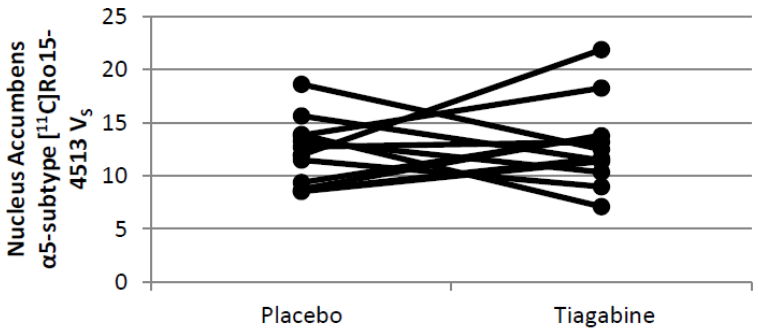


Figure 3: [^{11}C]Ro15-4513 spectra displayed as a sum of peaks from all regions of the brain in all individuals, with bands to show α1 and α5 subtype frequencies. Total summed peak height (s^{-1}) is plotted against β frequency (s^{-1}) on a logarithmic scale for ease of interpretation. There is a discernable reduction in curve integral in the prescribed α1 band associated with tiagabine administration.

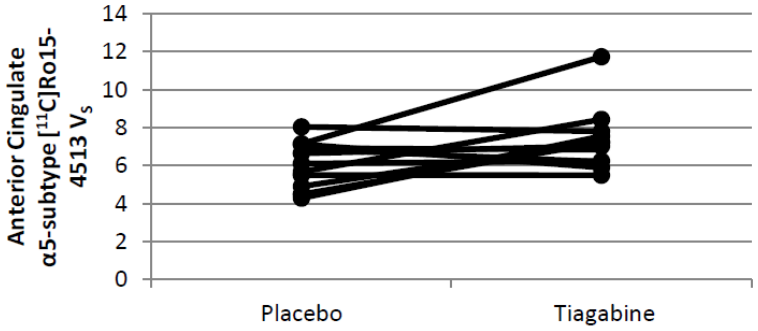
α5-subtype tiagabine challenge results

Tiagabine administration was associated with a significant increase of 23% in extra-synaptic α5-subtype [^{11}C]Ro15-4513 binding in the anterior cingulate gyrus and a 25% trend level increase in the hippocampus (*p*=0.06) (see Table 1 and Figure 4). There were no significant changes in α5-subtype [^{11}C]Ro15-4513 binding in the nucleus accumbens, parahippocampal gyrus or amygdala after tiagabine administration (all *p* values>0.1). *Post hoc* analysis indicated that tiagabine administration was associated with a 12% increase in whole brain α5-subtype [^{11}C]Ro15-4513 binding (mean±SD whole brain placebo and tiagabine α5-subtype V_S: 4.0±0.66, 4.36±0.80; p<0.0001).

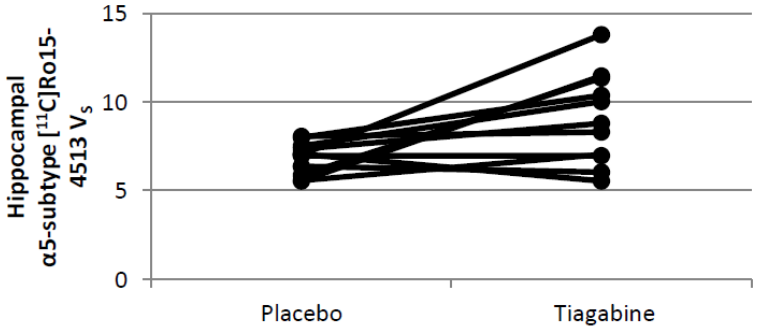
A.


Nucleus Accumbens α5-subtype [11C]Ro15-4513 Vs
25
20
15
10
5
0
Placebo
Tiagabine

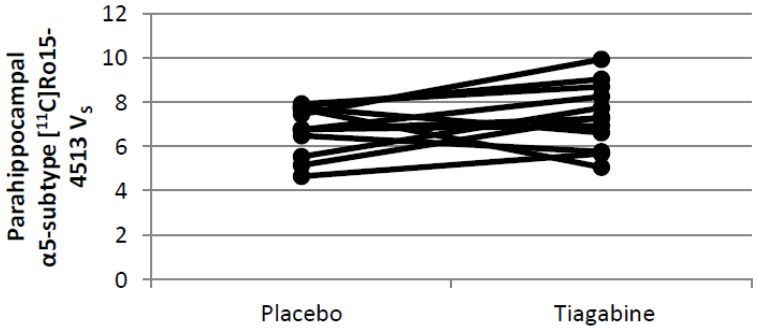
B.


Anterior Cingulate α5-subtype [11C]Ro15-4513 Vs
14
12
10
8
6
4
2
0
Placebo
Tiagabine

C.


Hippocampal α5-subtype [11C]Ro15-4513 Vs
15
10
5
0
Placebo
Tiagabine

D.


Parahippocampal α5-subtype [11C]Ro15-4513 Vs
12
10
8
6
4
2
0
Placebo
Tiagabine

E.

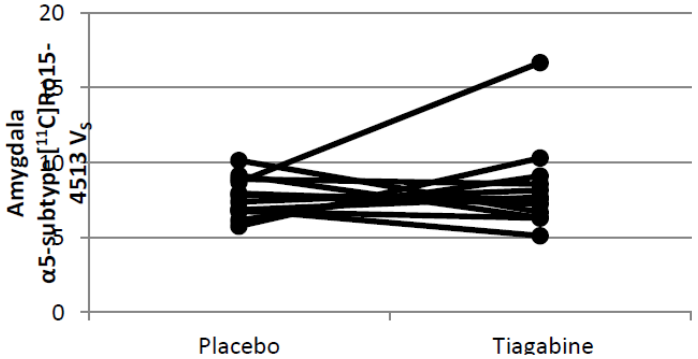

Amygdala α5-subtype [11C]Ro15-4513 Vs
20
15
10
5
0
Placebo
Tiagabine

Figure 4: Change in α5-subtype [^{11}C]Ro15-4513 binding after tiagabine administration in the nucleus accumbens (A), anterior cingulate gyrus (B), hippocampus (C), parahippocampal gyrus (D), and amygdala (E).

Correlations with memory performance and behavioural response

Given the suggested role of the hippocampal GABA-BZR in learning and memory in both healthy controls (Collinson et al., 2002; Crestani et al., 2002) and alcohol dependent populations (Lingford-Hughes et al., 2012), we explored whether changes in hippocampal α1- and α5-subtype binding after tiagabine administration correlated with baseline visuospatial memory or verbal memory performance. Better visuospatial memory performance, reflected by smaller percentage decreases in delayed recall ROCFT visuospatial scores compared to copy scores, was associated at a trend significance level with greater percentage increases in hippocampal α5-subtype binding after tiagabine administration ($r=0.54$, $p=0.08$). However, poorer visuospatial memory performance, reflected by larger percentage decreases in delayed recall ROCFT visuospatial scores compared to copy scores, was associated at a trend significance level with greater percentage decreases in hippocampal α1-subtype binding after tiagabine administration ($rho=0.53$, $p=0.10$). No correlations were found between Wechsler Memory Scale verbal memory scores and changes in hippocampal α1- or α5-subtype binding (p values>0.05). Tiagabine subjective behavioural effects did not correlate with percentage change in either α1- or α5-subtype [^{11}C]Ro15-4513 binding in any of the ROI's studied ($p>0.05$, correcting for multiple comparisons).

α1- and α5-subtype test-retest reliability and reproducibility

α1-subtype [^{11}C]Ro15-4513 binding reliability was excellent in the anterior cingulate gyrus (ICC>0.8), fair in the amygdala and parahippocampal gyrus (ICC>0.6) but poor in the nucleus accumbens and hippocampus (ICC<0.4) (see Table 2). α5-subtype reliability was excellent in the amygdala, anterior cingulate gyrus, nucleus accumbens, amygdala and parahippocampal gyrus (ICC>0.8) and fair in the hippocampus (ICC<0.6). Test-retest variability ranged between 17-38% for the α1-subtype and 0-22% for the α5-subtype.

	α1-subtype						α5-subtype					
Region	Test V_S	Test CV (%)	Retest V_S	Retest CV (%)	% VAR	ICC	Test V_S	Test CV (%)	Retest V_S	Retest CV (%)	% VAR	ICC
Nucleus Accumbens	0.43 ±0.66	153	0.54 ±0.92	170	24.3 ±174	0.08	11.5 ±5.1	44	8.8 ±2.2	25	21.7 ±18.9	0.83
Hippocampus	0.87 ±0.80	91	0.74 ±0.78	105	31.3 ±164	-0.02	7.7 ±3.0	39	6.2 ±1.6	26	17.6 ±31.0	0.41
Parahippocampus	0.87 ±0.67	77	0.85 ±0.96	113	23.6 ±163	0.62	6.2 ±1.7	27	6.2 ±2.7	43	0.4 ±53.6	0.82
Amygdala	0.62 ±0.54	87	0.72 ±0.72	100	38.0 ±134	0.63	8.3 ±3.2	39	7.5 ±2.5	33	8.6 ±24.1	0.90
Anterior Cingulate	1.69 ±1.80	107	1.57 ±1.87	119	17.4 ±169	0.87	5.2 ±2.9	56	5.7 ±3.5	61	6.4 ±38.0	0.84

Table 2: α1- and α5-subtype [^{11}C]Ro15-4513 test-retest reliability and reproducibility, and between-participants variation. Test-retest reliability is expressed as absolute variability (%VAR) and reliability as intraclass correlation coefficient (ICC). Between participants variation is expressed as percentage coefficient of variation (CV)=SD/mean x 100. Values are mean ±SD, ICC=Cronbach's Alpha.

Discussion

The main finding of our study is that synaptic α1-subtype [^{11}C]Ro15-4513 binding is sensitive to increases in endogenous GABA produced by a tiagabine challenge. We found that tiagabine administration resulted in significant reductions in α1-subtype [^{11}C]Ro15-4513 binding in the *a priori* regions of the anterior cingulate gyrus, amygdala, hippocampus, parahippocampal gyrus, and a reduction at a trend significance level in the nucleus accumbens. Additionally, we found significant global reductions in synaptic α1-subtype [^{11}C]Ro15-4513 binding throughout the brain. Reductions in α1-subtype binding after tiagabine administration ranged in magnitude from 61% in the amygdala to 29% in the parahippocampal gyrus and were greater than test-retest variability for every *a priori* region studied which suggests that they did not arise as a result of chance observations. Moreover, none of these reductions were accompanied by a significant change in regional plasma clearance K_1, which indicates that they are not a consequence of altered blood flow or ligand delivery. No significant relationships between cognitive measures and changes in [^{11}C]Ro15-4513 binding were found.

This is the first time to our knowledge that reductions in synaptic α1-subtype GABA-BZR PET tracer binding have been reported in humans after an acute GABA pharmacological challenge. Our findings concord with those of an autoradiographic study in primates where reductions in [^{11}C]Ro15-4513 binding were reported after an increase in brain GABA levels (Onoe et al., 1996). They are also consistent with our *a priori* hypothesis that an increase in synaptic GABA levels, following tiagabine blockade of GAT1, would reduce the affinity and thus the binding of the inverse

agonist [^{11}C]Ro15-4513 at synaptic α1-subtype GABA-BZR's. Our findings suggest that the "GABA shift", well described in-vitro (Braestrup et al., 1982; Onoe et al., 1996; Tallman et al., 1978), is measurable in humans *in vivo*. They also suggest that [^{11}C]Ro15-4513 binding at α1-subtype GABA-BZR's is sensitive to changes in phasic synaptic GABA neurotransmission.

Previous human neurochemical imaging studies using the GABA-BZR antagonist ligand [^{11}C]flumazenil have reported alterations in overall GABA-BZR binding after administration of agents which increase extracellular GABA levels. Weber and colleagues found a trend level increase in [^{11}C]flumazenil binding after acute administration of the irreversible GABA transaminase inhibitor vigabatrin (Weber et al., 1999). More recently, Frankle and colleagues found that acute administration of 16 mg tiagabine increased cortical and limbic [^{11}C]flumazenil binding (Frankle et al., 2009) and that an acute tiagabine dose of 0.25mg/kg (17.5mg tiabagine for 70kg participants) was required to produce significant increases in [^{11}C]flumazenil binding in the association cortex, sensory cortex and limbic regions (Frankle et al., 2012). Given that the affinity of GABA-BZR's antagonists are reported not to be altered by increases in GABA levels in vitro (Braestrup et al., 1982) these findings would suggest that [^{11}C]flumazenil is acting as a weak agonist *in vivo*. This is consistent with other *in vivo* studies where flumazenil appears to behave as a partial agonist (Higgitt et al., 1986; Miller et al., 1988) with a positive shift. Whether flumazenil is a partial agonist or antagonist, there is no current way of quantifying separate synaptic and extrasynaptic GABA-BZR binding from the [^{11}C]flumazenil signal and so one cannot know whether changes in binding are a reflection of altered GABA levels predominantly within the synapse. The findings from this present study extends the

human GABA-BZR neurochemical imaging literature by demonstrating that α1-specific binding is not only identifiable with [^{11}C]Ro15-4513 PET but that it is also sensitive to increases in limbic synaptic GABA levels.

We also found that tiagabine administration significantly increased overall brain extrasynaptic α5-subtype [^{11}C]Ro15-4513 binding, however significant increases in regional binding were only found in the anterior cingulate and at a trend level in the hippocampus but not in the other three regions studied. The increase in α5-subtype binding we found is entirely consistent with the compartmental arrangement of [^{11}C]Ro15-4513 binding. The reduced affinity of the α1-subtype after tiagabine administration, with ensuing reduced α1-subtype binding, results in an increase of the concentration of the free tracer in the brain. Hence the amount of tracer amenable to bind to the α5-subtype will increase resulting in an apparent increased affinity of the α5-subtype. The change in apparent affinity due to relative changes in the free tracer in tissue has been alternatively defined as increases in “reaction volume” by Delforge and colleagues (Delforge et al., 1996).

Our findings indicate that increases in limbic synaptic GABA levels are detectable in the living human brain using [^{11}C]Ro15-4513 PET. However, we would suggest that there are some constraints for using this technique in detecting differences between clinical populations. The test-retest results indicate that the α1-specific signal has relatively high test-retest variability (17-38%) with good reliability in the amygdala, parahippocampal gyrus and anterior cingulate gyrus, but poor reliability in the hippocampus and nucleus accumbens. Although the test-retest reliability estimates may have been affected by the small number of participants who completed this arm

of the study (n=4), relatively high variability in the α1-specific signal is likely to be a consequence of extracting a comparatively small α1-specific component from the [^{11}C]Ro15-4513 PET signal. These test-retest reliability estimates do not preclude comparisons in clinical populations but may limit the brain regions where change can be reliably detected. Other factors such as the expression of GAT1 and GAT3 uptake mechanisms may also add to the variability in the [^{11}C]Ro15-4513 α1-specific signal. A potential way of reducing variability and improving the tiagabine related α1-specific signal could be restrict analyses to regions of good reliability and to use a higher oral dose of tiagabine. We used a fixed oral dose of 15 mg tiagabine, equivalent to a mean dose of 0.18 mg/kg, which is less than the 0.25 mg/kg tiagabine doses reported by Frankle and colleagues as required to produce significant increases in [^{11}C]flumazenil binding (Frankle et al., 2012). However as several of our participants reported feeling markedly drowsy after receiving 15mg tiagabine, and as other studies have suggested that tiagabine has the potential to induce stupor (Azar et al., 2013; Hamandi et al., 2014), we would suggest that any increases in tiagabine dose should be made cautiously.

Although our results require replication and refinement, they have potentially important implications for the investigation of the human GABA-BZR system both in healthy and neuropsychiatric populations. They are particularly relevant for studying how alterations in synaptic GABA levels impact upon normal cognitive processes subserved by the limbic system such as salience, reward, emotional learning and memory; and to explore how increases in synaptic GABA levels differ in disorders where these processes may be abnormal such as anxiety disorders, affective disorders, schizophrenia, addiction and autism. Finally, our findings are also relevant

for the assessment of new treatments designed to increase limbic synaptic GABA levels in neuropsychiatric disorders.

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Conflict of Interest

The authors declare no conflict of interest.

References

Ahn, K., Gil, R., Seibyl, J., Sewell, R.A., D'Souza, D.C., 2011. Probing GABA receptor function in schizophrenia with iomazenil. Neuropsychopharmacology 36, 677-683.

Ashburner, J., Friston, K.J., 2005. Unified segmentation. Neuroimage 26, 839-851.

Azar, N.J., Bangalore-Vittal, N., Arain, A., Abou-Khalil, B.W., 2013. Tiagabine-induced stupor in patients with psychogenic nonepileptic seizures: nonconvulsive status epilepticus or encephalopathy? Epilepsy Behav 27, 330-332.

Baulac, S., Huberfeld, G., Gourfinkel-An, I., Mitropoulou, G., Beranger, A., Prud'homme, J.F., Baulac, M., Brice, A., Bruzzone, R., LeGuern, E., 2001. First genetic evidence of GABA(A) receptor dysfunction in epilepsy: a mutation in the gamma2-subunit gene. Nat Genet 28, 46-48.

Beck, A.T., Ward, C.H., Mendelson, M., Mock, J., Erbaugh, J., 1961. An inventory for measuring depression. Arch Gen Psychiatry 4, 561-571.

Braestrup, C., Schmiechen, R., Neef, G., Nielsen, M., Petersen, E.N., 1982. Interaction of convulsive ligands with benzodiazepine receptors. Science 216, 1241-1243.

Brunig, I., Scotti, E., Sidler, C., Fritschy, J.M., 2002. Intact sorting, targeting, and clustering of gamma-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. J Comp Neurol 443, 43-55.

Collinson, N., Kuenzi, F.M., Jarolimek, W., Maubach, K.A., Cothliff, R., Sur, C., Smith, A., Otu, F.M., Howell, O., Atack, J.R., McKernan, R.M., Seabrook, G.R., Dawson, G.R., Whiting, P.J., Rosahl, T.W., 2002. Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the alpha 5 subunit of the GABAA receptor. J Neurosci 22, 5572-5580.

Crestani, F., Keist, R., Fritschy, J.M., Benke, D., Vogt, K., Prut, L., Bluthmann, H., Mohler, H., Rudolph, U., 2002. Trace fear conditioning involves hippocampal alpha5 GABA(A) receptors. Proc Natl Acad Sci U S A 99, 8980-8985.

Cunningham, V.J., Jones, T., 1993. Spectral analysis of dynamic PET studies. J.Cereb.Blood Flow Metab 13, 15-23.

Delforge, J., Syrota, A., Bendriem, B., 1996. Concept of reaction volume in the in vivo ligand-receptor model. J Nucl Med 37, 118-125.

Doelken, M.T., Hammen, T., Bogner, W., Mennecke, A., Stadlbauer, A., Boettcher, U., Doerfler, A., Stefan, H., 2010. Alterations of intracerebral gamma-aminobutyric acid (GABA) levels by titration with levetiracetam in patients with focal epilepsies. Epilepsia 51, 1477-1482.

During, M., Mattson, R., Scheyer, R., Rask, C., Pierce, M., 1992. The Effect of Tiagabine HCI on Extracellular GABA Levels in the Human Hippocampus. Annual Meeting of the American Epilepsy Society, Seattle, Washington, USA.

Egerton, A., Demjaha, A., McGuire, P., Mehta, M.A., Howes, O.D., 2010. The test-retest reliability of 18F-DOPA PET in assessing striatal and extrastriatal presynaptic dopaminergic function. Neuroimage 50, 524-531.

Faul, F., Erdfelder, E., Lang, A.G., Buchner, A., 2007. G*Power 3: a flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Methods 39, 175-191.

Fink-Jensen, A., Suzdak, P.D., Swedberg, M.D., Judge, M.E., Hansen, L., Nielsen, P.G., 1992. The gamma-aminobutyric acid (GABA) uptake inhibitor, tiagabine, increases extracellular brain levels of GABA in awake rats. Eur J Pharmacol 220, 197-201.

Frankle, W.G., Cho, R.Y., Mason, N.S., Chen, C.M., Himes, M., Walker, C., Lewis, D.A., Mathis, C.A., Narendran, R., 2012. [C]flumazenil Binding Is Increased in a Dose-Dependent Manner with Tiagabine-Induced Elevations in GABA Levels. PLoS.One. 7, e32443.

Frankle, W.G., Cho, R.Y., Narendran, R., Mason, N.S., Vora, S., Litschge, M., Price, J.C., Lewis, D.A., Mathis, C.A., 2009. Tiagabine increases [11C]flumazenil binding in cortical brain regions in healthy control subjects. Neuropsychopharmacology 34, 624-633.

Gousias, I.S., Rueckert, D., Heckemann, R.A., Dyet, L.E., Boardman, J.P., Edwards, A.D., Hammers, A., 2008. Automatic segmentation of brain MRIs of 2-year-olds into 83 regions of interest. Neuroimage 40, 672-684.

Hadingham, K.L., Wingrove, P., Le, B.B., Palmer, K.J., Ragan, C.I., Whiting, P.J., 1993. Cloning of cDNA sequences encoding human alpha 2 and alpha 3 gamma-aminobutyric acidA receptor subunits and characterization of the benzodiazepine pharmacology of recombinant alpha 1-, alpha 2-, alpha 3-, and alpha 5-containing human gamma-aminobutyric acidA receptors. Mol.Pharmacol. 43, 970-975.

Hamandi, K., Myers, J., Muthukumaraswamy, S., 2014. Tiagabine-induced stupor - More evidence for an encephalopathy. Epilepsy Behav 31, 196-197.

Hammers, A., Allom, R., Koepp, M.J., Free, S.L., Myers, R., Lemieux, L., Mitchell, T.N., Brooks, D.J., Duncan, J.S., 2003. Three-dimensional maximum probability atlas of the human brain, with particular reference to the temporal lobe. Hum Brain Mapp 19, 224-247.

Henry, M.E., Jensen, J.E., Licata, S.C., Ravichandran, C., Butman, M.L., Shanahan, M., Lauriat, T.L., Renshaw, P.F., 2010. The acute and late CNS glutamine response to benzodiazepine challenge: a pilot pharmacokinetic study using proton magnetic resonance spectroscopy. Psychiatry Res. 184, 171-176.

Higgitt, A., Lader, M., Fonagy, P., 1986. The effects of the benzodiazepine antagonist Ro 15-1788 on psychophysiological performance and subjective measures in normal subjects. Psychopharmacology (Berl) 89, 395-403.

Hoftman, G.D., Volk, D.W., Bazmi, H.H., Li, S., Sampson, A.R., Lewis, D.A., 2013. Altered Cortical Expression of GABA-Related Genes in Schizophrenia: Illness Progression vs Developmental Disturbance. Schizophr Bull.

Kasugai, Y., Swinny, J.D., Roberts, J.D., Dalezios, Y., Fukazawa, Y., Sieghart, W., Shigemoto, R., Somogyi, P., 2010. Quantitative localisation of synaptic and extrasynaptic GABAA receptor subunits on hippocampal pyramidal cells by freeze-fracture replica immunolabelling. Eur J Neurosci 32, 1868-1888.

Kupers, R., Danielsen, E.R., Kehlet, H., Christensen, R., Thomsen, C., 2009. Painful tonic heat stimulation induces GABA accumulation in the prefrontal cortex in man. Pain 142, 89-93.

Lingford-Hughes, A., Hume, S.P., Feeney, A., Hirani, E., Osman, S., Cunningham, V.J., Pike, V.W., Brooks, D.J., Nutt, D.J., 2002. Imaging the GABA-benzodiazepine receptor subtype containing the alpha5-subunit in vivo with [11C]Ro15 4513 positron emission tomography. J Cereb Blood Flow Metab 22, 878-889.

Lingford-Hughes, A., Reid, A.G., Myers, J., Feeney, A., Hammers, A., Taylor, L.G., Rosso, L., Turkheimer, F., Brooks, D.J., Grasby, P., Nutt, D.J., 2012. A [11C]Ro15 4513 PET study suggests that alcohol dependence in man is associated with reduced alpha5 benzodiazepine receptors in limbic regions. J Psychopharmacol 26, 273-281.

Loup, F., Wieser, H.G., Yonekawa, Y., Aguzzi, A., Fritschy, J.M., 2000. Selective alterations in GABAA receptor subtypes in human temporal lobe epilepsy. J Neurosci 20, 5401-5419.

Makkar, S.R., Zhang, S.Q., Cranney, J., 2010. Behavioral and neural analysis of GABA in the acquisition, consolidation, reconsolidation, and extinction of fear memory. Neuropsychopharmacology 35, 1625-1652.

Malizia, A.L., Cunningham, V.J., Bell, C.J., Liddle, P.F., Jones, T., Nutt, D.J., 1998. Decreased brain GABA(A)-benzodiazepine receptor binding in panic disorder: preliminary results from a quantitative PET study. Arch Gen Psychiatry 55, 715-720.

Michels, L., Martin, E., Klaver, P., Edden, R., Zelaya, F., Lythgoe, D.J., Luchinger, R., Brandeis, D., O'Gorman, R.L., 2012. Frontal GABA levels change during working memory. PLoS One 7, e31933.

Miller, L.G., Greenblatt, D.J., Barnhill, J.G., Summer, W.R., Shader, R.I., 1988. 'GABA shift' in vivo: enhancement of benzodiazepine binding in vivo by modulation of endogenous GABA. Eur J Pharmacol 148, 123-130.

Montgomery, A.J., Thielemans, K., Mehta, M.A., Turkheimer, F., Mustafovic, S., Grasby, P.M., 2006. Correction of head movement on PET studies: comparison of methods. Journal of Nuclear Medicine 47, 1936-1944.

Muthukumaraswamy, S.D., Myers, J.F., Wilson, S.J., Nutt, D.J., Hamandi, K., Lingford-Hughes, A., Singh, K.D., 2013. Elevating Endogenous GABA Levels with GAT-1 Blockade Modulates Evoked but Not Induced Responses in Human Visual Cortex. Neuropsychopharmacology 38, 1105-1112.

Muthukumaraswamy, S.D., Myers, J.F., Wilson, S.J., Nutt, D.J., Lingford-Hughes, A., Singh, K.D., Hamandi, K., 2012. The effects of elevated endogenous GABA levels on movement-related network oscillations. Neuroimage 66C, 36-41.

Myers, J.F., Rosso, L., Watson, B.J., Wilson, S.J., Kalk, N.J., Clementi, N., Brooks, D.J., Nutt, D.J., Turkheimer, F.E., Lingford-Hughes, A.R., 2012. Characterisation of the contribution of the GABA-benzodiazepine alpha1 receptor subtype to [(11)C]Ro15-4513 PET images. J.Cereb.Blood Flow Metab 32, 731-744.

Onoe, H., Tsukada, H., Nishiyama, S., Nakanishi, S., Inoue, O., Langstrom, B., Watanabe, Y., 1996. A subclass of GABAA/benzodiazepine receptor exclusively localized in the limbic system. Neuroreport 8, 117-122.

Richards, D.A., Bowery, N.G., 1996. Comparative effects of the GABA uptake inhibitors, tiagabine and NNC-711, on extracellular GABA levels in the rat ventrolateral thalamus. Neurochem Res 21, 135-140.

Sanacora, G., Mason, G.F., Rothman, D.L., Behar, K.L., Hyder, F., Petroff, O.A., Berman, R.M., Charney, D.S., Krystal, J.H., 1999. Reduced cortical gamma-aminobutyric acid levels in depressed patients determined by proton magnetic resonance spectroscopy. Arch.Gen.Psychiatry 56, 1043-1047.

Shin, M.S., Park, S.Y., Park, S.R., Seol, S.H., Kwon, J.S., 2006. Clinical and empirical applications of the Rey-Osterrieth Complex Figure Test. Nat Protoc 1, 892-899.

Shrout, P.E., Fleiss, J.L., 1979. Intraclass correlations: uses in assessing rater reliability. Psychol Bull 86, 420-428.

Smolnik, R., Pietrowsky, R., Fehm, H.L., Born, J., 1998. Enhanced selective attention after low-dose administration of the benzodiazepine antagonist flumazenil. J Clin Psychopharmacol 18, 241-247.

Spielberger, C., Gorsuch, R., Lushene, P., 1970. Manual for the state-trait anxiety inventory. Consulting Psychologist Press, Palo Alto, CA, USA.

Sybirska, E., Seibyl, J.P., Bremner, J.D., Baldwin, R.M., al-Tikriti, M.S., Bradberry, C., Malison, R.T., Zea-Ponce, Y., Zoghbi, S., During, M., et al., 1993. [123I]iomazenil SPECT imaging demonstrates significant benzodiazepine receptor reserve in human and nonhuman primate brain. Neuropharmacology 32, 671-680.

Tallman, J.F., Thomas, J.W., Gallager, D.W., 1978. GABAergic modulation of benzodiazepine binding site sensitivity. Nature 274, 383-385.

Turkheimer, F., Moresco, R.M., Lucignani, G., Sokoloff, L., Fazio, F., Schmidt, K., 1994. The use of spectral analysis to determine regional cerebral glucose utilization with positron emission tomography and [18F]fluorodeoxyglucose: theory, implementation, and optimization procedures. J.Cereb.Blood Flow Metab 14, 406-422.

Tyacke, R., Robinson, E., Harris, A., Lingford-Hughes, A., Nutt, D., 2009. Paradoxical effects of GABA on the in vivo uptake of [3H]Ro15-4513 in rat brain. In: Nutt, D., Blier, P. (Eds.), British Association for Psychopharmacology Summer Meeting. Sage Science Press, Oxford, UK, p. A21.

Valentine, G.W., Mason, G.F., Gomez, R., Fasula, M., Watzl, J., Pittman, B., Krystal, J.H., Sanacora, G., 2011. The antidepressant effect of ketamine is not associated with changes in occipital amino acid neurotransmitter content as measured by [(1)H]-MRS. Psychiatry Res. 191, 122-127.

Weber, O.M., Verhagen, A., Duc, C.O., Meier, D., Leenders, K.L., Boesiger, P., 1999. Effects of vigabatrin intake on brain GABA activity as monitored by spectrally edited magnetic resonance spectroscopy and positron emission tomography. Magn Reson Imaging 17, 417-425.

Wechsler, D., 1981. The Wechster Memory Scale–revised. The Psychological Coporation, Harcourt Brace Jovanovich, Inc.