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The brain GABA-benzodiazepine receptor alpha-5 subtype in autism spectrum disorder: A pilot [¹¹C]Ro15-4513 positron emission tomography study

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1. Introduction

Autism Spectrum Disorder (ASD) is characterised by deficits in social reciprocity, communication impairments, and restricted, repetitive interests and behaviours (World Health Organization, 1993). Recent research suggests an approximate prevalence of 0.6-1.5% in the general population (Baron-Cohen et al., 2009; Schechter and Grether, 2008; Thomas et al., 2011; Zaroff and Uhm, 2011).

Numerous studies have reported differences in brain anatomy and function in individuals with ASD (e.g. see (Craig et al., 2007; Ecker et al., 2010; Hallahan et al., 2009)), but the underlying molecular basis of the condition remains unclear. This has led to a paucity of treatment targets. At present, therapeutic options for ASD are limited to medications used to alleviate specific symptoms, such as the licensed use of the antipsychotic risperidone for aggression and challenging behaviours (Matson et al., 2011)) and selective serotonin reuptake inhibitors in obsessive or repetitive behaviours (King et al., 2009). However, the efficacy of such pharmacotherapy has recently been questioned (McPheeters et al., 2011). There is, therefore, a need for better targeted, ASD-specific treatments, and this will only be possible on the basis of a better understanding of ASD pathophysiology.

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Conflict of Interest Statement

The authors declare no conflicts of interest.

ASD is now viewed as a heterogeneous set of disorders, which can be caused by various genetic, epigenetic and environmental factors, but emerging evidence suggests that an imbalance between excitatory glutamate and inhibitory gamma-amino-butyric acid (GABA) neurotransmission may form a final common pathway in ASD. In particular, defects in GABA transmission, leading to brain hyperexcitability, have been hypothesized to underlie the symptoms of ASD (Pizzarelli and Cherubini, 2011; Rubenstein and Merzenich, 2003; Yizhar et al., 2011).

GABA, the primary inhibitory neurotransmitter in the adult human brain, is synthesised from the excitatory neurotransmitter glutamate, via the action of glutamate decarboxylase (GAD) enzymes, GAD₆₅ and GAD₆₇. In the central nervous system, GABA is produced and released by inhibitory interneurons (Kubota et al., 2011; Tamamaki and Tomioka, 2010).

GABA acts on two main classes of membrane-bound receptors: ionotropic GABA_A receptors (ligand-gated Cl⁻channels), and metabotropic (G protein-coupled) GABA_B receptors. The GABA_A receptor family is the predominant type in the brain, and is the site of action of drugs such as benzodiazepines and several anaesthetics (Reynolds et al., 2003).

The GABA_A receptor is composed of five subunits arranged around a central pore (Nutt and Malizia, 2001). The subunits are diverse and different combinations of subunits give rise to GABA_A receptors with specific properties. Most brain GABA_A receptors contain α , β and γ subunits in a 2:2:1 stoichiometry (Tretter et al., 1997), although γ can be replaced by δ or ϵ subunits, and the σ subunit may substitute for the β subunit.

In man, Positron Emission Tomography (PET) has been critical in delineating the distribution and levels of GABA_A receptors in a variety of neuropsychiatric disorders including anxiety related disorders, benzodiazepine dependence (Nutt and Malizia, 2001), alcoholism, and epilepsy (Malizia and Richardson, 1995).

Evidence for abnormal GABA_A density in ASD comes from neuropathological studies. Blatt and colleagues reported reduced GABA_A receptors and benzodiazepine binding sites in the hippocampus (Blatt et al., 2001), and the cingulate cortex (Oblak et al., 2009; Oblak et al., 2010) of individuals with ASD. Other studies found altered GAD expression in ASD (Yip et al., 2007, 2008) and reduced neuronal cell size and increased cell packing density in GABAergic hippocampal neurons and interneurons, subiculum, entorhinal cortex, amygdala, medial septal nucleus, and mammillary bodies in ASD compared to controls (Kemper and Bauman, 1998).

Converging evidence for a GABA_A involvement in ASD comes from genetic studies. For instance, microduplications of the chromosome 15q11-13 locus have been observed in a proportion of people with ASD (Buxbaum et al., 2002; Cook et al., 1998; McCauley et al., 2004; Menold et al., 2001; Sebat et al., 2007; Shao et al., 2003). This region notably contains the genes coding for the GABA_A $\alpha 5$, $\beta 3$ and $\gamma 3$ subunits, and although duplication might be expected to lead to over expression of these receptor proteins, in vitro studies of a human neuronal cell line carrying a 15q duplication showed that this variant actually leads to *reduced* GABRB3 expression (Meguro-Horike et al., 2011).

Abnormal patterns of expression of 15q11-13 locus genes have been reported even in ASD cases without the mutation: in 4 of 8 cases of idiopathic ASD, levels of GABA_A α 5, β 3 and γ 3 were reduced as expression of the maternally inherited copies of these genes predominated (Hogart et al., 2007). Finally, reduced frontal and temporal cortical expression of mRNA in a network of genes highly expressed in GABA interneurons was observed in two samples of ASD (Voineagu et al., 2011).

There is, therefore, mounting evidence implicating the GABA system and in particular GABA_A receptors, generally in limbic areas, in ASD. However, there has only been one study which attempted to directly measure GABA_A receptors in the living brain in ASD. This SPECT (Single Photon Emission Computed Tomography) study reported decreases in GABA_A, especially in the frontal cortex in children with ASD (Mori et al., 2011). No study has yet examined adults with the condition.

We (Lingford-Hughes et al., 2002) have previously characterized the benzodiazepine receptor PET ligand [¹¹C]Ro15-4513 (Halldin et al., 1992) in rats and humans and showed that its uptake primarily has a limbic distribution. High levels of uptake were described in the anterior cingulate cortex, hippocampus, insular cortex, septal region and amygdala, with lower levels seen in the occipital cortex and cerebellum than that observed with the traditional PET benzodiazepine ligand [¹¹C]flumazenil (Lingford-Hughes et al., 2002).

These differences are explained by [¹¹C]Ro15-4513 having relative selectivity, namely approximately 10-fold higher affinity for the α 5 subtype of the GABA_A receptor compared with the other receptor subtypes in both humans and rodents. In addition, recent refinements in the analysis of [¹¹C]Ro15-4513 PET images have allowed us to further refine the signal, giving some discrimination between the high affinity α 5 receptor subtype and the highly abundant α 1 receptor subtype (Myers et al., 2012).

Therefore, we decided to use [¹¹C]Ro15-4513 PET to measure α 1 and α 5 GABA_A subtype receptor levels, with a particular interest in the α 5 subtype in limbic regions that have been implicated in ASD: the amygdala, the hippocampus, and the nucleus accumbens. In the light of the previous work discussed above, our primary hypothesis was that, compared to controls, individuals with ASD have a significant reduction in α 5 GABA receptor availability in these areas.

2. Methods

2.1. Participants

In this preliminary study, we included three male participants diagnosed with autism, meeting ICD-10 Research criteria, who scored above threshold for ASD in the Autism Diagnostic Observation Schedule (ADOS), and who had an IQ above 80. This IQ criterion was applied to avoid the possible confounding influence of IQ. We compared these individuals to data already acquired from three age-sex matched controls. All volunteers had capacity to consent to participation in the study; capacity was assessed by a qualified psychiatrist. English was the first language of all individuals. See Table 1 for participant clinical and demographic details.

We excluded people with a learning disability (mental retardation); people with diagnosed and treated ADHD, hyperkinesia or Tourette's syndrome. We also excluded people who were taking psychotropic medication with possible effects on the GABA system i.e. antiepileptic drugs, benzodiazepines and antidepressants (Bhagwagar et al., 2004); a history of dependence to alcohol or substances of abuse (except nicotine); a major mental illness; or a medical or chromosomal disorder known to be associated with ASD such as Fragile X Syndrome).

All participants were able to tolerate MRI with no history of claustrophobia or presence of a cardiac pacemaker, other electronic medical implant, or ferromagnetic metal foreign bodies.

This study was approved by the North London Research Ethics Committee 3 (REC reference number 10/H0709/90) and by the Administration of Radioactive Substances Advisory Committee (ARSAC); it was also internally approved by the Institute of Psychiatry and the Maudsley Research and Development Office (R&D2011/014) and the Imperial College London and Imperial College Healthcare NHS trust Joint Research Office (JROHH0151).

2.2. Imaging protocol

The PET tracer [^{11}C]Ro15-4513 was synthesised (Halldin et al., 1992) and purified by reverse-phase high performance liquid chromatography (Phenomenex uLTRACARB 7 ODS, 250 $\times$ 10mm). A bolus of [^{11}C]Ro15-4513 was administered through a cannula in the dominant vein of the antecubital fossa. There were no significant differences in dosimetry between the two groups (healthy mean $\pm$ s.d.: $40 \pm 5 \text{ GBq}/\mu\text{mol}$ in $4.14 \pm 0.45 \mu\text{g}$ of stable ligand; ASD mean $\pm$ s.d.: $33 \pm 11 \text{ GBq}/\mu\text{mol}$ in $5.19 \pm 1.9845 \mu\text{g}$ of stable ligand), compared using Student's t-tests (specific activity: $t=1.05$, $p>0.3$; stable ligand: $t=0.899$, $p>0.4$).

Scans were carried out using a Siemens ECAT EXACT HR+ (CTI/Siemens, model 962), to acquire 63 transaxial planes, each a 2.42mm slice (Spinks et al., 2000). A 10 minute ^{137}Cs transmission scan was acquired prior to each emission scan for correction for attenuation and scatter. Each [^{11}C]Ro15-4513 scan was divided into 24 time frames (1×30 , 4×15 , 4×60 , 2×150 , 10×300 , 3×600 seconds), reconstructed with filtered backprojection.

For the first 15 minutes of each scan, continuous measurement of blood radioactivity was sampled through a radial arterial cannula in each participant. Discrete blood samples were also taken approximately 4, 6, 8, 10, 20, 35, 50, 65, 80 and 90 minutes after [^{11}C]Ro15-4513 injection, and plasma was analyzed for radiolabeled metabolites. The plasma [^{11}C]Ro15-4513 concentration profile throughout the scan could then be estimated, using Clickfit, in-house software running in Matlab (The Mathworks, Natick, MA).

Each participant also underwent a T1-weighted MRI scan, with $1 \times 1 \times 1 \text{ mm}$ resolution, using a Philips 1.5T Gyroscan Intera scanner, for structural reference and localisation of any changes in PET signal.

2.3. Image Analysis

Reconstructed PET images were analysed on a Sun SPARC workstation (Sun Microsystems, Mountain View, CA, USA) using Analyze AVW version 9.0 (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN), Matlab (The Mathworks, Natick, MA) and SPM5 (available via <http://www.fil.ion.ucl.ac.uk/spm/>).

The structural MRI was coregistered, using a rigid-body technique, to the add-image, the weighted sum of all dynamic PET frames. Normalisation parameters were obtained by warping the coregistered structural MRI to 'Montreal Neurological Institute' space (International Consortium for Brain Mapping (ICBM)/MNI) using bias-corrected segmentation in SPM5. The inverse of these parameters was used to fit an object map of 83 regions of interest (ROIs) to each individual PET dynamic. Analyze 9.0 was used to check the goodness-of-fit of each object map, and to sample ROIs to collect time-activity curves (TACs) for each ROI.

Clickfit was used to carry out spectral analysis of each regional TAC collected from the original dynamic PET scans. TACs were described by a convolution of the plasma input function and a 1st-order polyexponential (Cunningham and Jones, 1993; Turkheimer et al., 1994). This analysis yields a spectrum of exponential parameters, each peak representing a component of the total signal from the tissue.

In order to allow us to distinguish between binding at the GABA_A $\alpha 1$ and $\alpha 5$ subtypes, this spectrum of component exponential parameters was then divided into ranges representing the different two major binding compartments of [¹¹C]Ro15-4513 (Myers et al., 2012).

In brief, the analysis relies on the fact that the temporal dynamics of binding at $\alpha 1$ and $\alpha 5$ differ; fast components are attributed to the rapidly equilibrating $\alpha 1$ subtype signal, between 0.0030 and 0.0040 sec⁻¹. In the case of the slower $\alpha 5$ receptor subtype binding site, the range of exponential decay values is approximately 0.00063 – 0.0030 sec⁻¹, and the V_T (distribution volume) of the ligand binding at this site calculated using the integral of this range of the spectrum, using the equation $V_T = \sum_{k=i}^j (\alpha_k / \beta_k - \lambda)$, where α_k is the k th peak height, β_k is the k th peak position, λ the decay constant, and i and j are the limits of the ranges given above. For more details on this spectral fitting method, readers should consult (Myers et al., 2012).

2.4. Statistical Analysis

Scan data and age-matching were assessed using two-tailed Student's t-statistics. A two-way repeated measures ANOVA was used to compare [¹¹C]Ro15-4513 V_T between ASD cases and healthy volunteers. Mean $\alpha 5$ V_T estimates (see Section 2.3) were also compared using Student's t-test in predefined ROIs: the left and right amygdala, hippocampus, and nucleus accumbens. All these are areas of the limbic system which are known to show high expression of the GABA_A $\alpha 5$ subunit. All have been previously implicated in ASD; see (Amaral et al., 2008; Blatt et al., 2001; Dichter et al., 2010).

All statistical analyses were conducted using GraphPad Prism (version 4.02 for Windows, GraphPad Software, San Diego, CA, USA, www.graphpad.com).

3. Results

Demographic and psychometric details for all six participants are shown in (Table 1). Participants were matched for age and gender

A two-way ANOVA showed significantly lower V_T across 83 regions in the brains of participants with ASD as compared to controls ($F=140.7$, $p<0.0001$). See Figure 1. Four regions survived stringent Bonferroni correction for multiple comparisons, the right and left nucleus accumbens ($t=8.61$, $p<0.001$; $t=8.03$, $p<0.001$, respectively) and the right and left subcallosal area ($t=4.62$, $p<0.001$; $t=6.84$, $p<0.001$, respectively). (See Figure 1.)

When the spectrum was analyzed in order to estimate V_T for the $\alpha 1$ and $\alpha 5$ receptor subtypes separately (see section 2.3), the source of this difference was identified. A two-way ANOVA across all regions revealed no differences in the $\alpha 1$ subtype range ($F=1.922$, $p>0.1$). See Figure 2. There was, however, a highly significant difference in the estimated $\alpha 5$ subtype V_T ($F=32.27$, $p<0.0001$). See Figure 3.

The three *a priori* selected limbic brain regions (see 2.4) were used to compare total V_T in ASD cases and healthy controls to further confirm the hypothesis. Two showed significant differences with Student t-tests, the nucleus accumbens ($t=3.360$, $p<0.05$) and the amygdala ($t=3.684$, $p<0.05$). See Figure 4. The hippocampus showed no significant differences, although $GABA_A$ was non-significantly lower in the ASD group ($t=1.916$, $p<0.07$).

4. Discussion

We found significantly lower [^{11}C]Ro15-4513 V_T across all 83 regions in the brain of participants with ASD compared with healthy volunteers ($p<0.0001$). Four regions survived stringent Bonferroni correction for multiple comparisons: the right and left nucleus accumbens and the right and left subcallosal area.

Further analysis suggested that these results were primarily due to lower levels of the $\alpha 5$ receptor subtype, rather than $\alpha 1$ subtype. Therefore, the results of this preliminary PET study in ASD, is consistent with evidence that ASD is characterised by a defect in GABA signalling via limbic $GABA_A$ receptors (see the Introduction).

Although these results are preliminary, we selected healthy volunteers that were matched in age and gender. We also took care not to include anyone with a history of substance use disorder, except for nicotine, or those taking medications that could modulate GABA or $GABA_A$ receptors (Bhagwagar et al., 2004). None had a history of seizures which have also been associated with alterations in $GABA_A$ (Laufs et al., 2011), and none suffered from mental retardation (intellectual disability).

To our knowledge, only one previous study has quantified $GABA_A$ density in the living ASD brain: Mori et al (Mori et al., 2011) found reduced accumulation of the $GABA_A$ -benzodiazepine ligand [^{123}I]iomazenil (which binds to the $\alpha 1$, 2, 3 and 5 subtypes in frontal cortex) using SPECT (Single Photon Emission Computed Tomography).

However, there are a number of important differences between our study and that of Mori et al. Firstly, Mori et al studied children with ASD (mean age, 7.3 ± 3.5 years), while we recruited adults only. Secondly, the patients in this study were sedated using triclofos, while all of our participants were medication free. Thirdly, most of the ASD children had a low IQ, with only 9 of the 24 ASD subjects having an IQ above 85. Finally, similarly to [^{11}C]flumazenil, it is likely that [^{123}I]iomazenil binding reflects the $\alpha 1$ subtype with limited discrimination of any other subtype. The advantage of using [^{11}C]Ro15-4513 is that we were able to discriminate $\alpha 1$ from $\alpha 5$, and hence probe the $\alpha 5$ subtype, which is present in high levels in the limbic cortex where dysregulation in ASD is thought to occur.

The $\alpha 5$ subunit-containing receptors constitute approximately 25% of GABA_A receptors in the hippocampus, and are also highly expressed in the anterior cingulate cortex and the ventral striatum. There is increasing evidence that these brain regions are critically implicated in the symptomatology of ASD (Ecker et al., 2012; McAlonan et al., 2005). The $\alpha 5$ subtype is localized primarily to extrasynaptic regions, on the cell bodies and apical dendrites of glutamatergic pyramidal neurons in the CA1 and CA3 hippocampal regions (Brunig et al., 2002); and mediate tonic inhibitory currents - rather than synaptic, transient, neurotransmission (Caraiscos et al., 2004). They have been implicated in reward, learning and memory (Nutt, 2006; Nutt et al., 2007).

The differences in GABA_A $\alpha 5$ levels found in this study may therefore be related to some of the social and emotional difficulties observed in people with ASD. For example, we observed significantly reduced GABA_A $\alpha 5$ in the nucleus accumbens, which is part of the ventral striatum. Ventral striatal abnormalities reported in previous studies of ASD include: abnormal anatomical developmental trajectories in the nucleus accumbens (Langen et al., 2009); impaired NAcc functional responses to social rewards (Scott-Van Zeeland et al., 2010); and abnormalities in white matter tracts connecting the frontal cortex to the nucleus accumbens (Langen et al., 2011). Since $\alpha 5$ -containing GABA_A receptors mediate tonic inhibition, a deficit in these receptors might lead to hyperexcitability and impaired information processing (Pizzarelli and Cherubini, 2011; Rubenstein and Merzenich, 2003). However, it is also possible that the reduced GABA_A $\alpha 5$ levels are an adaptation to, or downstream consequence of, other neurobiological changes, rather than being directly responsible for the symptoms of ASD. Further work is needed to examine this possibility.

This study has a number of limitations. Owing to the closure of the PET facility we were only able to collect data from a small number of individuals for this preliminary report, but we believe it is scientifically and ethically important to present these data in the public domain. Methodologically, we are confident that our analytical approach can dissociate $\alpha 1$ from $\alpha 5$ benzodiazepine receptor subtypes (Myers et al 2012), however given the small number of participants together with low signal to noise ratio of these methods of estimating V_T at the lower affinity $\alpha 1$ -containing subtype, we remain cautious. However, the lack of changes in receptor kinetics at either subtype suggests the differences in binding are probably due to down-regulation of receptor expression, supporting previous findings in gene assays (Fatemi et al., 2009).

Due to the small sample size, we could not examine possible correlations between GABA_A binding and particular symptoms of ASD, age, IQ, or symptoms of comorbidities frequently associated with ASD, such as anxiety disorders, OCD and depression. We were also unable to address the effects of possible neuroanatomical differences between people with ASD and controls, which might lead to partial volume effects in PET studies. However, the modest magnitude of the volumetric differences seen in most studies of high-functioning ASD (Ecker et al., 2012; McAlonan et al., 2005) suggests that it is unlikely that these could fully explain the present findings.

These preliminary results suggest that potentiation of GABA_A signalling, especially at GABA_A α5-subunit containing receptors, might potentially be a novel therapeutic target for ASD. Unselective GABA_A agonists and positive allosteric modulators, such as benzodiazepines, have undesirable features such as abuse potential and tolerance, but more selective modulators might avoid such limitations (Rudolph and Knoflach, 2011). Further research should extend this work in a larger sample of ASD individuals. It would also be interesting to use PET with the ligand [¹¹C]Ro15-4513 to measure GABA_A in disorders of known etiology characterised by ASD symptoms, such as Fragile X Syndrome (Rossi et al., 1995) and 15q11-13 duplication (Buxbaum et al., 2002; Cook et al., 1998; McCauley et al., 2004; Menold et al., 2001; Sebat et al., 2007; Shao et al., 2003).

5. Conclusion

In summary, we present preliminary evidence of reduced GABA_A α5 expression in adult males with ASD, consistent with the hypothesis that ASD is characterised by a defect in GABA signalling.

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Figure 1.

$[^{11}\text{C}]\text{Ro15-4513 } V_T$ estimated by spectral analysis in 83 regions of the brain in 3 healthy volunteers and 3 ASD patients. Error bars show standard deviation; regions are divided left and right. V_T is significantly lower in ASD patients than healthy volunteers across all regions, compared with a two-way ANOVA. Regions showing significant differences after Bonferroni correction for multiple comparisons are marked with an asterisk *. These regions include the nucleus accumbens and the subcallosal area.

Figure 2. $[^{11}\text{C}]\text{Ro15-4513 } V_T$ at the $\text{GABA}_A \alpha 1$ subtype, estimated by integrating spectral analysis between frequencies of approximately 0.0030 to 0.0050 sec^{-1} , in 83 regions of the brain in 3 healthy volunteers and 3 ASD participants. Error bars show standard deviation; regions are divided left and right. A two-way ANOVA shows no significant differences between the two subject groups.

Figure 3.

[^{11}C]Ro15-4513 V_T at the GABA $_A$ $\alpha 5$ subtype, estimated by integrating spectral analysis between frequencies of approximately 0.0006 to 0.0030 sec^{-1} , in 83 regions of the brain in 3 healthy volunteers and 3 ASD participants. Error bars show standard deviation; regions are divided left and right. A two-way ANOVA shows significantly lower V_T across all regions in ASD participants compared with controls. Regions showing significant differences after Bonferroni correction for multiple comparisons are marked with an asterisk *. These regions include the nucleus accumbens and subcallosal area.

Figure 4.

Three regions were selected *a priori* for analysis by Student's t-test, the nucleus accumbens (A), amygdala (B) and hippocampus (C). V_T estimated by spectral analysis in 3 healthy volunteers was compared with that in 3 ASD participants. In the nucleus accumbens and amygdala, there was a significantly lower V_T . The hippocampus showed no significant differences but $GABA_A$ was non-significantly lower in the ASD group.

Table 1

Participant demographic and psychometric data.

Group	Participant Number	Gender	Age	ADOS*	Full Scale IQ
ASD	ASD1	Male	43	14 (5 + 7 + 2)	117
	ASD2	Male	34	13 (3 + 7 + 3)	123
	ASD3	Male	41	11 (4 + 4 + 3)	127
Control	HC1	Male	40	n/a	n/a
	HC2	Male	39	n/a	n/a
	HC3	Male	37	n/a	n/a

* ADOS = Autism Diagnostic Observation Schedule – Revised. Total scores are given first. Subscale scores are given in parentheses as follows: (*Communication + Reciprocal Social Interaction + Stereotyped Behaviour/Restricted Interests*). Note that ADOS and IQ scores were only available for ASD individuals.